Assays of haemostasis in the diagnosis and monitoring of haemophilia

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Statement of Originality

This is to certify that the content of this thesis is my own work. This thesis has not been submitted for any other degree or purpose. I certify that the intellectual content of this thesis is the product of my own work, and that all assistance received in preparing this thesis and all sources have been acknowledged.

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Chapter 3 was published as:

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I designed the study, performed the assays, analysed the data and wrote the manuscript drafts.

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Summary

Optimal management of factor replacement therapies for persons with haemophilia requires accurate measurements of post-infusion factor levels to support dosing regimens that minimise the risks of bleeding events from underdosing, while preventing overdosing of expensive product. In the past 10 years there have been major additions to therapeutic options for haemophilia, including the development of extended half-life (EHL) factor replacement products and non-factor replacement therapies including bi-specific antibodies. Prior to these developments, clinical laboratories measured both plasma-derived and recombinant FVIII and FIX by one-stage clotting assays or by chromogenic assay for FVIII with reasonable and predictable accuracy. The main aims of the current work are to evaluate and validate some of the newer methods for product measurement, supported by field studies of measurement by Australian specialist haemostasis laboratories. Chapters 1 and 2 summarise important discoveries in the development of therapeutic options for haemophilics up to the present use of extended half-life therapies and the non-factor replacement therapeutic emicizumab. There is a description of the developmental stages of the current clotting and chromogenic-based assays for product measurement and how the era of new therapeutics impacts on the use of these assays, and assay descriptions of the tests used in this work. Chapters 3 is the first published detailed evaluation of the chromogenic FIX assays, supplemented by a field study of selected Australian haemophilia reference laboratories in the application of one the two available commercial chromogenic FIX assays. In Chapters 5 and 6, two extended half-life FVIII replacement therapies, Eloctate and Adynovate, respectively, were the subject of field studies of Australian specialist haemostasis laboratories, coinciding with their introduction as therapeutic agents for haemophilia A. This work allowed these laboratories to validate the measurement accuracy of their current reagents, details of which were not published in previous reports. In Chapter 7 there is a single centre study exploring measurement

characteristics of the extended half-life FVIII replacement product efanesoctocog alfa. This work includes new in vitro stability studies at multiple temperatures and time points, plus adaptation of FVIII clotting assays of the reagent used for product potency assignment to non-affiliated analysers. A published review article (Chapter 8) summarises available and potential strategies for measuring some newer therapeutic agents for FVIII deficiency, namely the non-factor replacement therapies emicizumab and Mim8, the recombinant porcine FVIII, Obizur, and efanesoctocog alfa. Chapter 9 is a published article on first field study of emicizumab measurement, including participants from five Australian states. The study also includes new data on assay linearity covering the therapeutic window, detailed assessment of assay precision on spike samples and ex vivo samples, and assessment of quality control material freeze/thaw stability. A field study of haemostasis reference laboratories in the measurement of FVIII antibodies using chromogenic FVIII Bethesda assays is presented in Chapter 10. Antibody were measured in the presence and absence of emicizumab. This study was performed because the introduction of emicizumab in Australia has necessitated the use of bovine-component chromogenic FVIII assays, which is new to some testing laboratories. Chapter 11 briefly summarises the important findings of the combined work.

Declaration of authorship

This thesis with publications is submitted to the University of Sydney in fulfilment of the requirement for the Degree of Doctor of Philosophy (PhD). I, Geoffrey Wilson Kershaw, declare that the work presented in this thesis is original except as acknowledged in the text. I hereby declare that I have not submitted this work, either in part or in full, to any other institution. The research presented in this thesis was conducted by me under the guidance of my primary supervisor professor Vivien Chen and auxiliary supervisors.

Geoffrey Kershaw

30.06.2025

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statement above is correct.

Vivien M. Chen

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Publications related to the thesis

1. Kershaw G, Dissanayake K, Chen VM, Khoo TL. Evaluation of Chromogenic FIX assays by automated protocols. Haemophilia 2018; 24:492-501
2. Kershaw G, Chen VM, Cai N, Khoo TL. Australian comparative field study evaluating the activity of recombinant factor VIII Fc fusion protein (Eloctate®) Haemophilia 2020:26(5)
3. Kershaw G, Dix C, Chen VM, Cai N, Khoo TL. Emicizumab assay evaluations and results from an Australian field study of emicizumab measurement. Pathology 2022; 54:755-762
4. Kershaw G, Dix C. Measuring Emicizumab Levels in the Hemostasis Laboratory. In Favaloro E, Lippi G, eds. Hemostasis and Thrombosis Methods and Protocols 2nd Edn. New York: Springer Science 2023; 2663:589-595
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Presentations related to the thesis

Oral presentations

- Chromogenic FIX assays. THANZ Scientific Workshop Sydney Convention Centre. October 28th 2017.

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- Laboratory monitoring of EHLs & new therapeutics in haemophilia. Coagulation challenges meeting. Adelaide 4th May 2019. Novo Nordisk sponsored meeting.

- Laboratory Impact of New Hemophilia Therapies. Masterclass presenter. ISTH 2019 July 6-10th, 2019. Melbourne Convention Centre.

- FVIII and FIX assays in the era of new therapeutics for haemophilia. RCPA Haemostasis and Thrombosis Workshop Melbourne July 5th 2019. Melbourne Convention Centre.

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Poster presentations

- Kershaw G, Chen VM. Quantitative and Qualitative Differences Exist between APTT reagents in Clot Waveform Analysis in Haemophilic Plasma Spiked with Normal Plasma and rFVIII Products. PB1311. ISTH SSC Dublin. Res Pract Thromb Haemost. 2018;2(Suppl. 1):118.

- Kershaw G, Dissannayake K, Chen VM, Khoo L. A multi-laboratory validation of the Rossix chromogenic FIX assay. WFH 2020 World Congress

- Geoffrey Kershaw, Caroline Dix, Vivien M Chen, Nancy Cai, Teh-Liane Khoo. Measuring plasma emicizumab levels: results from an Australian field study. Blood 2021 Adelaide Convention Centre.

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List of Abbreviations

ADA	anti-drug antibodies
AHA	acquired haemophilia A
AHF	anti-haemophilic factor
APTT	activated partial thromboplastin time
bCSA	Chromogenic substrate assay with bovine components
BDD	B domain deleted
BSA	bovine serum albumin
BU	Bethesda units
CSA	chromogenic substrate assay
CV	coefficient of variation
ECAT	External Control for Assays and Tests
Efa	Efanesoctocog alfa
EHL	extended half life
EQA	external quality assurance
FII	factor II
FIIa	activated factor II
FIX	factor IX
FIXa	activated factor IX
FV	factor V
FVIII	factor VIII
FVIIIa	activated factor VIII
FXa	activated factor X
HA	haemophilia A
HB	haemophilia B
HIV	human immunodeficiency virus
ITI	immune tolerance induction
KCT	kaolin clotting time
OSA	one-stage clotting assay
pdFIX	plasma-derived factor IX
pdFVIII	plasma-derived factor VIII
pNA	para-nitroaniline
PTT	partial thromboplastin time
PTTK	partial thromboplastin time with kaolin
RCPAQAP	Royal College of Pathologists of Australasia quality assurance programs
rFIX	recombinant factor IX
rFVIII	recombinant factor VIII
rpFVIII	recombinant porcine FVIII
SHL	standard half-life
VWF	von Willebrand factor

CHAPTER 1

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1. Definition and classification of hereditary haemophilia A and haemophilia B

Haemophilia A (HA) and Haemophilia B (HB) are rare bleeding disorders caused by deficiency of plasma proteins factor VIII (FVIII) and factor IX (FIX), respectively (1). Both HA and HB have X-linked inheritance, hence predominantly males are affected, though in rare cases female carriers or females with two affected X chromosomes are classified as having haemophilia if a bleeding phenotype is present (1). Differing classification systems for haemophilia severity had existed prior to the year 2001, based on laboratory-determined factor levels, clinical bleeding, or a combination of both (2, 3). A classification for haemophilia, based solely on baseline plasma factor VIII or FIX levels, was published in 2001 by the Scientific Subcommittee on FVIII and FIX of the Scientific Standardisation Committee (SSC) of the International Society on Thrombosis and Haemostasis (2). Three groupings were in this classification: severe haemophilia is defined as an individual having factor levels of <1% of normal; moderate, 1% to 5% of normal; and mild, >5% to 40% of normal. This SSC classification system has been widely adopted, allowing for better comparability among clinical studies because of the standardized definitions. The units of measurement for factor levels can also be expressed as International Units (IU) relative to International Standards (IS) that have been assigned in international collaborative studies (4). Thus, 100% factor, traditionally defined as the amount of factor in a pool made from >20 donors, can be expressed as 100 IU/dL or 1.00 U/mL, with all three modes of expression still used in clinical laboratories and scientific publications. The classification based on plasma factor levels highlights the need for laboratories to produce accurate results at the diagnostically important lower levels (3).

2. History of replacement therapies for haemophilia

2.1 Whole blood and blood plasma

Therapeutic options for haemophilia prior to the mid-1960s were mostly limited to fresh frozen plasma, sometimes whole blood, with bed rest and ice packs (5). In the 1950s the need for repeated transfusions of plasma at frequent intervals to maintain the hemostatic level of plasma anti-haemophilic factor (AHF), or FVIII, had been well established in the treatment of hemophilic hemorrhages (6).

2.2 Cryoprecipitate and plasma-derived FVIII

In the late 1950s Poole and Robinson performed detailed measurements of FVIII of donor plasma during the collection, preparation and storage process, to evaluate why fresh frozen plasma was often ineffective (6). They found a significant loss of FVIII during its preparation and its storage of more than 50% and estimated the time a patient's post-infusion FVIII level spent above 5% of normal ranged from 3 to 8.5 hours. The same authors by chance observed that the AHF content of the last few drops of plasma left in a bottle after a transfusion was greater than that of the freshly thawed unit. This led to the discovery that AHF was associated with the presence of cold-precipitated fibrinogen which usually remains as a precipitate even after all the ice has melted (7). A major therapeutic breakthrough followed a years later in 1965 with publication of a method for cryoprecipitate production from whole blood donation that yielded protein content of seven times that of whole plasma, and which was successful in treating four individuals with bleeding using a total of 41 units of the new product (8). Each unit comprised 3 mL of precipitated material made up with 10 mL of normal

saline to a final infusion volume of 13 mL. The authors suggested maintaining FVIII levels in the range 30-60% to treat bleeds. Others soon reported successful clinical outcomes in treating bleeding patients using the same procedure to produce cold precipitated AHF (9). A method modification was used to salvage cryoprecipitate from expired stored whole blood that was then re-suspended in autologous plasma, which could then be given to haemophiliacs (10). The use of cryoprecipitate continues to be a mainstay of treatment in many parts of the world (11). Further advances in plasma fractionation and commercial adaptations yielded lyophilized clotting factor concentrates that could raise missing clotting factor to normal levels (12). In the United States (US), between 1970 until the early 1980s, mortality among persons with haemophilia declined substantially due to increased availability of clotting factor replacement products for treating life-threatening bleeds, and improved medical management provided by specialised haemophilia treatment centres (HTCs) that were established in the mid-1970s (13). It has been estimated that life expectancy in haemophiliacs rose from around 40 years of age in 1960 to 60 years by 1980, but the increasing demand for plasma came with risks of transmitting blood-borne diseases, particularly in the US where some paid blood donors had a high risk of carrying blood-borne diseases (12). Clotting factor concentrates were being prepared from plasma pools of up to 20,000 donors with inadequate donor screening and infective agent testing (12). A study of 727 individuals with HA or HB enrolled in nine US treatment centres during 1987 and 1988 found 53% to have human immunodeficiency virus (HIV) infection and 98% of these also were infected with hepatitis C virus (14). In the United Kingdom (UK) more than 1000 individuals with HA were HIV positive by 1987, most likely due to use of imported plasma products from the US when local volunteer donations through the National Health Service were insufficient to meet the demand

(15). Death rates in the UK among patients with severe haemophilia rose from 8 per 1,000 during 1977-84 to peak at 38 per 1,000 by 1991-92, with the increase confined to patients who tested seropositive for HIV, where the death rate peaked at 81 per 1,000 (16). HIV infection in haemophilic patients in the US was majorly reduced in 1985 following introduction of heat-treated factor concentrates (12), but there was a subsequent case report in 1986 of HIV seroconversion in a previously intreated individual with mild HA who had received high doses of heat-treated FVIII concentrate (17). The demonstration that heat-treatment may not be fully effective in eliminating viral-transmission gave further impetus to the development of recombinant (rFVIII) treatment (11). There was, however, continued improvement in heat treatment protocols for plasma-derived products, plus the addition of solvent-detergent treatments which were highly efficacious in inactivating hepatitis B and C virus and HIV (18).

2.3 Relationship between anti-haemophiliac factor and von Willebrand factor

In 1971 it was demonstrated by Zimmerman and colleagues that a rabbit antibody raised to AHF detected normal levels of antigen in 12 of 13 individuals with HA, and reduced levels in plasma from 11 individuals with von Willebrand Disease (VWD) (19). The material was initially called FVIII-related antigen and now known to be von Willebrand factor (VWF). The authors suggested that VWD may be a disease in which true deficiency of AHF exists and speculated as to whether the AHF-like material in classic haemophilia is non-functional through a defect in structure or due to presence of an inhibitor. Two years later Arthur Bloom and colleagues proposed that a high molecular weight molecule of autosomal inheritance produced the VWF activity, and which is complexed to a smaller molecule with X-linked inheritance that produces the anti-haemophiliac factor activity (FVIII) (20). In 1979 Tuddenham was able to separate

FVIII and demonstrate it retained full coagulant function in the absence of VWF (21). The understanding that FVIII and VWF were separate proteins under separate genetic control laid the early foundation for production of recombinant FVIII (rFVIII), which could then be infused into individuals with HA and be expected to bind to that individual's VWF. The VWF molecule itself acts as a carrier molecule for FVIII, protecting FVIII from otherwise rapid degradation, but FVIII is still subject to dominant VWF-dependent clearance, with VWF having a half-life of ~15 hours (22).

2.4 FVIII gene cloning and the first recombinant FVIII

The discovery that FVIII and VWF were separate proteins was followed by the cloning of the FVIII gene and led to the successful use of rFVIII replacement therapy. The FVIII genetic map analysis was published in *Nature* in 1984 by Gitschier et al., showing it comprised 186 kb and 26 exons, making it the largest gene to be characterized at the time (23). DNA clones were then used to produce biologically active FVIII in cultured mammalian cells, with the FVIII shown to shorten the clotting time of haemophilic plasma and be capable of binding to VWF (24). In 1989 two adult patients with severe HA were successfully treated for up to 12 months with a rFVIII product that showed similar efficacy to plasma-derived FVIII (25). Neither patient developed the serious complication of antibodies to rFVIII which was attributed to the rFVIII being structurally identical to plasma-derived FVIII. This small study supported the continuation of clinical trials with rFVIII. Two first two rFVIII products, Recombinate and Kogenate, were undergoing clinical trials simultaneously from the late 1980s. Recombinate became the first rFVIII approved by the FDA for use in treating HA (26). Risk of inhibitor development was of major concern with the introduction rFVIII therapies and was a major focus of clinical studies (27). A multi-centre trial of 95

previously untreated children with severe (defined as <2% FVIII), moderate, or mild haemophilia A was conducted between 1989 and 1992 with the rFVIII, Kogenate (28). The product was well tolerated and response to treatment was excellent. Inhibitors to FVIII developed in 16 patients, eight with titres of <10 Bethesda units and eight with >10 Bethesda units (28). Another study of previously untreated patients (PUPs) with rFVIII (Recombinate) found the product to be well tolerated, with a high correlation between predicted and actual plasma levels measured by the one-stage clotting assay (OSA) (29). A cumulative inhibitor incidence of 23.9% among the 75 patients who had received at least one dose of rFVIII, which was considered comparable to inhibitor incidence in contemporary prospective studies of inhibitor incidence after infusion of plasma-derived FVIII.

2.5 FIX gene cloning and the first recombinant FIX

The first FIX gene clones were isolated in 1982 (30) and the complete nucleotide sequence reported in 1984 (31). As with FVIII gene cloning, the FIX gene cloning formed the basis of the development of recombinant FIX (rFIX) replacement products, capable of eliminating the risks of hepatitis and HIV infection associated with the use of plasma-derived FIX. The first rFIX product licensed for use to treat HB was Benefix in 1997, with post marketing surveillance studies further characterising Benefix as safe and effective (32). A second rFIX replacement product for treatment and prophylaxis in HB, Rixubis, was licensed in 2013, and was shown to have similar pharmacokinetic properties to Benefix (33). Both products are currently used for prophylaxis and treatment of bleeding episodes in HB.

2.6 Development of modified and extended half-life replacement therapies

Modified and unmodified rFVIII and rFIX products are summarized in Tables 1 and 2, respectively. The first rFVIII and rFIX concentrates had amino acid sequences copied exactly from plasma derived FVIII and FIX. Once it had been shown that the risk of increased inhibitor development by use of recombinant products was no greater than that of pdFVIII and pdFIX, attention turned to the production of replacement products that could be made more efficiently, and/or had post-infusion half-lives greater than full length recombinant products. The extended half-life (EHL) products were expected to improve quality of life of recipients on prophylaxis due to the requirement for fewer intra-venous injections to maintain factor levels above that needed to prevent spontaneous bleeds. Some rFVIII products have been modified by removal of most of the B-domain of FVIII, made possible after recognition that FVIII activity is retained after its removal (34). These are known as B-domain deleted (BDD) products, and both standard half-life (SHL) and EHL forms exist. For FVIII, three main types of modification have been employed to extend product half-life by retarding clearance. One approach is PEGylation, where polyethylene glycol (PEG) chains are added to the FVIII molecule, as applied to Adynovate, Jivi and Esperoct (35-37). A second approach is fusing the fragment crystallizable (Fc) to FVIII, as applied to Eloctate (38). The third approach, used in ALTUVIIIO, is to use multiple modifications in a single molecule, including Fc fusion and FVIII-binding to the D′D3 domain of VWF, the D′D3 binding designed to allow the molecule to circulate in plasma independently of VWF, thereby breaking the VWF half-life ceiling (39). For FIX, modifications are PEGylation as used for Refixia, Fc fusion used for Alprolix, and albumin fusion used for Idelvion (40-42). A major consideration in using these modifications to extend product half-life is the potential impact they may have on laboratory assays used to measure FVIII or FIX

levels in post-infusion samples. Some of the modifications have indeed been shown to cause significant over- or under-estimation in plasma levels of FVIII and FIX by the available laboratory assays, which has created uncertainty as to which assay to use to measure each product (43-46). Accurately measuring these FVIII and FIX products is critical to achieve factor levels that can effectively maintain hemostasis, while preventing the rare but potential thrombosis when over-dosed with product (47). Hence there is need for understanding product-methodology interactions at the clinical laboratory level to ensure clinically useful factor levels. Importantly, there are chromogenic substrate assays (CSA) for both FVIII and FIX, which can complement clotting-based assays, thus providing a necessary alternate assay system for measuring some products.

Table 1. Factor VIII replacement products. Information from references: (43, 45, 48, 49).

Brand name / drug name	**Product type**	**Type**	**Mean half-life (h)**	**Company**	**Suitable reagents for measurement**	**Not recomm-ended**
Biostate	Plasma derived	SHL		CSL Behring	OSA CSA	
Advate / octocog alfa	Recombinant, full length	SHL		Shire	OSA CSA	
Xyntha / moroctocog alfa	Recombinant, BDD	SHL		Pfizer	OSA	CSA
ReFacto / moroctocog alfa	Recombinant, BDD	SHL		Pfizer	CSA, OSA (using ReFacto Standard)	
Adynovate / rurioctocog alfa pegol	Recombinant, full length, PEGylated	EHL	14.3	Shire	OSA, CSA	
Eloctate / efmoroctocog alfa	Recombinant, BDD, IgG_1Fc fusion	EHL	18.8	Bioverat-ive	OSA, CSA	
Jivi / damoctocog alfa Pegol	Recombinant, BDD-PEGylated	EHL	18.2	Bayer	Actin FSL, Pathromtin, Synthasil, CSA	Actin FS, CK Prest, APTT-S, PTT-A
Afstyla / lonoctocog alfa	Recombinant, BDD, single chain	EHL	14-16	CSL Behring	CSA only	OSA
Esperoct / Turoctocog alfa pegol	Recombinant, BDD-PEGylated	EHL	19	Novo Nordisk	Actin, Actin FS, Pathromtin	APTT-SP, PTT-A
ALTUVIIIO / Efanesoctocog alfa	Recombinant, BDD-Fc-VWF-XTEN	EHL	42	Sanofi / Sobi	Actin FSL, some others	CSA, several OSA reagents

SHL, Standard half-life; EHL, Extended half-life; OSA, one-stage assay; CSA, chromogenic substrate assay

Table 2. Factor IX replacement products. Information from references: (43, 45)

Brand name / drug name	Product type	Type	Mean half-life adults (h)	Company	Suitable reagents for measurement	Not recomm-ended
Benefix / nonacog alfa	Recombinant	SHL		CSL Behring	OSA CSA	
Rixubis / nonacog gamma	Recombinant	SHL		Shire	OSA CSA	
Refixia / nonacog beta pegol	Recombinant PEGylated	EHL	93-96	Novo Nordisk	CSA, SynthaFax	Most OSA reagents
Alprolix / eftrenonacog alfa	Recombinant FIX-IgG_1Fc fusion	EHL	56.7	Pfizer	CSA, OSA	Kaolin OSA
Idelvion / albutrepenon-acog alfa	Recombinant Albumin fusion FIX-Fp	EHL	91.6	CSL Behring	Pathromtin	CSA, SynthaFax Actin FSL, Actin FS

SHL, Standard half-life; EHL, Extended half-life; OSA, one-stage assay; CSA, chromogenic substrate assay

2.7 Development of the bi-specific antibody emicizumab, a non-factor replacement therapy for haemophilia A

Coinciding with development of EHL rFVIII products over the past 10 years was the emergence of the bi-specific antibody emicizumab, first demonstrated to be efficacious against ongoing bleeds in a non-human primate model (50). Subsequent clinical trials in humans showed markedly reduced annual bleeding rates in haemophilics both with and without inhibitors (51, 52). The advantages of emicizumab over FVIII replacement include sub-cutaneous dosing and long half-life of around 30 days. Emicizumab simultaneously binds FIXa and FX leading to FXa generation (53). This FVIIIa-like

activity of emicizumab in plasma leads to marked shortening of the activated partial thromboplastin time, and to a marked overestimation of FVIII and other intrinsic pathway factor levels when measured by OSA (54). For this reason, FVIII assays and FVIII inhibitor titres must be performed by bovine-component chromogenic FVIII assays, which do not cross-react with emicizumab (55). Measurement of emicizumab itself can be made using FVIII-deficient substrate plasma spiked with emicizumab as a calibrator (56). This means introduction of a new assay into clinical haemostasis laboratories, for which a requirement exists for a multi-centre study to ensure assay validation.

3. Assays used for the diagnosis of haemophilia and monitoring of FVIII and FIX replacement therapies and some non-factor replacement therapies.

3.1 Development of the activated partial thromboplastin time

The activated partial thromboplastin time (APTT) is an assay where the time to clot formation of citrate-anticoagulated platelet-poor plasma is measured in seconds (57). The APTT has been considered a global assay of haemostasis because it requires the presence of multiple clotting factors at relatively normal levels to be present to ensure clot formation in a 'normal' time. The clotting factors required for clot formation measurable by the APTT constitute the 'Intrinsic Pathway' or 'Contact Factor Pathway' shown in Figure 1. The end point of the APTT is conversion of soluble fibrinogen to insoluble fibrin stands by the enzymic action thrombin. Deficiencies of one or more clotting factors within the intrinsic pathway, including FVIII or FIX, can result in delayed thrombin formation, hence delayed fibrin formation manifesting as a prolonged

APTT. An adaptation of the APTT is used for measurement FVIII and FIX levels using specific clotting factors OSA.

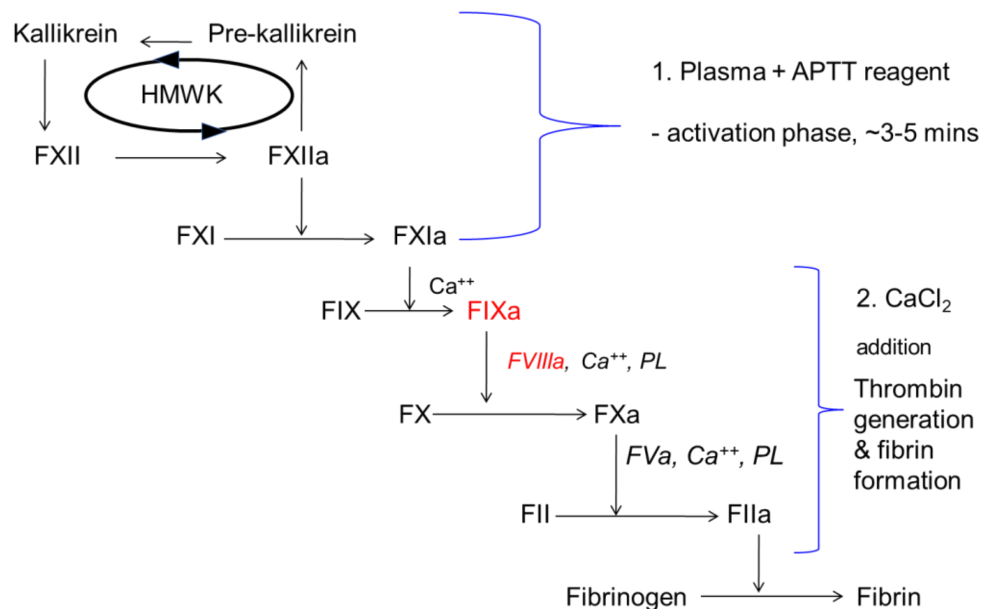

Figure 1. Schematic of the intrinsic pathway of coagulation showing the location of the activated forms of FVIII and FIX (in red). The activation phase brings clotting factors to a uniform level of activation to FXIa. The addition of calcium chloride allows the cascade to go to completion with the formation of a fibrin clot.

The basis of the current APTT and OSA was work by Langdell, Wagner and Brinkhous (58), who described a technique to both diagnose haemophilia and measure AHF levels. Their assay was named the partial thromboplastin time (PTT). The PTT was a presumptive test for the diagnosis of haemophilia, based on the observation that in haemophilic plasma the prothrombin time, using 'complete' thromboplastins, is normal whereas PTT was prolonged. The PTT technique involved incubation platelet poor citrated test plasma with partial thromboplastin in glass tubes, incubating at 37°C for a few minutes. The mixture was re-calcified with time to clot formation measured using a tilt-tube technique and a stopwatch. The 'partial thromboplastin' the authors used had been prepared from ultracentrifugation of rabbit brain extract, which essentially removed most of the thromboplastin, hence eliminating reactivity with factor VII. The

PTT did have some technical difficulties, including variable results according to whether new or re-washed glass tubes were used, and the washing method itself. In 1958, Margolis described the kaolin clotting time (KCT) as a method of detecting various clotting factor deficiencies (59). Unlike the PTT, the KCT did not use an exogenous source of phospholipid, or partial thromboplastin, but did employ pre-activation of test plasma with kaolin before the re-calcification step. This internal activator made the assay independent of tube size and reagent volumes, reducing the variability of the PTT seen with uncontrolled contact factor activation. It was Pollock and Proctor, who in 1961 published a method combining both the partial thromboplastin of the PTT with the kaolin activation of Margolis' KCT into a single test system known as the partial thromboplastin time with kaolin, or PTTK, the first version of an 'activated' PTT, or APTT (60). The method allowed for the creation of a narrower, shorter and more predictable reference interval created from testing normal plasma samples, which proved to be around 30 to 45 seconds. This allowed for improved screening of patient plasma for clotting factor deficiencies, and adaptation to a more reliable method of measurement of individual plasma clotting factors levels when adapted to the OSA. The original PTT had a disconcerting wide normal range depending on the type of glass tubes used, rendering the assay unsuitable for more general us in clinical laboratories (61). Over time, these developments led to more widespread use of the APTT test as a screening test for factor deficiencies, and for measuring intrinsic pathway factor levels in diagnostic laboratories. In subsequent decades reagent manufacturers introduced many commercial APTT reagents for use in those laboratories. When used in standardised automated/semi-automated protocols, in conjunction with quality management system, these reagents behave reliably and predictably. The standardised test set up for APTT consists of adding to a cuvette equal

volumes of test plasma and APTT reagent, incubating the mixture at 37°C for a fixed time interval of a few minutes to allow for contact factor activation, followed by addition of one volume of pre-warmed $CaCl_2$. The reference intervals for APTT reagents typically occupy a window within the 20–45 s region, for example, 25– 37 s, depending on the analyser/APTT reagent combination (62). For plasma severely deficient in FVIII or FIX the APTT can extend to more than 100 seconds, and this may be the first laboratory observation made at the time of diagnosis of severe HA or HB.

3.2 Activated Partial Thromboplastin Time reagents

Normal practice internationally is for laboratories to purchase APTT reagents from one or more reagent suppliers. There are currently around 40 different APTT reagents commercially available. Individual APTT reagents composition varies between manufacturers, and between different reagents produced by a single manufacturer. The reagents can vary according to the type of activator, buffering, phospholipid source and concentration. Characteristics of different APTT reagents are summarised in Table 3. Reagent composition affects the response of the reagent to three areas of clinical interest in test plasma samples: factor deficiency sensitivity, lupus anticoagulant sensitivity and heparin sensitivity (62). In the context of haemophilia testing, all APTT reagents should ideally have sufficient sensitivity to deficiencies of FVIII and FIX to mild deficiencies of FVIII and FIX, but analysis of individual reagents show this is not always the case (63). Furthermore, adaptation of the APTT standard protocol to an OSA should allow accurate quantification of FVIII and FIX levels in diagnostic samples ranging from severely deficient to levels seen at diagnosis. The APTT reagent choice has practical implications for measuring some FVIII and FIX replacement products, as some EHL

molecules react differently with different reagents to the extent that recovery rates are overestimated or underestimated to unacceptable degrees.

Table 3. Characteristics of some APTT reagents used in haemostasis testing for sample screening and for one-stage clotting assays. Activator and phospholipid information was obtained from the individual product inserts.

APTT reagent	**Manufacturer**	**Activator**	**Source of Phospholipid**	**$CaCl_2$ concentration**
Actin	Siemens	Ellagic acid	Rabbit	0.025M
Actin FS	Siemens	Ellagic acid	Soy	0.025M
Actin FSL	Siemens	Ellagic acid	Soy, chicken	0.025M
Pathromtin	Siemens	Silica	Soy	0.025M
Cephascreen	Diagnostica Stago	Polyphenols	Rabbit	0.025M
CK Prest	Diagnostica Stago	Kaolin	Rabbit	0.025M
PTT A	Diagnostica Stago	Silica	Rabbit	0.025M
Triniclot aPTT S	Diagnostica Stago	Silica	Porcine, chicken	0.020M
PTT LA	Diagnostica Stago	Silica	Rabbit	0.025M
Synthasil	Werfen	Silica	Synthetic	0.020M
Synthafax	Werfen	Ellagic acid	Synthetic	0.020M
APTT SP	Werfen	Silica	Synthetic	0.025M

Cephen 5	Hyphen Biomed	Silica	Soy	0.025M
Cephen LS	Hyphen Biomed	Silica	Soy and synthetic	0.025M

3.3 Coagulation clotting end-point detection systems

The clotting end-point detection systems differ between coagulation analysers, which potentially adds to assay variability between systems in both APTT clotting times and in APTT-based OSA for clotting factors including FVIII and FIX. The two dominant systems used by laboratories analysers are the optical-based methods and viscosity-based methods. In both cases, the clotting endpoint occurs when sufficient thrombin has been generated to convert soluble fibrinogen to insoluble fibrin. In optical systems, the appearance in the test cuvette of fibrin strands causes an increase in opacity of the plasma-reagent mixture, as measured by an increase in absorbance, or a decrease in light transmission, of a single wavelength of light passed through the cuvette. Readings are typically taken at 0.1 second intervals. This optical data can be presented graphically as waveforms, including the primary clot wave, and two mathematically determined derivative waves. The Sysmex system takes the time at which 50% of the total optical density change occurs within the primary clot wave, which is typically near the centre of the steep change in the primary clot wave. The Werfen system takes the time of the second derivative peak as the clotting endpoint, which is always towards the start of the primary clot wave. While both systems are essentially measuring fibrin formation optically, the different algorithms potentially add to the variability between analysers in measuring both APTT and clotting factor levels by OSA. In mechanical or viscosity-based clot detection systems, when plasma clots a change in viscosity of the liquid

happens as the first strands appear. Placement of a metal ball in the cuvette which oscillates back and forth under the influence of a magnetic field allows the clot endpoint to be detected when the amplitude of the ball movement is reduced below a pre-defined limit. Both optical-based and viscosity-based clot detection systems are utilized in many diagnostic laboratories, including those participation in the field studies forming part of this work.

3.4 Calibration standards for FVIII and FIX assays

The need for a standard for FVIII was recognized during the 1960s with first cryoprecipitate, then intermediate purity FVIII concentrates used for treatment (64). The first IS for FVIII was created in 1971 (65). Most testing laboratories calibrate their coagulation analysers with commercial calibration plasma presented in lyophilized form and for which FVIII and FIX value assignment is traceable to International Standards (IS). Current IS are assigned a value in IU after multi-centre international collaborative studies (4). ISs are primary reference preparations, not intended for routine daily use in laboratories. The International Society on Thrombosis and Haemostasis (ISTH) Scientific and Standardization Subcommittee (SSC) produces a secondary coagulation standard plasma (SSC Plasma). This secondary standard is value-assigned relative to the IS, and is used by vitro diagnostic manufacturers to calibrate commercial plasma calibrators used by clinical laboratories to calibrate their analysers for FVIII and FIX measurements (4).

3.5 Factor VIII and Factor IX assays by one-stage clotting method

Timely and accurate measurement of FVIII and FIX by OSA is fundamental to haemophilia diagnosis and successful management of factor replacement therapy in individuals with HA or HB. The current automated assays are modifications of the original parallel line bioassay of the PTT and the PTTK (58, 60). The assay's components are suitable sample diluent, an APTT reagent, such as those listed in Table 3, a source of single factor deficient plasma, deficient in either FVIII or FIX, and calcium chloride solution as the clot initiation reagent. Clinical requirements for the OSA in a diagnostic setting are satisfied if the assay can measure factor levels as low as 1 IU/dL and distinguish between 1 IU/dL and < 1 IU/dL. By premixing the factor deficient plasma with diluted test plasma of unknown factor levels, the degree of shortening in the clotting time of the factor deficient plasma will be proportional to the level of factor in the diluted test plasma. The final factor level is determined from a calibration line constructed from serial dilutions of a calibration plasma tested under the same conditions.

3.6 Factor VIII assay by chromogenic substrate method

The CSA was first developed in the 1980s and uses purified components of the coagulation pathway, and removes the need fibrin clot formation (47). The automated chromogenic substrate assay for FVIII measures uses colour formation as a marker for the quantity of FVIII in the test sample. The steps used in the assay are shown in Figure 5. The method focusses on the central portion of the intrinsic pathway. In the first of

two stages, Diluted test plasma is mixed and incubated with activated FIX (FIXa) and factor X (FX) in the presence of thrombin, calcium ions and phospholipids. FVIII in the test sample is converted to its active form (FVIIIa) by the action of thrombin. The FVIIIa links FIXa and FX on a phospholipid surface leading to the creation of activated FX (FXa). After a suitable incubation period, a chromogenic substrate for FXa consisting of a small peptide linked to para-nitroanaline (pNA) is added to the mixture. Free pNA is released and the amount of colour formed between two fixed time points is read at 405nm. The assay can use the same calibration plasma as for OSA, provided a WHO-referenced assigned value for the chromogenic method is available. A commercial plasma calibrator that has an assigned value for CSA must therefore be used. There are at least six different CSA kits available for purchase for the purpose of measuring clinical samples. Some of these assays are affiliated with instrument manufacturers, others not, but in general any CSA kit can be adapted to different analysers. Some applications for FVIII CSA require both 'high' and a 'low' calibration curves to achieve better accuracy at low levels.

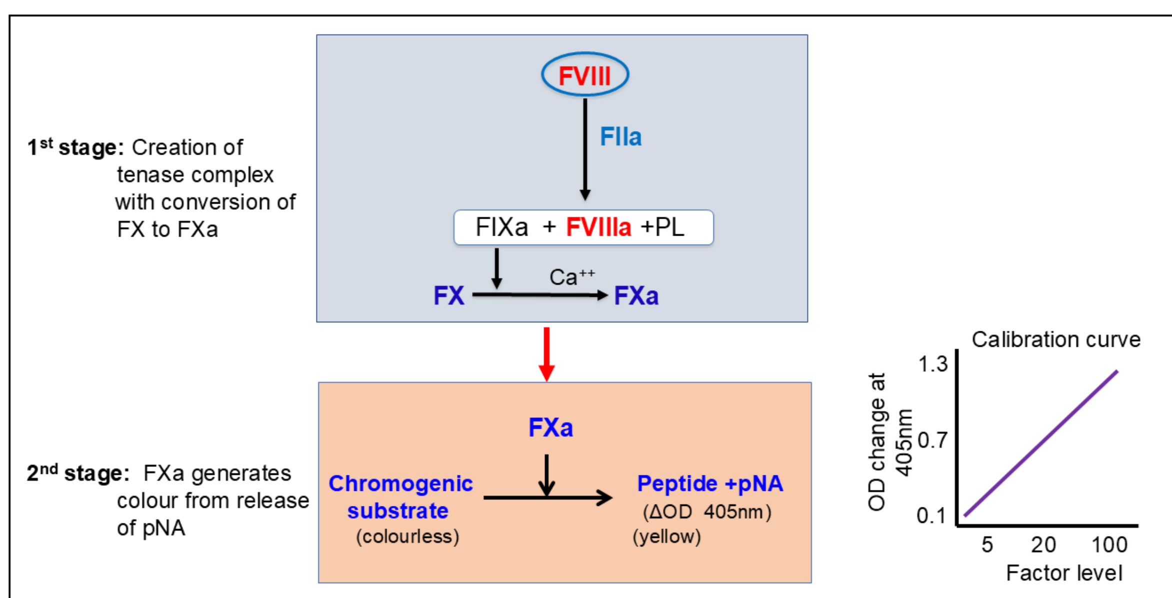

Figure 2. Schematic of the chromogenic FVIII assay. All assay components except FVIII are sourced from the test kit vials. In the first stage thrombin activates FVIII to create the tenase complex which then converts FX to FXa. In the second stage, the FXa releases para nitroanaline (pNA) from a peptide, generating a yellow colour at a rate directly proportional to the amount of FVIII in the test plasma.

3.7 Factor IX assay by chromogenic substrate method (FIX:Cr)(CSA)

There are two main commercial suppliers of FIX chromogenic kits, the Hyphen Biomed and Rossix tests (Figure 3). The FIX CSA resembles the FVIII CSA in that it targets the central part of the intrinsic pathway and is a two-stage test (Figure 4). Also, like its FVIII counterpart, it provides an additional option for measuring some FIX EHL products not measurable by FIX OSA with some APTT reagents. The assay requires conversion of plasma FIX to FIXa by the action of activated FXI (FXIa). The kits differ slightly in how they measure FIX, mainly related to the process FVIII activation by thrombin. Both versions of the assay can be automated on different platforms. The same plasma calibrators are used for FIX CSA as for FVIII CSA, with OSA FIX calibrator value used as the CSA value. FIX CSA may require both ‘high’ and ‘low’ calibration curves to maintain accuracy at all levels.

Biophen FIX Cr	Rossix FIX Cr
R1 FX, FVIII, fibrin inhibitor	RA: FX, FVIII, FV(bovine), fibrin inhibitor
R2 FXIa, FIIa, PL, Ca	RB: FXIa, FII, PL, Ca
R3 FXa substrate	Sub: FXa Substrate, IIa inhibitor
R4 Tris-BSA Buffer	Diluent: Buffer with heparin antagonist

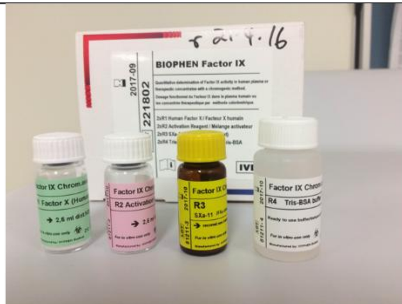

Figure 3. Reagent composition of two commercial chromogenic FIX assays. The tests differ in the components highlighted in red text. In the Biophen test FVIII in reagent 1 (R1) is activated directly by FIIa in reagent 2 (R2). In the Rossix test, FV in reagent A (RA) converts FII in reagent B (RB) to FIIa, which then activates FVIII.

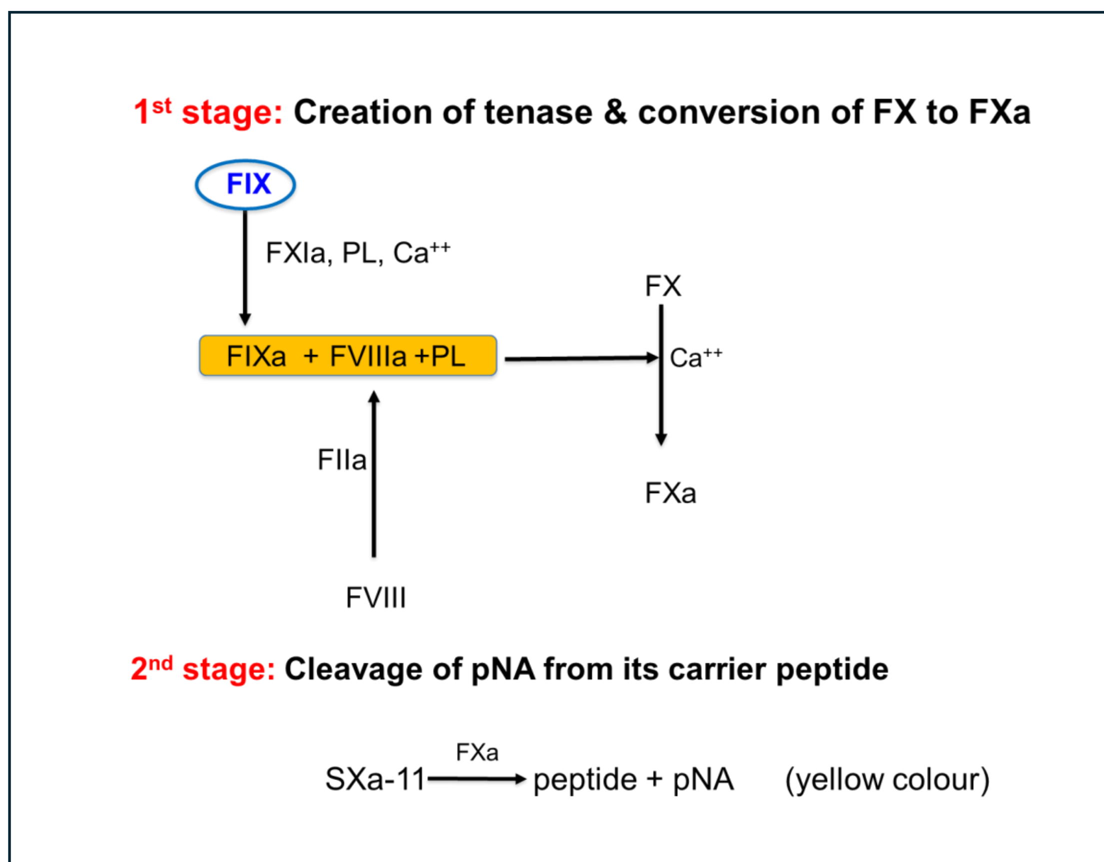

Figure 4. Schematic of the chromogenic FIX assay. In the first stage FVIII is converted to FVIIIa by the action of FIIa. FIX from the test sample is converted to FIXa under the action of FXIa. The IXa then converts FX to FXa. In the second stage the FXa cleaves free pNA from its carrier producing a yellow colour measured at 405nm.

3.8 Emicizumab assays

Assays for the FVIII mimetic emicizumab are mainly modifications of the standard OSA for FVIII in a test system that does not contain FVIII (66). The FVIII CSA can also be used to measure emicizumab in plasma provided the test kit contains both human FX and human FIXa (66). Most CSA kits contain at least one bovine component, usually bovine FX, which renders the kits insensitive to emicizumab making these kits unsuitable for assaying the drug, but suitable for assaying FVIII levels in the presence of the drug. The standard OSA protocol for FVIII requires some modifications to make it suitable for emicizumab assays. Firstly, the test sample standard predilution in buffer must be increased from 1/10 to either 1/40 or 1/80 because emicizumab action in binding FX to FIXa is immediate, not requiring pre-activation as would FVIII in the same test system. The increased dilution increases the clotting times to a more workable range in seconds for construction of the calibration curve. The second modification is the use of product-specific calibrators and control plasma comprising FVIII deficient plasma spiked with known concentrations of emicizumab. Assays for emicizumab will be subject to interference in the presence of FVIII in the same sample, for which different strategies have been investigated to correct for the overestimation of emicizumab by the presence of FVIII (67).

3.9 FVIII inhibitors and their measurement by the Bethesda method

FVIII inhibitors are IgG antibodies that bind to and neutralize the effectiveness of therapeutically infused FVIII. While FVIII antibodies may arise as an immune response to infused FVIII treatment products in individuals with congenital HA (68), or they can arise as autoantibodies in individuals without a bleeding history, leading to the condition of acquired haemophilia A (AHA) (69). The development of FVIII inhibitors is a major complication of treatment with replacement products (70). A 1999 review of inhibitor development by Scharrer et al. concluded that inhibitors develop in around 30% of severe HA cases, and the risk of inhibitor development was no different between recombinant and plasma-derived treatment products (71). The recombinant products considered in these studies were the first two developed, Recombinate® and Kogenate®, both full-length FVIII molecules matching the structure of wild type FVIII. The Bethesda method for FVIII inhibitor titres is designed to detect the presence of anti-FVIII antibodies in the plasma of individuals with HA (72, 73). A standardised Bethesda method of measuring FVIII inhibitor titre strength was published by Kasper et al in 1975 (74), and modifications introduced by Verbruggen et al to improve specificity at lower titres (72). The Bethesda method requires mixing dilutions of test plasma in equal volume with pooled normal plasma which serves as a source of FVIII at ~100 IU/dL. After two hours incubation in a 37°C the FVIII level in each tube is measured by either OSA or CSA (Figure 5). If the residual FVIII is 70% or lower than an inhibitor has been detected, where a 50% residual defines 1.0 Bethesda unit (BU). The exact titre is calculated mathematically or graphically.

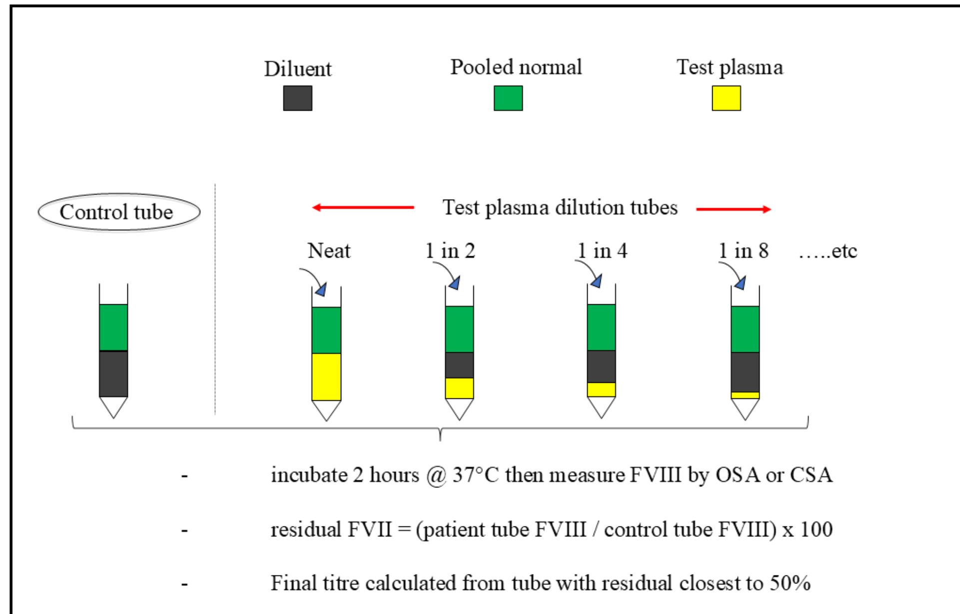

Figure 5. Schematic of the Bethesda assay for FVIII inhibitors. Serial dilutions of test plasma are made in a suitable diluent then mixed with an equal volume of pooled normal plasma. Comparison of the FVIII remaining in each tube with a control tube FVIII allows calculation of the final inhibitor titre.

The introduction of rFVIII therapies including EHL has not diminished the need for inhibitor screening as antibodies can cross-react with replacement products or even arise consequent to a product switch (68). The introduction of emicizumab has assisted in management of persons with inhibitors, since the anti-FVIII do not bind to emicizumab. Determining the FVIII inhibitor status is still needed in CHA treated with emicizumab in case additional FVIII infusions are needed. In AHA, inhibitor titres are needed to monitor the disappearance of an inhibitor during immunosuppressive therapy. Protocols have been successfully developed using emicizumab to treat AHA (75), which consequently renders clotting assays unusable for Bethesda assays to monitor FVIII inhibitor strength. Measurement of residual FVIII in the presence of emicizumab requires a FVIII CSA that contains bovine FX, or both bovine FX and FIXa components, to prevent cross-reactivity with emicizumab (76). Both the FVIII OSA and CSA can be used with the standard test protocols in Bethesda assays in the absence

of emicizumab. Validation of the bovine CSA for use in Bethesda assays in samples containing emicizumab is required to ensure result accuracy, since the CSA assay is relatively new for this application, Bethesda assays previously being performed exclusively by OSA. As with the OSA for emicizumab, a bovine chromogenic FVIII assays as part of a Bethesda assay is new to many laboratories, who would benefit from a multi-centre study to validate their procedures.

3.10 Multi-laboratory field studies

Field studies in haemophilia testing have become increasingly important in the past decade to the introduction of new therapies, and the associated requirement for laboratories to measure these products. The first major published field study for a rFVIII EHL was by Sommer et al in 2014 for the measurement of Eloctate (77). There have since been several more comparative studies published for different FVIII and FIX EHLs, partly driven by an ISTH SSC recommendation that manufacturers supply guidance to clinical laboratories as to which assays types/reagents can be used for measuring post-infusion recoveries of their products (78). In Australia, the FVIII products include the EHLs Eloctate and Adynovate, plus the FVIII mimetic emicizumab. A major part of the current work was to conduct such field studies of measurement of factor or non-factor replacement therapies among Australian haemophilia-associated reference laboratories, and other specialized haemostasis laboratories. These studies are important as they provided the testing laboratories exposure to a hitherto not seen product and/or allow validation of their testing methods for these products. External quality assurance programs for measuring some EHL FVIII therapies are either not available or not available locally. An example schema for

conducting field studies is shown in Figure 6. The principle is to issue de-identified frozen vials of product to multiple laboratories, request measurement of the product under controlled conditions, then return the results to the issuer for collation and summarizing. The requirements for a successful field study are many, as summarised below:

1. Identification of the treatment product that needs to be measured. This will often include FVIII or FIX replacement products that have been newly introduced either in a clinical trial formal, or more often been recently licensed for use in Australia and will require laboratory measurement. Some of these products may have some existing company-sponsored study data available on their measurement characteristics, and others not.
2. Establish a source of the product used for the field study. This is usually achieved by requesting the manufacturer to supply vials of clinical material of known concentration from which spiked test samples can be prepared.
3. Obtain a source of diluent for the product to be spiked into. This is typically normal plasma deficient in the specific factor to be measured, FVIII or FIX in this case. Large batches of a single lot are important to ensure consistency of sample content between the testing laboratories. For emicizumab test sample preparation the diluent needs to be FVIII deficient plasma. The spike calculations require careful consideration, usually requiring lot-specific potency information from the supplier. This is critical as lot-specific values can differ by up to 10% from the label value stated on the product packaging. Multiple spike levels need to be prepared to cover the clinically important range.
4. Ultra-low freezer storage facilities to hold samples before shipment, and an established courier network for distribution of samples.

5. Documentation includes invitations to participate, testing instructions, result sheets, method questionnaires, and contact information for follow up communication with participants. Testing instructions sometimes need to be more detailed than normal, as in the case of emicizumab assays and chromogenic FIX assays, where very few sites had previously used these assays.
6. Collation and analysis of results for report writing. The report provides feedback for the participants who can use the field study report as part of their in-house method validation process.

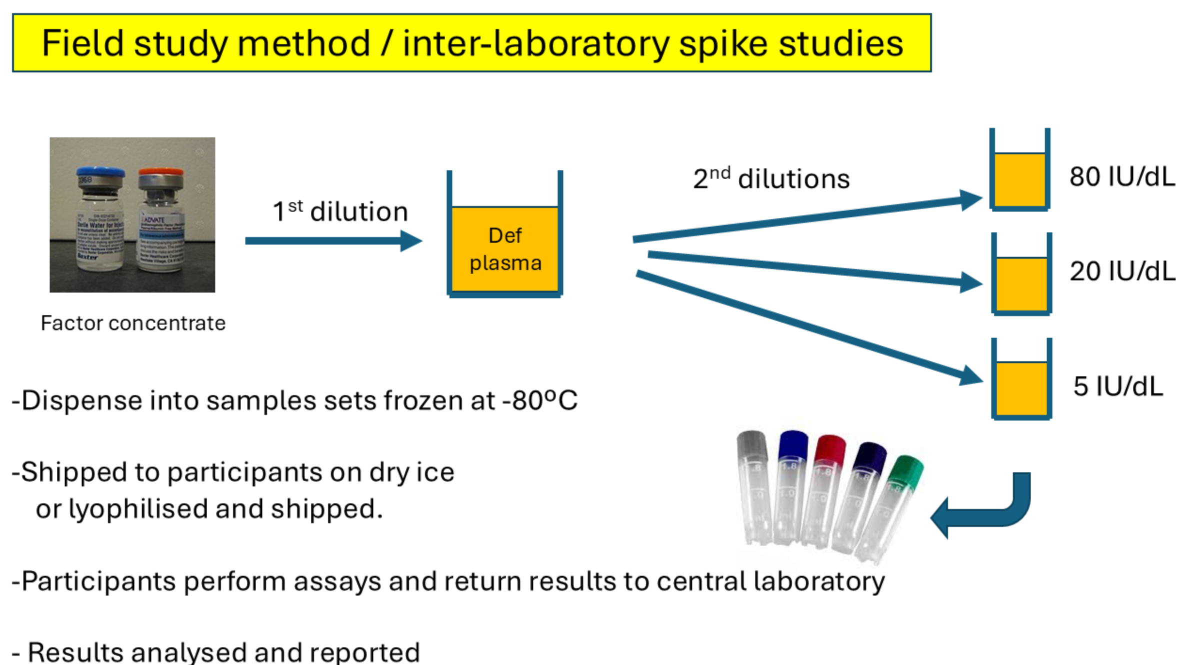

Figure 6. Schematic of a field study for measurement of replacement products. The product is spiked into factor deficient substrate plasma in two steps to reach final concentrations, which are then dispensed into to cryovials before freezing and distributing to participants.

4. The impact of the rFVIII and rFIX modifications on laboratory measurement of factor levels.

When either plasma-derived, or full length unmodified rFVIII or rFIX replacement products are used in haemophilia treatment, measurement in post-infusion plasma samples generates reasonable agreement between OSA and CSA, or between the different APTT reagents used for OSA. One area where diagnostically important differences exist between OSA and CSA is in non-severe haemophilia, where large differences between assay types have been noted, and related to specific gene mutations (79). The modifications to rFVIII and rFIX molecules that allow for extended half-lives can have unpredictable consequences on the accuracy of OSA and/or CSA in the measurement of those products in post-infusion samples. When new EHL rFVIII or rFIX factor replacement products are introduced into a country or geographical area the haemostasis laboratory must determine whether their existing assay with plasma standards will suffice for product measurement, or whether alternate strategies are required. This information is mostly sourced from published field studies where they exist and following published recommendations (1, 43, 45, 80). Example recommendations are shown in Tables 1 and 2. Some limitations of field studies are that not all APTT reagents are represented in each study, due to the large number of different reagents in use. More than one field study for any given product is justifiable, to fill in the gaps in APTT reagents assessed, and to confirm the original study's findings. There are situations where repeat field studies for the same product have sufficiently contradictory outcomes to prompt the need for more studies, as seen with the rFVIII Adynovate (81-83). The suitability of APTT reagents for measuring is sometimes correlated to activator type. For FIX-Fc (Alprolix), reagents with ellagic acid activators gave slightly higher recoveries the silica-bases reagent, but both are deemed acceptable, in contrast to kaolin-based APTT reagents, which significantly underestimate FIX levels (41). For other products, such as N8-GP (Esperoct) the silica-

based APTT reagent Pathromtin give recoveries close to target whereas the silica-based APTT-SP majorly under-recover (84). Furthermore, the study found the same APTT reagent, Synthasil, gave significantly different recoveries testing the same samples on different analyser types. These studies reinforce the need for individual laboratories to access local field studies and/or external quality assurance programs which include the same rFVIII and rFIX product types they are expected to measure. When a laboratory's current factor assay is deemed unsuitable for measuring one or more replacement products in several alternative approaches can be considered to ensure accurate post-infusion measurement (85), as briefly described in the following sections, 4.1 to 4.4.

4.1 Product-specific reference standards

In principle, a product-specific calibrator would allow laboratories to measure a replacement product by their existing APTT reagent for OSA or their current CSA, since any variable reactivity with specific reagents would be automatically corrected for. A discussion of the relative merits of product-specific calibrators from a WHO meeting was published in 2020 (86). Benefits included the application of the 'like versus like' principle to better harmonise assays used by different clinical laboratories, with generation of results in IU correlated to patient outcomes in pre-licensure clinical trials. Potential cons would be the need for the laboratory to maintain multiple reference materials (calibrators), the need for all stakeholders to 'buy in' to the process, and a mechanism to make reference material widely available to clinical laboratories (86). A precedent for the use of a product-specific calibrator came with the introduction of Refacto AF, a BDD rFVIII replacement product. Large discrepancies had been observed in FVIII measurements between OSA and CSA using standard plasma calibrators, which was resolved with the introduction of a product-specific calibrator in Europe

(87). In Australia, the same product, under the name Xyntha, was potency assigned by OSA, and is measured by OSA without a product-specific calibrator. At present the ready availability of product-specific calibrators seems limited to the ReFacto standard, and standards for the non-factor replacement product emicizumab.

4.2 Use of correction factors

A correction factor is either a constant or an equation that is applied to a factor assay result to bring the result into line with the result expected if the original potency assignment method was used for measurement. For example, the manufacturer of the single chain BDD rFVIII (Afstyla) has recommended use of CSA for measurement, or use of OSA provided a correction factor of 2 is applied to the results (88). This approach to OSA measurement has been questioned, particularly measuring trough levels, due to variability between different APTT reagents, some non-linearity between dilutions, and potential lot-to-lot changes in drug sensitivity of APTT reagents (43, 89).

4.3 Switching assay type between clotting and chromogenic assays

A simple switch from an OSA to CSA, or a switch in APTT reagent brand to one shown to give an acceptable recovery can be made. An example of this is for the PEGylated rFIX Refixia and rFIX-FP (Idelvion), where very few APTT reagents give acceptable recoveries, but both commercially available FIX CSA were acceptable for Refixia, but showed some overestimation with Idelvion (90, 91). It is essential to standardize and improve reliability of measurements of these concentrates as diagnosis and treatment monitoring is based on these results (91).

4.4 Other laboratory issues arising from using new treatment products.

The practical issues facing specialist haemostasis laboratories when testing haemophilia treatment products that do not give acceptable results with the laboratory's usual assays:

- the maintenance of multiple APTT reagent types and their associated calibration curves and test protocols; maintenance of CSA for FVIII and FIX
- a method of identifying the product type being given to each patient to ensure the correct choice of test, which can be an issue for reference centres receiving significant numbers of referred samples
- having a strategy for performing Bethesda assays in the presence of emicizumab
- having a strategy for performing factor assays when more than one product type is present in the plasma, for example emicizumab plus a replacement FVIII, or emicizumab plus endogenous FVIII in patients with AHA
- maintaining staff expertise in an area that is becoming increasingly complicated with the advent of new therapeutics

Currently in Australia there is a tender system for replacement rFVIII and rFIX EHLs that restricts the number of different products that can be used. This benefits testing laboratories by limiting the number of assay issues that would otherwise arise if all available EHLs were in use. As the tender is renewed about every four years, there is ongoing potential for product switching which may require changes to local test procedures. The EHL replacement therapies and the non-factor therapy emicizumab currently in use still required local validation, which was a large focus of the current studies. Some haemophilia treatment centres in Australia have participated in international clinical trials of replacement products that were not in general use, and for

which method adjustments were needed to obtain result accuracy, a case in point being efanesoctocog alfa. The expertise thus gained was invaluable in initiating further studies when emicizumab was made widely available.

Aims and Outline

This work was conducted during a period of rapid change in the therapeutic options for the treatment of HA and HB. In particular, the introduction into Australia of EHL FVIII and FIX products plus the introduction of the non-factor replacement product emicizumab, has necessitated a reappraisal as to current methods and their applicability to measuring the new products. This work was aimed at assessing applicability existing methods for measurement of new treatment products plus introducing and validating newer tests into Australian haemostasis laboratories that are needed for clinical management of haemophilia.

The specific aims were:

(1) To evaluate the performance characteristics of chromogenic FIX assays including diagnostic accuracy, reference intervals, precision studies and recovery studies of several FIX replacement products. This was followed with a multi-laboratory study to determine inter-laboratory agreement of the new assay in measuring FIX levels in sets of samples comprising differing FIX products.

(2) To validate the use of different OSA and CSA FVIII assays for measuring the EHLs Eloctate and Adynovate by means of Australia-wide field studies. The study allowed laboratories to compare their performance in measuring these newly introduced FVIII EHL replacement products. This type of study is important as these EHL FVIII products were not available in external quality assurance

programs, and performance of some specific APTT reagents was not previously reported.

(3) To explore laboratory characteristics of the efanesoctocog alfa (ALTUVIIIO), and its measurement in plasma in preparation for its potential introduction into Australia as a therapeutic option for HA. This includes the adaptability of the potency assignment reagent, Actin FSL APTT, to non-affiliated analysers. This study is needed due to the high levels if inaccuracy that have been reported with many commonly used reagent/analysers systems currently used in many Australian haemophilia reference laboratories for monitoring of efanesoctocog therapies.

(4) To set up and evaluate multiple OSA and a CSA protocol for measuring the bi-specific antibody and FVIII mimetic emicizumab. This included determining test linearity within the therapeutic interval, assay precision studies, and adaptability. The study was then expanded to an Australia-wide field study of emicizumab measurement to determine interlaboratory accuracy and variability between sites covering a large range of reagent/analyser combinations.

(5) To determine assay agreement in measuring FVIII inhibitor titres in the presence of emicizumab by use of bovine-component chromogenic FVIII assays. This was carried out as a multi-laboratory field study of selected Australian haemophilia reference laboratories. The study included samples from congenital HA and AHA spiked with emicizumab.

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CHAPTER 2

Methods

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Preface

This chapter briefly summarises the haemophilia assays used in the main studies and the broad approach taken in setting up the field studies. More detail of sample preparation is contained within the individual method chapters.

Ethics approvals

Ethics approvals for collection of patient samples obtained from the Ethics Committee of the Royal Prince Alfred Hospital, Sydney. Collection of additional citrate tubes from Red Cross blood donors was by a fee-for-service contractual arrangement with Australian Red Cross Blood Transfusion Service. Collection required researcher presence at the collection centre with provision of study information to each donor. Written informed consent from each donor was obtained before commencement of blood collection.

1. Citrated plasma collection from controls

Blood from Red Cross donors used for FIX reference intervals was collected by clean venipuncture using a 16-gauge needle into 3.2% citrated vacuum tubes from Becton Dickenson. Whole blood samples were transported at room temperature to the hospital laboratory for double centrifugation, dispensing into cryovials, then freezing at -80° until testing.

Blood from healthy staff volunteers and patients was collected by clean venipuncture using a 21-gauge needle into 3.2% citrated vacuum tubes from Becton Dickenson. Samples were double centrifuged, dispensed into cryovials and frozen at -80°C until tested.

Double centrifugation was 10 minutes at 2,500 x g at 20°C, followed by transferring plasma to a clean container and re-centrifugation at the same settings. The plasma above any pellet was transferred to pre-labelled cryovials or screw-capped sample tubes for freezing.

2. Automated one-stage clotting factor assays for FVIII and FIX

The methods described below were used at Royal Prince Alfred Hospital in Sydney, referred to as the ‘central laboratory’. Field study participants used their local standard factor assay protocols, unless the study required use of specific method modifications. Such modifications were required for the chromogenic FIX assay, emicizumab assay, and Bethesda assay using chromogenic FVIII reagents. Reagent details were captured and reported for all field studies as required.

3.1 Diagnostica Stago STA-R analyser.

The standard FVIII and FIX one-stage clotting assays (OSA) on Stago STA-R analyser are a fully automated system where plasma was serially double diluted from 1/10 in Owren-Koller buffer, then mixed with an equal volume of factor deficient substrate plasma, APTT reagent and $CaCl_2$. Each test reagent had a standard 50µL volume added to the cuvettes. Clot endpoint detection was viscosity based, determined by cessation of movement of a metal ball within the cuvette oscillating under a magnetic field as fibrin is formed. Calibration line fit was linear on log-log transformed X and Y axes. Manufacturer guidelines were followed for affiliated reagents. Different reagent brands were used over the course of the studies with necessary adjustments made to APTT activation times according to manufacturer guidelines. Third party reagent vials non-standard sizes required transfer to alternate vials or placement of sleeves around the vials to fit reagent positions. Calibration

plasma was Siemens Standard Human Plasma (SHP) or Diagnostica Stago Unicalibrator. Test values exceeding the calibration curve range for OSA were retested at 1/20 dilution for high values or 1/5 for low values

2.2 Sysmex CS2500 analyser.

Several different Siemens APTT reagents were used as per the manufacturer guidelines built into the analyser software in a fully automated system. Plasma was tested at multiple dilutions with a 1/20 standard dilution with Siemens OVB. Diluted plasma was mixed with equal volumes of factor deficient plasma, APTT reagent and 0.025M $CaCl_2$. Each test reagent had a standard 40µL volume added to the cuvettes. Clot endpoint was detected optically at 660nm, and defined as the time taken for the primary clot wave to reach 50% of the total absorbance change in the fibrin formation phase. Calibration line fit was point-to-point on log-log transformed X and Y axes. Third party APTT reagents required adjustment to the test protocol for the recommended activation time. Reagents in non-standard vials were transferred to alternate vials, or had sleeves placed around the vial for secure placement in reagent racks. Test values exceeding the calibration curve range were retested at 1/40 dilution for high values or 1/10 for low values.

2.3 Instrument Laboratory ACLTOP analysers

These were fully automated protocols. Manufacturer guidelines were followed for all Instrument Laboratory (IL) reagents. Plasma was tested at multiple dilutions in IL Factor Diluent with a standard dilution of 1/10. This was followed with addition of factor deficient plasma, APTT reagent and $CaCl_2$. All three IL APTT reagents, Synthasil, Synthafax and APTT SP had individual and different volume ratios for sample: factor deficient plasma: APTT reagent: $CaCl_2$. This was important to note when third party reagents were adapted to the ACLTOPs. Clot endpoint was detected

optically at 671nm and defined as the time taken for the 2nd derivative curve to reach its maximum peak height during the fibrin formation phase. This occurs at the start of the primary clot wave formation. The Y-axis contains plots milli-absorbance unit change with time on the X-axis. Calibration line fit was by 2nd order polynomial with $1/\sqrt{X}$ and $\sqrt{Y}$ axes transformations, which allowed use of a zero calibration point on this system. Test values exceeding the calibration curve range were retested at 1/20 dilution for high values or 1/5 for low values.

3. Chromogenic substrate assays for FVIII

The central laboratory used the Hyphen Biomed and Siemens kits as required, both according to manufacturer guidelines with some minor modifications. The Hyphen FVIII test 1st stage incubation time was increased from 300s to 450s to enhance detection of mild HA discrepant phenotypes, based on previously published work. For the Siemens kit, two changes were made to the calibration line to improve assay precision in the mild haemophilia range. An additional calibration point was added, and the calibration line fit was changed from linear to point-to-point. All chromogenic FVIII assays were calibrated with the Siemens Standard Human Plasma (SHP) using the WHO-referenced chromogenic FVIII value. At the time of testing the Diagnostical Stago calibration plasma did not have a chromogenic FVIII assigned value.

4. Chromogenic substrate assays for FIX

5.1 Rossix Chromogenic FIX

The Rossix FIX test was adapted to the Sysmex CS2500 and STA-R analysers using the reagent volumes and incubation times in the package insert. Assistance was sought and received from the manufacturer of the Rossix test for the Sysmex and STA-R adaptations. The sample needle washing steps in the supplied protocol required slight

modification due to inconsistent results. Alternate reagent vials were used as needed to fit the Sysmex reagent racks. High range and low range protocols were set up on the Sysmex in a fully automated process. For the Rossix test on the STA-R, a low protocol was created by manually pre-diluting one part of calibration plasma in 9 parts of FIX deficient plasma and including a 0 IU/dL calibration point (buffer only). Calibration curve fit for the Rossix test on the STA-R was third order polynomial for the high range protocol and second order polynomial for the low range protocol. SHP was used to calibrate the assay using on the Sysmex analyser and Unicalibrator was used on the STA-R analyser. The FIX OSA package insert value were used as specific chromogenic FIX values were not available.

4.2 Hyphen Biomed chromogenic FIX

The Hyphen chromogenic FIX assay (Hyphen BioMed, Neuville-Sur-Oise, France) was performed on the CS2500 with SHP as calibrator. Protocols were obtained via the kit manufacturer with the Hyphen test modified for point-to-point calibration. Fully automated ‘high’ and ‘low’ range protocols were created. Several attempts were made to adapt the Hyphen FIX test to the STA-R analysers to allow additional comparative studies but were discontinued due to reduced FIX recovery levels in quality control material testing.

5. Emicizumab assays

The emicizumab assays were adaptations of existing OSA for FVIII. The standard sample dilutions were changed from 1/10 to 1/40 on the STA-R analyser, from 1/20 to 1/80 on the Sysmex CS2500 analyser, and from 1/10 to 1/80 on the ACLTOP750 analyser. Calibration plasma and two levels of control plasma were FVIII deficient plasma spiked with emicizumab purchased from r^2 diagnostics. Additional

emicizumab calibration and control kits were purchased by the central laboratory for distribution to all field study participants. Interference in normal OSA and other APTT-based clotting assays from emicizumab carryover was avoided by running samples in separate batches on the Stago and Sysmex analysers. The ACLTOP analyser did not have a carry-over issue based on information supplied by the manufacturer (Werfen). An additional emicizumab assay was created using the modified protocol of Hyphen chromogenic FVIII assay run on the Sysmex CS2500 analyser.

6. Field studies

7.1 FVIII spiked sample preparation.

Eloctate, Advate and Adynovate lyophilised concentrates were reconstituted as per manufacturer instructions for spiking into congenital FVIII deficient plasma. A multi-step process was used to generate the final sample target concentrations. Targets were calculated from the lot specific potency values of each product, obtained from the product box by requesting the information from the manufacturer. All diluted products were dispensed into cryovials and stored at -80°C until shipment on dry ice.

6.2 FIX sample preparation

FIX replacement products Benefix and Rixubis were spiked into congenital FIX deficient plasma to final concentrations based on the lot-specific potency values. The calibration plasma SHP was spiked to 5 IU/dL and 1 IU/dL, and the normal control plasma Control N (Siemens) was reconstituted and dispensed into cryovials undiluted. Cryovials of plasma were rapidly frozen by placing into a -80°C freezer where they remained until shipping by overnight transport on dry ice.

6.3 Emicizumab spiked sample preparation

A clinical vial of emicizumab (Hemlibra) was supplied by Roche with label value of 30 mg/mL from which all final spike concentrations of 5, 10, 20, 40, 80 and 120 µg/mL were calculated. These targets did not need adjusting because the lot-specific assigned value was 29.9 mg/mL. The drug was spiked into immune-depleted FVIII deficient plasma purchased from Precision Biologics. The emicizumab-spiked pools were dispensed into cryovials and rapidly frozen at -80°C until tested in the central laboratory or shipped to field study participants.

6.4 FVIII inhibitor titres in the central laboratory by Bethesda method in emicizumab- containing samples.

Test samples containing > 5 IU/dL were heat inactivated for 20-30 min at 56°C then hard spun to remove precipitated fibrinogen before creation of serial doubling dilutions in 4% albumin. Dilutions were mixed with an equal volume of pooled normal plasma (Precision Biologics), incubated 2 hours at 37°C then assayed for FVIII on the Sysmex CS2500 analyser using the Siemens bovine-component chromogenic FVIII assay. Residual % FVIII was calculated from the FVIII present in a control tube consisting of pooled normal mixed in equal volume with 4% albumin.

6.5 Field study participant Bethesda assays

Field study participants were asked to perform Bethesda assays with their usual test set-up, but to read the final FVIII levels using a bovine-component chromogenic FVIII assay. Participants were requested to return their results plus individual sample dilution calculation for full assay interpretation by the study coordinator.

7. Quality control

For all central laboratory FVIII and FIX OSA two levels of commercial assayed quality control plasma were tested at least once on each day of testing on all analysers by all reagent protocols. The central laboratory was enrolled in the Royal College of Pathologists of Australasia Quality Assurance Programs (RCPAQAP) and/or the External Control for Assays and Tests (ECAT) Haemophilia modules for factor VIII and FIX assays during the testing period.

8. Data and Statistics

All data was entered into Microsoft Excel spreadsheets for statistical analyser. Further statistical analysis and all graphs were created with GraphPad Prism software (V5-V10).

CHAPTER 3

Evaluation of chromogenic factor IX assays by automated protocols

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CHAPTER PREFACE

This work has been published as:

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This study was the first published evaluation of the two commercially available chromogenic FIX assays. The study included work-up of the assays on two automated analysers, establishing reference intervals, test reproducibility, diagnostic accuracy, and performance in measuring SHL and EHL FIX replacement products. Geoffrey Kershaw did most of the working including design and planning the study, sample collection, sample testing and writing the manuscript. Kumari Dissanayake performed samples collection and testing, data entry and analysis. Liane Khoo and Vivien Chen assisted in study design, result analysis and contributing to the final manuscript.

The study received funding from John Lloyd Clinical Excellence Fund of the Australian Haemophilia Centre Directors Organisation.

ABSTRACT

Introduction: Chromogenic substrate assays (CSA) to measure Factor IX (FIX) have recently become commercially available. However, information on their performance characteristics and use in diagnostic haemostasis laboratories remains limited. **Aim:** To evaluate the Hyphen Biomed (Hyphen) and Rossix FIX CSAs on fully automated coagulation analysers and compare them to the FIX one-stage assay (OSA). This study

was conducted in a tertiary referral haemostasis laboratory associated with a haemophilia treatment centre.

Methods: Automated CSA protocols were adapted to the Sysmex CS2500 (CS2500) and Diagnostica Stago STA-R (STA-R) analysers. Samples assayed were from healthy volunteers, haemophilia B patients and FIX deficient plasma spiked with either plasma derived, recombinant or extended half-life FIX products.

Results: Reference intervals for Hyphen and Rossix assays were 73 IU/dL to 164 IU/dL and 73 IU/dL to 168 IU/dL respectively on the CS2500 analyser; and 84 IU/dL to 165 IU/dL for the Rossix assay on the STA-R. Repeatability across all method/analyser combinations resulted in CVs ranging from 0.8% to 5.4%. Between run reproducibility gave CVs less than 6.7% for all method/analyser combinations. In spiked samples, FIX recoveries were mostly within an acceptable limit of 100±25% for BeneFIX®, Rixubis® and Alprolix® with some differences between CSAs.

Conclusion: Both commercial factor FIX CSA kits can be adapted for Stago and Sysmex automated coagulation analysers. Reagent cost and workflow practices will need to be considered. These assays are potentially more consistent than OSA in measurement of replacement FIX products in haemophilia B patients.

INTRODUCTION

Haemophilia B (HB) is an X-linked recessive bleeding disorder caused by a deficiency in coagulation factor IX (FIX). Measurement of plasma FIX levels is required for the diagnosis and classification of patients with HB, the post-infusion monitoring of FIX replacement therapy, and for potency labelling of FIX replacement products. Assays of FIX activity have been almost exclusively performed by one-stage APTT-based clotting assay (OSA) [1,2]. Considerable inter-laboratory variability in FIX estimates exists due to: variability in APTT activator (silica, ellagic acid or kaolin); phospholipid source; type of factor deficient plasma; analyser clot detection principles; and different assay designs even when the same reagents and analysers are used [1,4,5]. Recently, two commercial chromogenic substrate FIX assays (CSA) have been developed. CSA are two-stage assays: the first stage being the generation of activated factor X (FXa) which is dependent on the activity of the plasma FIX, the second being colour generation from chromogenic substrate by FXa. These kits can be adapted to run on automated coagulation analysers currently used for OSA.

While reduction in variability through use of chromogenic assays for measurement of some FVIII products has recently been reviewed [5], less is known about FIX products. Barrowcliffe [6] reported that a chromogenic assay under-estimated potency of recombinant FIX (rFIX) relative to OSA. More recent studies have shown FIX estimates for rFIX to be significantly lower by CSA than for OSA, but differences were not observed when testing plasma derived FIX (pdFIX) products [7,8]. Potency discrepancies for both recombinant and extended half-life (EHL) FIX products have been described between the OSA and CSA and for different OSAs related to APTT reagents [9].

In view of the potential applications of measuring FIX by CSA for both diagnosing HB and monitoring FIX replacement therapy this study had two aims. Firstly, to evaluate the performance of two commercial chromogenic FIX assays adapted to automated coagulation analysers including establishing reference intervals, reproducibility studies, and measurement of FIX in deficient samples; and secondly, to compare the CSA to OSA in spike-recovery studies for different FIX replacement products.

MATERIALS AND METHODS

Sample collection. Nine parts blood was collected into one part 0.109M tri-sodium citrate anticoagulant with platelet poor plasma stored in cryovials at -80°C until tested. Aliquots were rapidly thawed at 37°C prior to testing and analysis was completed within 3 hours.

Reference intervals. Blood was drawn from 128 donors (70 males, 58 females, median age 38 years, range 18-79 years) after informed consent from staff volunteers (n=38) and blood donors attending Australian Red Cross Blood Transfusion Service (ARCBTS) (n=90). Use of ARCBTS donor samples was approved by the ethics committee of ARCBTS. The study had approval by the Ethics Committee of the Royal Prince Alfred Hospital, Sydney (X12-0238).

Patient samples. Citrated plasma from 35 patients with FIX deficiency were included in the study consistent with the laboratory's quality procedures for validation of new assays, comprising 17 different patients with HB, 10 receiving oral vitamin K antagonists, and eight with liver dysfunction.

Reproducibility. Replicate precision (repeatability) was performed with commercial control plasma or FIX deficient study samples. Between run reproducibility was assessed using commercial control plasma.

Spiked plasma recovery study. Single lot numbers of two rFIX concentrates were obtained for the study: Benefix® (Pfizer, New York, USA) and Rixubis® (Baxalta, Lessines, Belgium). A pdFIX concentrate, Monofix®, was obtained from CSL (Melbourne, Australia). Products were reconstituted with supplied diluents, then spiked into immuno-depleted FIX deficient plasma (Precision Biologics, Dartmouth, Canada) to nominal FIX concentrations 5, 20, 40, 80 and 120 IU/dL with storage in cryovials at -80ºC until tested. FIX-deficient plasma pre-spiked to 80, 20, and 5 IU/dL with extended half-life (EHL) product, rFIX-Fc (Alprolix®) were donated by Biogen Idec (Zug, Switzerland). Samples spiked with Monofix®, Benefix® or Rixubis® were tested on three different test days by each of three CSA protocols and three OSA protocols. Alprolix® was tested by fewer protocols due to limited supply of material. Final percent recoveries of nominal spike levels for Benefix® and Rixubis® were adjusted to reflect the manufacturer's potency assignment of the specific lots used in the study, since these differed slightly from the label value. For Monofix® the percent recovery was calculated from nominal spike levels without further adjustment.

One stage FIX assays. The OSA for FIX was performed by the laboratory's standard method on the STA-R analyser (Diagnostica Stago, Asnieres, France) (STA-R) with Triniclot aPTT S (TCoag, Wicklow, Ireland) as activator and congenitally deficient FIX substrate plasma (Helena Laboratories, Beaumont, Texas, USA) and Standard Human Plasma (SHP), (Siemens, Marburg, Germany) as calibrator. Samples were assayed at a minimum of two dilutions. A FIX 'low' range curve was created for testing samples with <5 IU/dL FIX activity by bench pre-dilution of one part SHP to nine parts of FIX

deficient plasma. Two additional OSAs were created using the activators Synthafax (Instrument Laboratory, Bedford, MA, USA) and Actin (Siemens, Marburg, Germany).

Chromogenic FIX assays. The Rossix chromogenic FIX assay (Rossix; Molndal, Sweden) was performed on the Sysmex CS2500 analyser (Sysmex, Kobe, Japan) (CS2500) with software validated by Siemens (Marburg, Germany) using SHP as calibrator, and on the STA-R analyser with Unicalibrator (Diagnostica Stago, Asnieres, France) as calibrator. The Hyphen chromogenic FIX assay (Hyphen BioMed, Neuville-Sur-Oise, France) was performed on the CS2500 with SHP as calibrator. Protocols were obtained via the kit manufacturers with Hyphen test modified to use point-to-point calibration. Standard sample dilution for high range calibrations was 1/80 for Rossix and 1/100 for Hyphen. For Rossix and Hyphen CS2500 tests fully automated low range protocols were created by additional automated calibration plasma pre-dilution of 1/5 and inclusion of a 0 IU/dL calibration point (buffer only). Calibration curve fit for Rossix high and low range protocols on the CS2500 were point-to-point. For the Rossix test on the STA-R, a low protocol was created by manually pre-diluting one part of calibration plasma in 9 parts of FIX deficient plasma and including a 0 IU/dL calibration point (buffer only). Calibration curve fit for the Rossix test on the STA-R was third order polynomial for the high range protocol and second order polynomial for the low range protocol.

Reagents and buffers for all protocols were equilibrated for 30 minutes on board before calibration. Fresh calibrations were performed daily for all CSA and OSA protocols [10]. The Rossix kit contains human (F)actors VIII, X, XIa and FII, plus bovine FV. Activation of FVIII is by FIIa formed through FV-mediated activation of FII during the incubations. The Hyphen kit contains human FVIII, FX, FXIa and FIIa, the latter

directly activating FVIII. Test plasma FIX is converted to FIXa to complete the tenase complex. Both kits contain a substrate for FXa.”

Calibrator assessment. To assess the effect of calibrator differences on spike recoveries, FIX levels in three additional lots of SHP and Unicalibrator were measured by the three CSA protocols and one OSA protocol (Triniclot aPTT S), calibrated with the 4th International Standard for Factors II, VII IX and X, 09/172 (4th IS, National Institute for Biological Standards and Control, Potters Bar, UK).

Cost analysis. Cost per test was calculated in Australian dollars from actual average number of tests obtained, where for the CSA, ‘test’ included calibrators, controls and test samples. The cost of calibrators, controls and cuvettes was not considered, as these were equivalent across methods.

Statistical Analysis. Results were analysed in Excel (Microsoft Corporation) and GraphPad Prism 6 (Graphpad Software, La Jolla, CA, USA). Tests of Gaussian distributions were made by D’Agostino and Pearson omnibus test. Reference intervals were determined by parametric and non-parametric methods [11].

RESULTS

Reference intervals and precision. Reference intervals values for each CSA protocols and the OSA are plotted in Fig. 1, and the calculated reference intervals listed in Table 1. Parametric reference intervals for the three CSAs and one OSA were calculated from $\log_{10}$ transformed data after all distributions failed the D'Agostino & Pearson omnibus normality test. The non-parametric FIX reference intervals representing the 2.5th to 97.5th percentile of the distribution were 73-158 IU/dL for the Rossix assay on the

Sysmex analyser, 84-165 IU/dL for the Rossix assay on the STA-R analyser, and 73-164 IU/dL for the Hyphen assay on the Sysmex analyser, with lower limits agreeing to within 1 IU/dL of those determined by parametric method (Table 1). There was no difference in FIX levels between male and female donors by all protocols (*P*>0.10, Mann-Whitney test). Repeatability studies gave CVs of 1.7% to 3.8% for the high curve assays and 0.8% to 5.4% for the low curve assays (Table 2). Between run CVs ranged from 1.8% to 4.5% for the two assays performed on the Sysmex analyser, and from 4.0 to 6.7% for the Rossix test on the STA-R analyser (Table 2).

Table 1. Plasma factor IX reference intervals by one-stage assay and three chromogenic assay protocols

	OSA STA-R FIX (IU/dL)	Rossix CSA STA-R FIX (IU/dL)	Rossix CSA CS2500 FIX (IU/dL)	Hyphen CSA CS2500 FIX (IU/dL)
N	128	128	128	128
Geometric mean	113	115	107	105
Reference interval				
Parametric method				
Lower limit (90%CI)	78 (74-81)	85 (82-88)	72 (69-76)	72 (69-75)
Upper limit (90%CI)	164 (156-172)	154 (148-160)	158 (150-166)	153 (146-160)
Non-parametric method				
Lower (90%CI)	77 (68-85)	84 (83-90)	73 (69-79)	73 (67-76)
Upper (90%CI)	171 (159-191)	165 (142-186)	168 (147-183)	164 (143-185)

OSA, one stage assay; CSA, chromogenic substrate assay; CI, confidence interval

Table 2. Repeatability and between run reproducibility of chromogenic FIX assays. Samples were assayed using both high range and low range calibration curves. Testing was performed over at least four test days for each sample and within three hours of reagent loading.

		Repeatability								Between run			
Rossix	Calibration	high	high	high	high	low	low			high	high	low	low
CS2500	n	10	10	10	10	10	10			18	17	10	12
	FIX(IU/dL)	141	92	55	34	7.4	4.0			104	37	18.1	9.6
	CV(%)	2.3	2.8	2.4	2.1	5.4	3.8			4.5	3.4	1.8	2.8
Rossix	Calibration	high	high	high	high	high	high	low	low	high	high	low	low
STA-R	n	10	10	10	10	8	8	6	6	14	14	10	10
	FIX(IU/dL)	101	54	40	28	11.7	6.5	9.3	5.2	102	45	11.1	4.9
	CV(%)	3.8	3.7	2.9	2.0	2.2	5.0	4.0	3.2	6.4	6.7	6.2	4.0
Hyphen	Calibration	high	high	high	high	low	low			high	high	low	Low
CS2500	n	10	10	8	10	10	10			22	21	10	10
	FIX(IU/dL)	146	102	69	39	9.5	4.3			104	39	15.5	7.8
	CV(%)	2.5	3.1	2.0	1.7	0.8	2.3			3.5	2.7	3.6	3.5

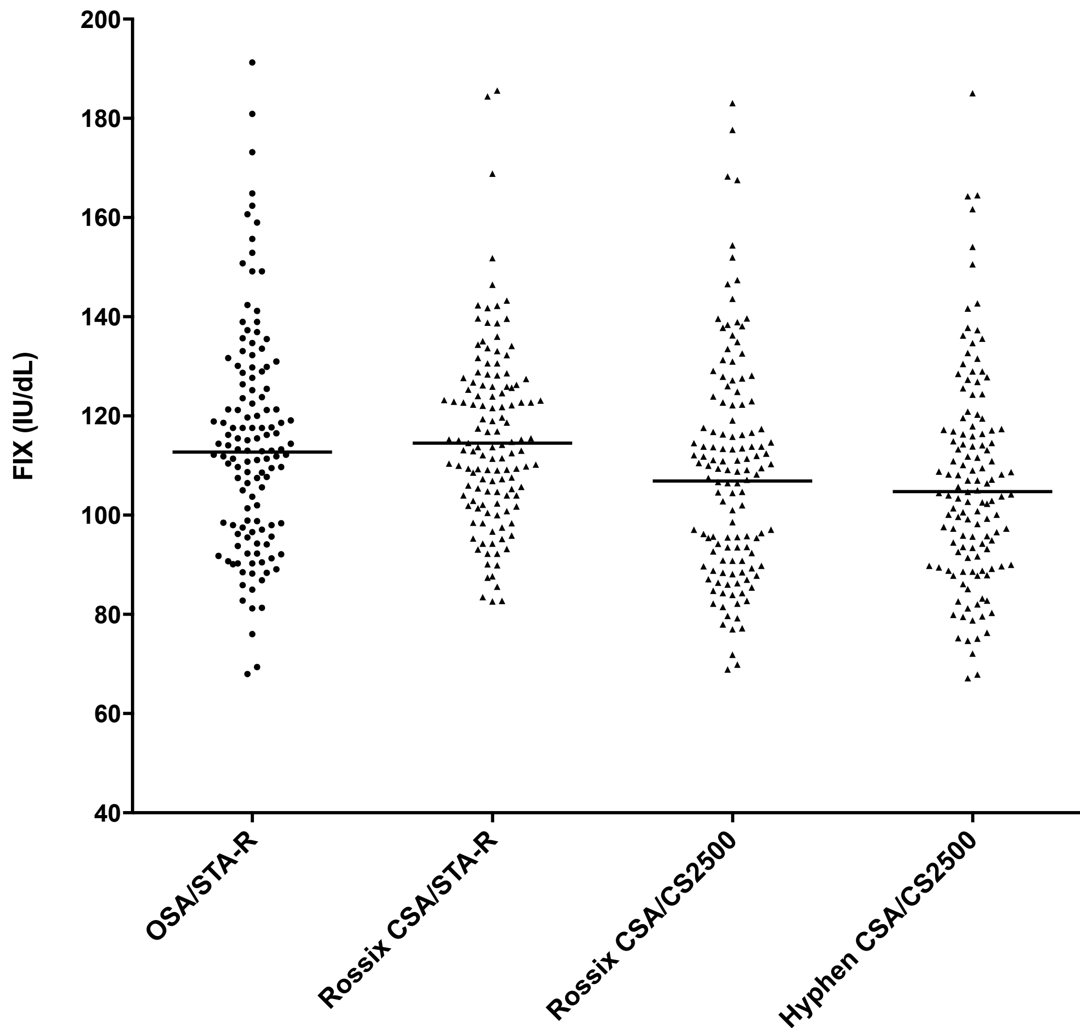
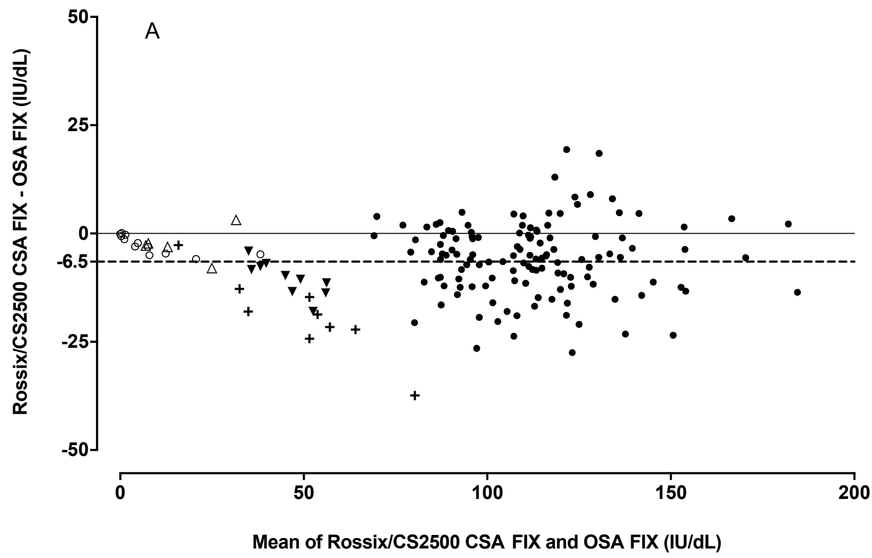


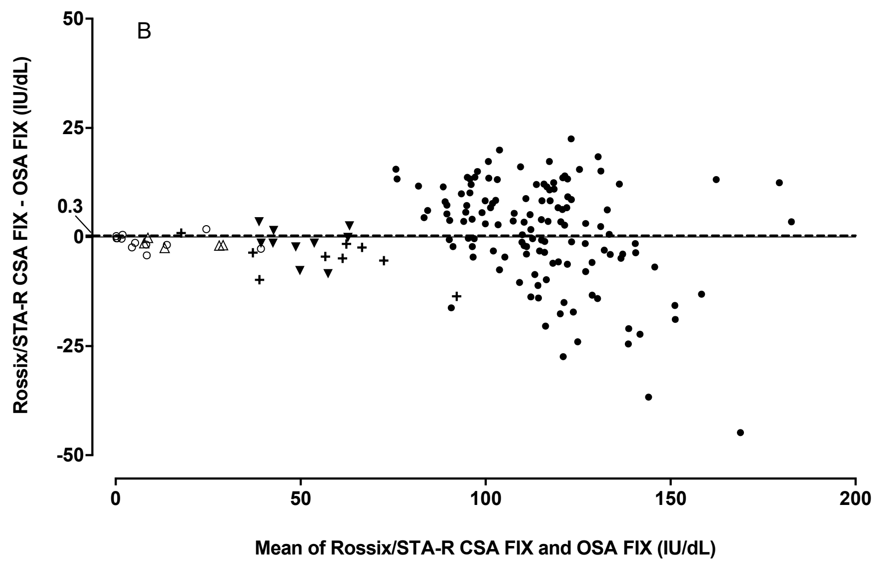
Figure 1: Reference interval data for FIX (n=128). The one-stage assay (OSA) was performed on the STA-R analyser with Triniclot aPTT S activator. The chromogenic substrate assay (CSA) from Rossix was performed on the STA-R and CS2500 analysers. The CSA from Hyphen was performed on the CS200 analyser. Geometric means are shown as horizontal lines.

Comparability. Comparability of each CSA with the OSA in normal and clinical samples was assessed by the method of Altman and Bland [12] shown in Fig. 2. The Rossix and Hyphen tests performed on the Sysmex analyser had mean biases of minus 6.5 IU/dL and minus 8.3 IU/dL respectively (Fig 2). The Rossix test on the STA-R analyser had a mean bias of 0.3 IU/dL. For individuals with HB there was general agreement between methods, with levels obtained by the OSA and Rossix CSA test higher than by the Hyphen test (Table 3). Four

individuals with FIX CSA of 0.0 to 0.4 IU/dL were reported as <0.5 IU/dL as were four individual tests results which gave 'linearity' errors with the CS2500 due to undetectable FIX as dOD values were close to the zero calibration point (buffer only) (Table 3). The FIX levels in the HB group showed generally good agreement between chromogenic methods, with levels obtained by the OSA and Rossix test slightly higher than by the Hyphen test. In 11 samples from recently untreated HB individuals two individuals classified as mild HB (>5-40 IU/dL) with the Rossix test were classified as moderate HB (1-5 IU/dL) by the Hyphen test (samples 3 and 4, Table 3). All three samples classified as severe HB (<1 IU/dL) by OSA were also classified as severe HB by all the three chromogenic protocols (samples 6, 8 and 10, Table 3). One sample (sample 7) was classified as moderate HB by OSA and Rossix/STA-R, and as severe HB by both CS2500 CSA protocols.

Spike recoveries. Mean recoveries by the Rossix test on the Sysmex and STA-R analysers were acceptable (within 75-125% of label value) for Benefix® and Rixubis® at all five spike levels from 120 IU/dL to 5 IU/dL. Recovery of Alprolix® by the Rossix test on the CS2500 was acceptable at 98.8% to 125.0% of spike level for the three spike levels (Fig 3). By comparison, pdFIX (Monofix®) recoveries tended to be higher, with ranges 126.9% to 137.8% on the CS2500 and 137.5% to 162.0% on the STA-R analyser. Mean recoveries by the Hyphen test tended to be lower than Rossix recoveries at each spike level for each product (Fig 3). Hyphen recovery ranges were 60.3% to 80.2% (Rixubis®), 65.4% to 90.9% (Benefix®), 99.3% to 139.9% (Monofix®) and 68.7% to 89.3% (Alprolix®). For each product, the lowest recovery by the Hyphen test was at the 5 IU/dL spike level, with most other recovery levels being acceptable. For the OSA, recovery of Benefix® and Rixubis® with Triniclot aPTT S and Actin APTT reagents yielded similar profiles, with a tendency to over-recovery that increased in degree the lower the spike level. For Synthafax, recovery of Benefix® and Rixubis® was


A
Rossix/CS2500 CSA FIX - OSA FIX (IU/dL)
50
25
0
-6.5
-25
-50
0
50
100
150
200
Mean of Rossix/CS2500 CSA FIX and OSA FIX (IU/dL)


B
Rossix/STA-R CSA FIX - OSA FIX (IU/dL)
50
25
0.3
0
-25
-50
0
50
100
150
200
Mean of Rossix/STA-R CSA FIX and OSA FIX (IU/dL)

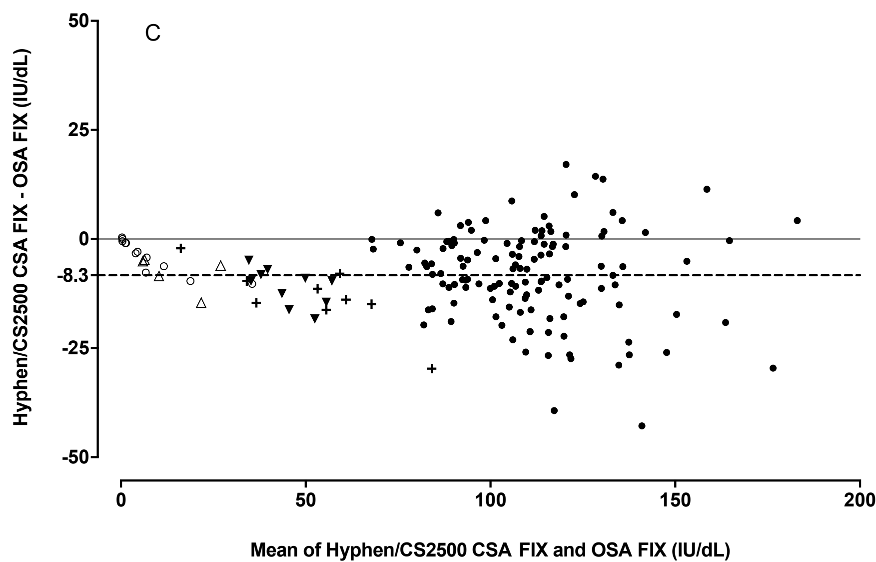

C
Hyphen/CS2500 CSA FIX - OSA FIX (IU/dL)
50
25
0
-8.3
-25
-50
0
50
100
150
200
Mean of Hyphen/CS2500 CSA FIX and OSA FIX (IU/dL)

Figure 2: Altman and Bland plots of three FIX chromogenic assays with one-stage FIX assay: Rossix assay/CS2500 analyser (panel A); Rossix assay/STA-R analyser (panel B); Hyphen assay/CS2500 analyser (panel C). Graphs show reference interval donors (solid circles), warfarinised patients (solid triangles), patients with liver dysfunction (crosses), haemophilia B patients untreated for >7 days (open circles), haemophilia B patients on prophylaxis (open triangles). The dashed lines show average bias across all samples for each method pairing.

Table 3. FIX levels in samples from patients with haemophilia B by chromogenic assays and one-stage assays. Results marked with an asterisk indicate samples which gave an analyser 'linearity' error due to very low optical density change, so were assigned a value of <0.5 IU/dL.

	Test protocol				
	STA-R Trini OSA	STA-R Rossix CSA	CS2500 Rossix CSA	CS2500 Hyphen CSA	
Sample	FIX (IU/dL)	FIX (IU/dL)	FIX (IU/dL)	FIX (IU/dL)	Recent FIX therapy
1	14.7	12.9	10.1	8.5	>7 days untreated
2	5.6	3.2	2.6	2.3	>7 days untreated
3	9.1	7.4	6.0	4.9	>7 days untreated
4	10.5	6.3	5.5	2.8	>7 days untreated
5	23.6	25.4	17.7	14.0	>7 days untreated
6	<1.0	<0.5	<0.5	<0.5	>7 days untreated
7	1.8	1.4	<0.5*	0.9	>7 days untreated
8	<1.0	<0.5	<0.5*	<0.5*	>7 days untreated
9	5.9	4.6	3.7	3.0	>7 days untreated
10	<1.0	<0.5	<0.5	<0.5*	>7 days untreated
11	40.6	37.9	35.8	30.3	>7 days untreated
12	1.6	2.1	1.3	0.7	Severe HB, Benefix® prophylaxis
13	8.8	8.6	6.5	4.0	Severe HB, Benefix® prophylaxis
14	8.4	7.0	5.6	3.3	Severe HB, Benefix® prophylaxis
15	28.5	27.0	21.0	14.4	Severe HB, Benefix® prophylaxis
16	14.5	12.0	11.4	6.0	Moderate HB, Benefix®prophylaxis
17	30.0	28.1	33.1	23.9	Severe HB, Monofix® prophylaxis

Trini, Triniclot aPTT S reagent; OSA, one-stage assay; CSA, chromogenic substrate assay

acceptable at spike levels from 120 IU/dL down to 20 IU/dL, with over-recovery at the 5 IU/dL spike level. For Alprolix® recovery was acceptable with Triniclot aPTT S at all three spike levels, but there was over-recovery with the OSA using Synthafax. The three OSAs gave significant over-recovery of the 5 IU/dL spike level for all products tested (except Triniclot

aPTT S and Alprolix®, where recovery was close to 100%), in contrast to the CSAs, where recoveries at 5 IU/dL were more varied but generally closer to predicted levels (Fig. 3). The FIX levels (mean % of assigned value ±sd) in three lots of calibration plasma measured against the 4th IS were for Unicalibrator 90.6±1.7 by OSA/STA-R, 90.5±3.1 by Rossix/STA-R, 87.3±1.9 by Rossix/CS2500 and 81.6±2.0 by Hyphen/CS2500. For SHP values were 98±4.6 by OSA/STA-R, 100.2±5.6 by Rossix/STA-R, 96.3±1.6 by Rossix/CS2500 and 91.2±2.4 by Hyphen/CS2500 (Figure S1).

General observations and costings. The CSA protocols were straightforward to perform on both analysers after the initial implementation and familiarization period. Sample analysis times were ~12 minutes and ~10 minutes for the Rossix test on the CS2500 and STA-R analysers, respectively, and ~6 minutes for Hyphen test on the CS2500. Tests achieved per half kit averaged: 46 ($4.89/test) for Rossix/CS2500; 39 ($5.77/test) for Rossix/STA-R; and 42 ($4.17/test) for Hyphen/CS2500.

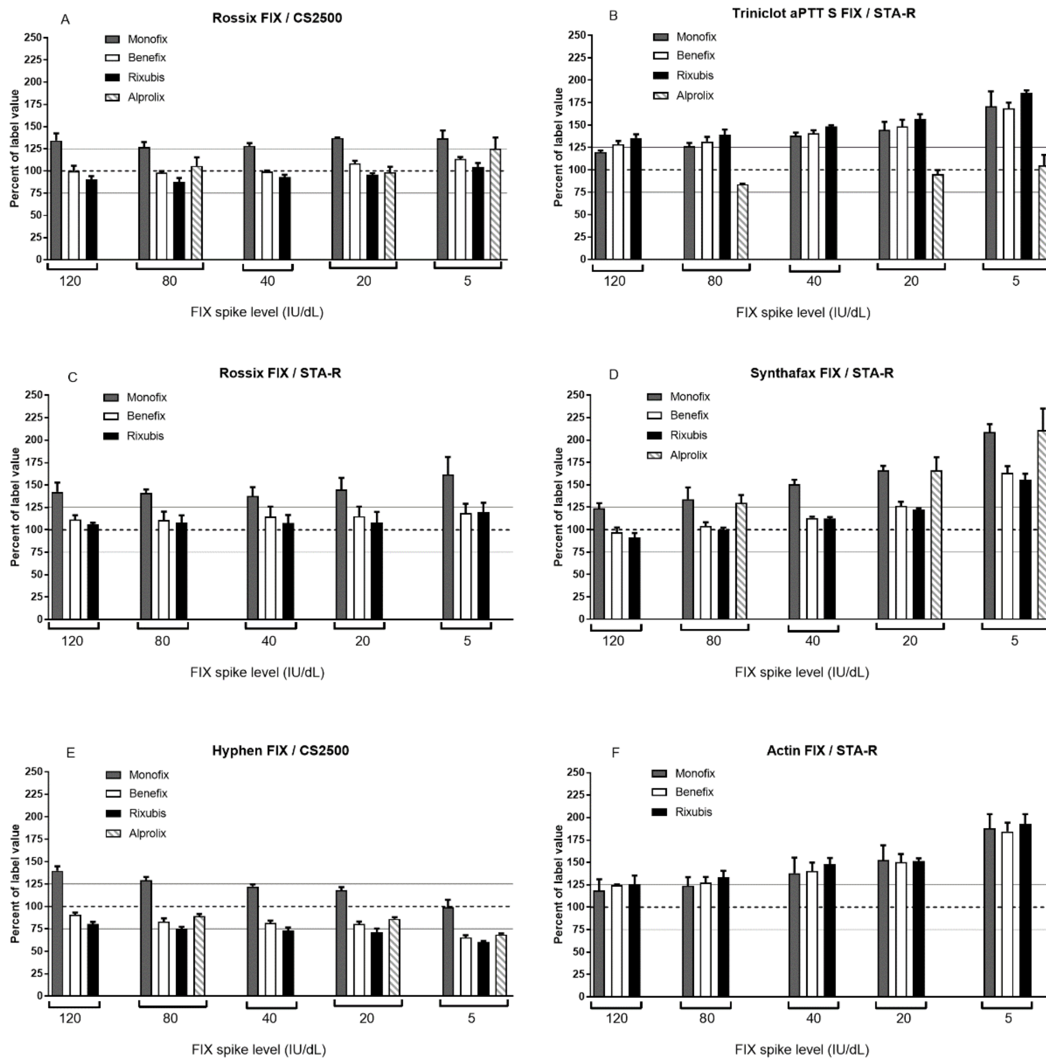


Figure 3: Recovery of FIX in spiked samples by chromogenic assays and one-stage clotting assays. Samples were spiked to five levels with plasma derived FIX (Monofix®), recombinant FIX (Benefix® and Rixubis®) or three levels with FIX-Fc (Alprolix®). Chromogenic assay/analyser combinations: Rossix/CS2500 (A); Rossix/STA-R (C); Hyphen/CS2500 (E). One-stage clotting assays were performed on the STA-R analyser with: Triniclot aPTT S reagent (B); Synthafax APTT reagent (D); Actin APTT reagent (F). Column heights are mean recoveries showing 1SD bar calculated from three independent runs for each product type, except for Alprolix® in panels A and E, where two independent runs were performed. The dashed line represents 100% of target recovery level. The solid lines represent at 75% and 125% of target recovery levels.

DISCUSSION

Currently FIX measurement for the diagnosis of haemophilia B and monitoring of FIX replacement therapy is almost exclusively performed by OSA, using various APTT reagents which differ in activator type and phospholipid composition, leading to variability in results. The FIX CSA may have potential advantages over OSA in both haemophilia B diagnosis and monitoring replacement therapies with fewer reagent choices and different methodology to OSA. Hence, this study was undertaken as a general evaluation of the Rossix and Hyphen CSA for FIX performed on the CS2500 and the Rossix test performed on the STA-R, which included comparison with OSA in recovery estimates of pdFIX and rFIX spiked samples.

Both upper and lower non-parametric reference interval limits by CSA appeared appropriate and were similar to limits for OSA performed on the same reference samples (Table 1). The lower limit of the reference interval (2.5^{th} percentile) by Rossix test on the STA-R was slightly higher than Rossix or Hyphen test limit on the CS2500 possibly related to use of a different calibration plasma or to differences in CSAs adaptations between automated analysers. All CSA assays showed high levels of precision making them suitable for use in haemostasis laboratories using these two analysers.

In the samples from 11 untreated HB individuals all three samples called severe by OSA were called severe by all CSA platforms. However, in five individuals classified as non-severe by OSA, there was reclassification by at least one CSA: from mild to moderate (4 samples) or moderate to severe (1 sample). These re-classifications were consistent with the negative bias of Rossix and Hyphen CS2500 tests relative to OSA as calculated from the total sample set. At low levels of FIX some discordance is reasonably expected between different FIX assay types of this complexity. There is also a limitation in drawing conclusions about discrepancies when a single OSA is used as a 'gold standard' in view of the known variability between different

OSAs related to APTT reagent differences [13]. Kilberg and colleagues recently reported discrepancies between CSA and OSA for measurement of native FIX in a sub-group of non-severe Haemophilia B with FIX by CSA observed to be more than twice the OSA level [14]. They speculated that this was related to a particular causative mutation. In our study, the discrepancies seen in untreated HB samples (samples 2 and 4, Table 3), occurred in the opposite direction, with a CSA value being less than half the OSA value. These are less likely to be related to causative mutations since there was a general trend for lower levels by CSA in this group, and the effect was not observed in all CSA protocols. Nevertheless, future planned studies of correlation with mutation status and bleeding phenotype may prove useful.

It is clinically important to have accurate recovery estimates of replacement products to prevent under- or over-dosing of patients with HB in the post-surgical setting and during routine prophylaxis. Therefore, assays are required where recovery estimates closely align with the assay used for potency assignment by the manufacturer. The recovery estimates in this study were interpreted against an arbitrary target of 100±25% of spike level. There was uniform over-recovery of pdFIX by all FIX assays in the current study, the reason for which is not clear. Recoveries of two rFIX products (Benefix® and Rixubis®) and the EHL product (Alprolix®) demonstrated that CSAs generally give closer and more consistent agreement to target levels compared to any of the three OSA protocols employed, where two of three APTT reagents tended to over-recovery for Benefix® and Rixubis® (Fig. 3). This pattern of similar recovery of pdFIX and discrepant recovery of rFIX products using plasma calibration standards between OSA and CSA by Hyphen test has been previously reported [7]. Consistent with the present study, in a collaborative study assessing post-infusion FIX assays, median CSA results were around 25% lower than median OSA results (*P*<0.01) with Benefix® [8]. In the present study, there was tendency for over-recovery of Benefix® and Rixubis® by the Rossix test on the STA-R (Fig 3c) in comparison to the CS2500 (Fig 3a), that could largely be explained by calibrator

differences. The Unicalibrator lots (Rossix CSA/STA-R) averaged 90.5% of assigned value for FIX relative to the 4th IS while SHP lots (Rossix/CS2500) averaged 96.3% of assigned value. These calibrator differences may have also contributed to the different lower reference interval limits for the Rossix test performed on the STA-R versus the CS2500 (Table 1). Alprolix®, Benefix® and Rixubis® are assigned potency by OSA against WHO International Standards for FIX concentrates [4,15,16] while commercial calibrators are assigned against WHO International Standards for plasma FIX, which is another potential source of variation between nominal spiked values and measured recovery. Within the field, such variability may be unavoidable as clinical laboratories routinely employ commercial calibrators for measurement of product recovery.

CSA may be preferable to OSA for a few reasons. Firstly, CSA showed better linearity in percent recovery over the full range of spike levels, with OSA tending towards greater over-recovery at the 5 IU/dL spike level. It is recognised that OSA may showed reduced linearity in FVIII assays at the low end of the curve due to relative dilution of calibration plasma compared with test plasma. This can be overcome by prediluting calibrator in FVIII deficient plasma and may differ according to buffer and APTT reagent [17]. A similar phenomenon may be contributing to the relative over-recovery in our study with FIX OSA. CSA may be less prone to this artefact. Better accuracy of CSA FIX trough levels may translate to more appropriate frequency of dosing during prophylaxis.

Secondly, having a single CSA that can measure all FIX replacement products including EHL-FIX-Fc using plasma standards would have practical benefits from the testing laboratory's perspective. These include removing the need for selecting specific APTT reagents for specific products, or use of product-specific calibrators. Similarly, in a recent study of EHL FIX product, N9-GP [18], recovery was deemed acceptable (±30% of target) at four spike levels

from 90 IU/dL to 3 IU/dL by both Rossix and Hyphen CSA, while for OSA, only two of eight APTT reagents (Synthafax and DG Synth) gave acceptable recovery at 90 IU/dL and 60 IU/dL. The same study also found recovery of samples spiked with Benefix® was acceptable by the Rossix CSA at all four spike levels and acceptable by the Hyphen CSA at the 90 IU/dL and 60 IU/dL with the Hyphen kit. Percent recoveries of Benefix® were similar to, though generally lower than recoveries in this study including observed higher recovery by the Rossix test relative to the Hyphen test. The differences between the studies may relate to the calibration plasma used (SSC lot 4 versus Unicalibrator /SHP), and a different analyser type at one of two sites. The over-recovery of N9-GP by most silica-based APTT reagents has recently been explained by Rosen et al, who found that N9-GP was prematurely converted to activated FIX (FIXa) during the contact activation phase of the clotting assay [19]. Another recent 3-site study measuring recovery of N9-GP at four spike levels using Rossix and Hyphen CSA concluded these assays were able to measure N9-GP using a ±30% of target as acceptable [20]. The suitability of CSA for measuring the third current EHL product, rFIX-Fp remains unclear [21]. Thirdly, there are many different APTT reagents used in OSA compared to the two available CSA, potentially allowing better assay consistency between laboratories where CSAs are adopted. A field study of laboratories using CSA for FIX product recovery estimates would be useful in establishing the consistency of CSAs and selected OSAs in different sample types.

The important issue of cost needs to be considered before the introduction of a new assay. Our cost per test estimates were similar between all CSA protocols. In this study assays were performed on freshly reconstituted and loaded reagents run to vial completion, but in the ‘real world’ of haemostasis testing, the cost per patient sample would be higher as vials would rarely be used to completion. A recent study modelling potential usage patterns of FIX CSA

kits suggests costs per patient test could be reduced by batch testing of samples combined with aliquoting and freezing unused kit reagents [22], though validation of freeze-thaw strategy was not within the scope of the current study. In addition, when properly validated, the use of stored calibration curves creates another option for reducing the cost per test of CSAs.

The Rossix and Hyphen CSA overall performed well in accurately measuring the spiked level of recombinant and EHL product across all levels of spiked FIX, with some minor differences. Of the protocols evaluated here the Rossix test performed on Sysmex CS2500 gave the best overall performance in spike recovery studies.

CONCLUSION

The Rossix and Hyphen chromogenic assays for FIX can be adapted to Stago and Sysmex coagulation analysers and calibrated with plasma standards. The two CSAs for FIX evaluated here show good basic performance characteristics with respect to determination of reference intervals, test reproducibility, adaptability to automated analysers, detection of FIX deficiency, and in the measurement of different types of FIX replacement products.

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DISCLOSURES

GK has received a speaking fee from Biogen

LK has received a speaking fee from Biogen

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CHAPTER 4

An Australian field study validating the Rossix chromogenic FIX assay for measuring FIX replacement products

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CHAPTER PREFACE

This chapter builds on the previous chapter evaluation chromogenic FIX assays. This study was aimed at establishing the assay in an additional four interstate haemophilia reference laboratories. Validation of FIX assays was achieved through supplying the laboratories with the Rossix chromogenic FIX kits, protocols for implementing the assay on their coagulation analysers, and assessing interlaboratory agreement by means of a large centrally prepared plasma sample set. The sample set included normal FIX from donors, commercial calibrators and control, recombinant standard half-life FIX and extended half-life FIX. General issues around the use of chromogenic FIX assays are discussed.

Geoffrey Kershaw designed the study, prepared the samples sets, prepared analyser protocols and test instructions for distribution to each site, tested the samples, statistically analysed the results and wrote the chapter plus a study report distributed to the participant laboratories and the Australian Haemophilia Centre Directors Organisation (AHCDO). Vivien Chen and Liane Khoo contributed to setting up the study with products and funding, study design reviewing the results and study write-up. The study received funding from the John Lloyd Clinical Excellence Fund of AHCDO.

ABSTRACT

Background. Chromogenic assays for FIX have been used for the measurement of FIX standard half-life (SHL) and extended half-life (EHL) FIX replacement products. This study aimed to establish protocols in selected Australian reference laboratories and validate its use in measuring both spiked plasma samples and ex vivo plasma samples containing FIX replacement products.

Methods. Rossix chromogenic FIX kits were bulk purchased for use in five haemophilia reference laboratories in five Australian states. Each laboratory tested a 17-sample set of frozen plasma samples on each of three days. Samples sets contained SHL FIX products Benefix and Rixubis, EHL product Alprolix, diluted commercial calibrator and control plasma, and plasma from healthy volunteers.

Results. All laboratories successfully adapted the assay to their analysers. Chromogenic FIX assay levels showed good mean recovery levels for all spiked sample types across the six protocols in five laboratories. Mean recoveries were within 25% of target for the 20 IU/dL and 80 IU/dL spiked samples. Between-laboratory reproducibility was excellent for all samples of 20 IU/dL or higher, with CVs in the range 3%-10%, which included spiked samples and ex vivo samples. The ex vivo samples for normal donors and patients receiving Benefix or Alprolix had similar variability to spiked samples at equivalent levels.

Conclusion. The Rossix chromogenic FIX assay was successfully adapted to three different haemostasis analyser types, The STA-R series, Sysmex CS, and ACLTOP. The FIX chromogenic assay can potentially be used to measure SHL and some EHL FIX products, and to detect discrepant phenotypes in haemophilia B.

INTRODUCTION

Congenital haemophilia B (HB) is an X-linked bleeding disorder where defects in the factor IX (FIX) gene lead to absence or deficiency of clotting factor IX (FIX) (1). In recent decades therapy for HB has largely been through the intravenous infusion of standard half-life (SHL) FIX concentrates, either plasma derived or (r)ecombinant FIX, given prophylactically or on demand. The most widely used assay for measuring FIX activity levels in plasma is the one-stage APTT-based clotting assay (OSA) (2-4). The assay is applied both as a diagnostic test for haemophilia B (HB), and for monitoring levels of replacement FIX. The HB severity is classified by OSA as severe (levels <1 IU/dL), moderate (1 to <5 IU/dL) or mild (5-40 IU/dL) by international guidelines (5). In recent years, two chromogenic substrate assays (CSA) for FIX have become commercially available and can be adapted to run on automated haemostasis analysers (6). Chromogenic FIX assays may offer an alternative to the OSA for monitoring FIX levels of SHL and extended half-life (EHL) FIX products. The FIX CSA test kits from Rossix (Rossix AB, Mölndal, Sweden) and Hyphen (Hyphen-Biomed, Neuville-sur-Oise, France) in a general evaluation were found to have good reproducibility, and showed acceptable recovery estimates in spiked samples containing the SHL rFIX products Benefix (Pfizer, New York, USA), Rixubis (Takeda, USA) and the EHL Alprolix (FIX-Fc, Sanofi-Aventis) (7). There is variability in measurement accuracy of different rFIX EHL products by both OSA and CSA. Some extended half-life (EHL) rFIX products have also become available in recent years, with their use differing according to geographic region. The EHL FIX products are the Fc fusion protein Alprolix, Idelvion (rFIX-FP, CSL Behring) and a pegylated product Refixia (FIX-GP, Novo Nordisk). Other studies have shown FIX CSAs suitable for estimating FIX levels of Refixia and Alprolix with some differences between kits (6, 8, 9). The measurement of Idelvion, however, is overestimated by CSA , and to varying degrees all three FIX EHL products have limitations as to which APTT reagents are acceptable for use in OSA.(6, 9) In the context of the potential introduction of new EHL FIX replacement products into Australia

the FIX chromogenic assay may play a role in monitoring these products, for example where locally used APTT reagents do not give accurate recovery estimates. Alprolix is currently in use in Australia for HB replacement therapy, while Refixia and Idelvion have been limited to clinical trial subjects.

Because the FIX CSA not currently used clinical haemostasis laboratories in Australia, this study aimed to introduce and validate the Rossix FIX assay in five haemophilia reference laboratories by means of a field study measuring FIX SHL and EHL products and normal plasma samples. This required assisting participant reference laboratories to establish the assay on their coagulation analysers, followed by testing a large diverse sample set to assess both accuracy and inter-laboratory agreement in assayed levels of chromogenic FIX. The study was coordinated by the Institute of Haematology at Royal Prince Alfred Hospital (central laboratory) in conjunction with Anzac Research Institute Concord, University of Sydney.

METHODS

Test samples and FIX replacement products

Test samples consisted of a mixture of ex vivo samples, samples spiked to specific target levels and a control plasma. Samples characteristics of the 17-sample set are summarised in Table 1. The ex vivo samples came two from two healthy volunteers and two patients with severe HB receiving prophylaxis with either Benefix or Alprolix. The HB donors had sample collected at

Table 1. Summary of sample types contained in the sample sets sent to participants.

Sample ID	Sample type	Source/level
F9-1	Healthy volunteer	Ex vivo plasma of unknown FIX level
F9-2	Healthy volunteer	Ex vivo plasma of unknown FIX level
F9-3	Siemens N control	Commercial control. FIX target of 110 IU/dL
F9-4	Benefix	Spiked to 80 IU/dL
F9-5	Rixubis	Spiked to 80 IU/dL
F9-6	Benefix	Spiked to 20 IU/dL
F9-7	Rixubis	Spiked to 20 IU/dL
F9-8	Benefix	Spiked to 5 IU/dL
F9-9	Rixubis	Spiked to 5 IU/dL
F9-10	Benefix	Spiked to 1 IU/dL
F9-11	Rixubis	Spiked to 1 IU/dL
F9-12	Siemens SHP	Spiked to 1 IU/dL
F9-13	Alprolix	Ex vivo sample peak level
F9-14	Alprolix	Ex vivo sample trough level
F9-15	Benefix	Ex vivo sample trough level
F9-16	Benefix	Ex vivo sample peak level
F9-17	Siemens SHP	Spiked to 5 IU/dL in FIX deficient plasma

SHP, Standard Human Plasma

two time points each, representing trough and peak FIX levels. Donors gave informed written consent, and the study was approved by the Research Ethics and Governance Office of RPAH (Protocol X18-0535). Vials of standard half-life rFIX products Benefix (Lot X38492, 250 IU) and Rixubis (Lot TNA17007AJ, 250 IU) were purchased from the Pfizer and Takeda,

respectively. Benefix and Rixubis vials were reconstituted as per insert instructions then spiked into congenital FIX deficient plasma (George King Biomedical, Overland Park, Kansas, USA) in two steps to reach final concentrations of 1, 5, 20 and 80 IU/dL. Dilutions were calculated using the lot-specific potency values, which differed from the nominal value of 250 IU/vial. It was originally planned to include Refixia samples in the study, but it was not possible to obtain the material to do the spiking. Vials of Siemens Standard Human Plasma (SHP Lot 503265, Siemens, Marburg. Germany) were spiked into FIX deficient plasma to final concentrations of 1 and 5 IU/dL for additional assessment of low FIX level recoveries versus spiked product samples. Vials of Siemens (N)ormal control plasma (Lot 507761) was reconstituted, pooled and aliquoted as an additional normal sample. Its FIX target was the package insert value of 110 IU/dL. Cryovials of plasma were rapidly frozen by placing into a minus 80°C freezer where they remained until shipping by overnight transport on dry ice.

Chromogenic FIX kits and test protocols

A single lot of Rossix chromogenic FIX kits were bulk purchased by the central laboratory (RPAH) for distribution to the participating haemophilia reference laboratories. Four interstate laboratories were invited to participate in the study. The invited laboratories had no previous experience with chromogenic FIX assays. In total, samples were tested on six platforms: four Stago Sta-R series analysers, one Sysmex CS2500 analyser and one ACLTOP700 analyser. Analyser-specific assay protocols were sent to each site with the kits to assist implementation of the assay. The protocols used for the Sysmex CS2500 ‘high’ and ‘low’ curves and the STA-R ‘high curve’ were as previously used in the Chromogenic FIX evaluation.(7) For better precision, Sta-R series users were asked to run samples with values expected to be <10 IU/dL at 1/20 dilution, as opposed to the standard 1/80 dilution used for higher FIX level samples. Laboratories were asked to report FIX levels correct to one decimal place. The protocol for the

ACLTOP was obtained from the kit distributor. Participants were given sufficient Rossix kits to allow for initial familiarisation and practice runs as well as testing the survey samples. Some technical advice was supplied on reagent handling, a recommendation to use fresh calibration on each day of testing, and to complete testing within three hours of loading reagents. All sites except the central laboratory were blinded as to the contents of the samples. The FIX calibration values were from the plasma calibrator package insert by OSA method, since separate chromogenic assigned values were not available. Interstate shipping of chromogenic FIX kits was by overnight transport in insulated packaging containing freezer packs.

FIX clotting assays

The central laboratory (RPAH) performed FIX assays on the 17-sample set by three different OSA protocols on each of three days for comparative analysis to the chromogenic results. Protocols were: (i) TriniClot aPTT S reagent with Helena FIX deficient plasma on the Sta-R analyser; (ii) Synthasil APTT reagent with Helena deficient plasma on the Sysmex CS2500 analyser; (iii) Actin FS APTT reagent with Siemens deficient plasma in the Sysmex CS2500 analyser. All OSAs were calibrated with SHP.

Statistics

Descriptive statistics were performed in Microsoft Excel and GraphPad Prism V6. The focus was mainly on mean and percent product recoveries relative to target values, plus precision studies using coefficients of variation.

RESULTS

Assay implementation and excluded data

All four interstate sites reported receiving sample sets in frozen condition. Two interstate sites reported no issues with the chromogenic assay test set-up or calibration and processed the three sets of samples on different days as instructed. A third site had initially implemented an incorrect protocol causing failure of calibration. After consulting the central laboratory, the correct protocol was implemented after which calibration was successful, and the samples processed without further issues. The remaining site calibrated the test successfully but used incorrect dilution (1/80 instead if 1/20) on seven results with values around 5 IU/dL. These seven data points were excluded as a group from the analysis due to potential lack of accuracy. The same site reported in whole numbers instead on one decimal place, causing some loss of precision noticeable mainly in the 1-5 IU/dL FIX range. This data was not excluded. All chromogenic FIX protocols were calibrated with their current lot of SHP and all sites tested the 17-sample sets on each of three different days.

Assays precision

Between assay CVs for CSA for spiked samples or ex vivo samples with FIX values ≥ 20 IU/dL ranged between 5.7% and 11.2% (Figure 1). The CVs for the OSA on the same samples were slightly lower in all cases, ranging from 2.1% to 9.3%. The CSA CVs on four samples between 3.7 and 5 IU/dL ranged from 9.3% to 16.6%. The highest CSA CVs of 35.3% and 40.7% were predictably at the lowest spiked level of 1 IU/dL for Benefix and Rixubis, respectively (Figure 1). The EHL Alprolix was measured at peak and trough levels in ex vivo samples with between assay CVs of 10.0% and 16.6%, respectively, higher than for Benefix and Rixubis at similar levels, largely caused by a single result of three runs being discordant at one site.

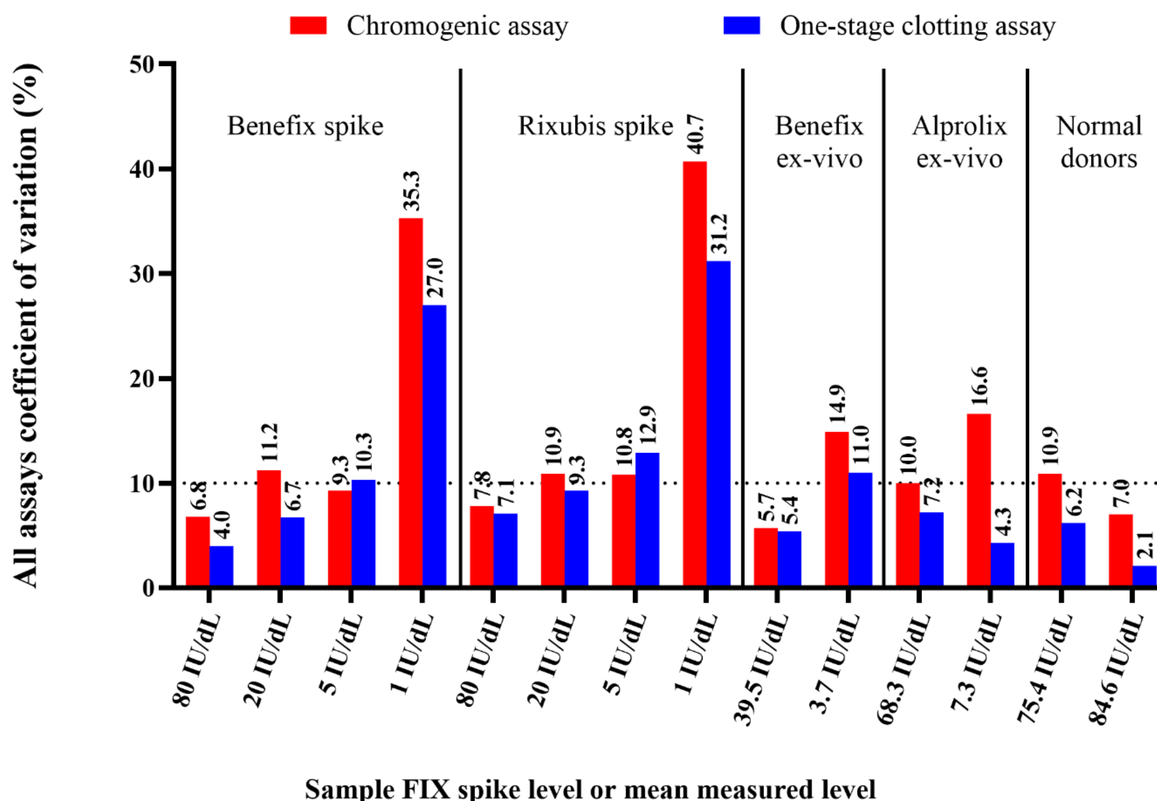

Figure 1. Inter-assay reproducibility for chromogenic and one-stage FIX assays in Benefix/Rixubis-spiked samples and ex vivo samples. Coefficient of variation values above each bar were calculated from all individual runs: 15-18 runs from six different sites/assays for chromogenic tests; 9 runs for one-stage assays from the central laboratory.

FIX chromogenic assay recoveries of spiked samples

Mean FIX CSA recovery for SHL product Benefix at 80 IU/dL and 20 IU/dL were 66.3 IU/dL and 17.7 IU/dL, representing 88.5% (range 74.4-98.9%) and 88.2 % (range 76.5-107.0%) of the spike targets (Table 2). Rixubis had similar mild underestimation of spike levels with corresponding mean recoveries of 67.3 IU/dL and 17.3 IU/dL, representing 84.1% (range 74.3-96.8%) and 86.7 % (range 73.0-102.0%) of the spike targets. For the N control plasma and the SHP spikes of 5 IU/dL and 1 IU/dL mean CSA recoveries were close to target at 106.6, 5.1 and 1.2 IU/dL, respectively (Table 2). The distribution of individual assay points as a percent of target is shown in Figure 2 (Panel A), and as IU/mL in Figure 3. This data shows good linearity

of CSA across the range of values from 80 IU/dL to 1 IU/dL, with a marked increase in spread of values at the 1 IU/dL level.

FIX one stage assay recoveries of spiked samples

The Benefix and Rixubis mean recoveries in the spiked samples of 80, 20 and 5 IU/dL were uniformly above the target levels for both products with mean recoveries ranging from 118.8% for Benefix at 80 IU/dl to 136.2% for Rixubis at 5 IU/dL (Table 2). Of the three individual OSA protocols the two from the Sysmex analyser gave good linearity for the full range of spike levels with mean recoveries within 20% to 30% of target (Figure 2, Panels B, C). The Sta-R protocol showed a definite upward trend with decreasing spike level, with the 5 and 1 IU/dL showing over-recovery (Figure 2, panel D).

CSA/OSA ratios in ex-vivo samples and control samples

Distinct patterns were seen in the CSA/OSA ratios related to sample type. Mean CSA/OSA in healthy volunteer samples 1 and 2 and the N control sample were at unity (range 0.99-1.01) (Table 2). Spiked Benefix and ex vivo Benefix samples had similar CSA/OSA ratios, in the range 0.66-0.78 for all levels. In contrast, Alprolix measurements were higher by CSA than OSA, with the CSA/OSA equal to 1.28 in both peak and trough samples.

Table 2. Factor IX levels for the Rossix chromogenic substrate assays and one-stage assays expressed as means and percent of target. The chromogenic assays are from 15-18 individual runs across six test systems. One-stage assays are from nine runs across three test systems.

		Chromogenic Assays		One-Stage Clotting Assays		
Product/ Sample type	Factor IX target IU/dL	Mean (range) IU/dL	Mean (range) % of target	Mean (range) IU/dL	Mean (range) % of target	Mean ratio CSA/OSA
Benefix	80	66.3 (59.5-79.1)	82.9 (74.4-98.9)	89.4 (83.8-93.6)	111.8 (104.8-117.0)	0.74
	20	17.7 (15.3-21.4)	88.5 (76.5-107.0)	23.7 (21.3-26.5)	118.7 (106.5-132.5)	0.75
	5	4.3 (3.7-5.1)	85.3 (74.0-102.0)	6.5 (5.9-7.9)	130.3 (118.0-158.0)	0.65
	1	0.9 (0.3-1.5)	94.4 (30-150)	1.4 (1.1-2.1)	144.1 (106.7-210.0)	0.66
Rixubis	80	67.3 (59.4-77.4)	84.1 (74.3-96.8)	96.7 (87.7-107.5)	120.8 (109.6-134.4)	0.70
	20	17.3 (14.6-20.5)	86.7 (73.0-102.0)	24.9 (21.9-29.6)	124.6 (109.5-147.8)	0.70
	5	4.2 (3.6-5.3)	84.0 (72.0-106.0)	6.8 (5.6-8.2)	136.2 (112.0-164)	0.62
	1	0.9 (0-1.4)	86.7 (0-140)	1.4 (1.0-2.2)	144.8 (100.0-220.0)	0.60
N control	110	106.6 (93.6-116.1)	96.9 (85.1-105.5)	105.4 (101.1-112.7)	95.8 (91.9-102.5)	1.01
SHP	5	5.1 (4.3-6.1)	101.7 (86.0-122.0)	6.0 (5.5-7.3)	120.3 (110.0-146.0)	0.85
SHP	1	1.2 (0.3-2)	116.1 (30-200)	1.3 (1.0-2.1)	133.3 (100.0-210.1)	0.87
Healthy volunteer 1	N/A	75.4 (57.8-90.0)	N/A	75.8 (71.5-85.5)	N/A	0.99

Healthy volunteer 2	N/A	84.6 (72.3-97.0)	N/A	83.9 (81.7-86.8)	N/A	1.01
HB/Benefix	Trough	3.7 (3.0-4.9)	N/A	4.9 (4.4-6.0)	N/A	0.76
HB/Benefix	Peak	39.5 (34.7-43.0)	N/A	50.4 (46.6-54.3)	N/A	0.78
HB/Alprolix	Trough	7.3 (5.6-11.0)	N/A	5.7 (5.4-6.1)	N/A	1.28
HB/Alprolix	Peak	68.3 (48.0-81.4)	N/A	53.3 (46.7-59.1)	N/A	1.28

CSA, chromogenic substrate assay; OSA, one-stage clotting assay; N/A, not applicable; HB, severe haemophilia B receiving prophylaxis; SHP, Standard Human Plasma

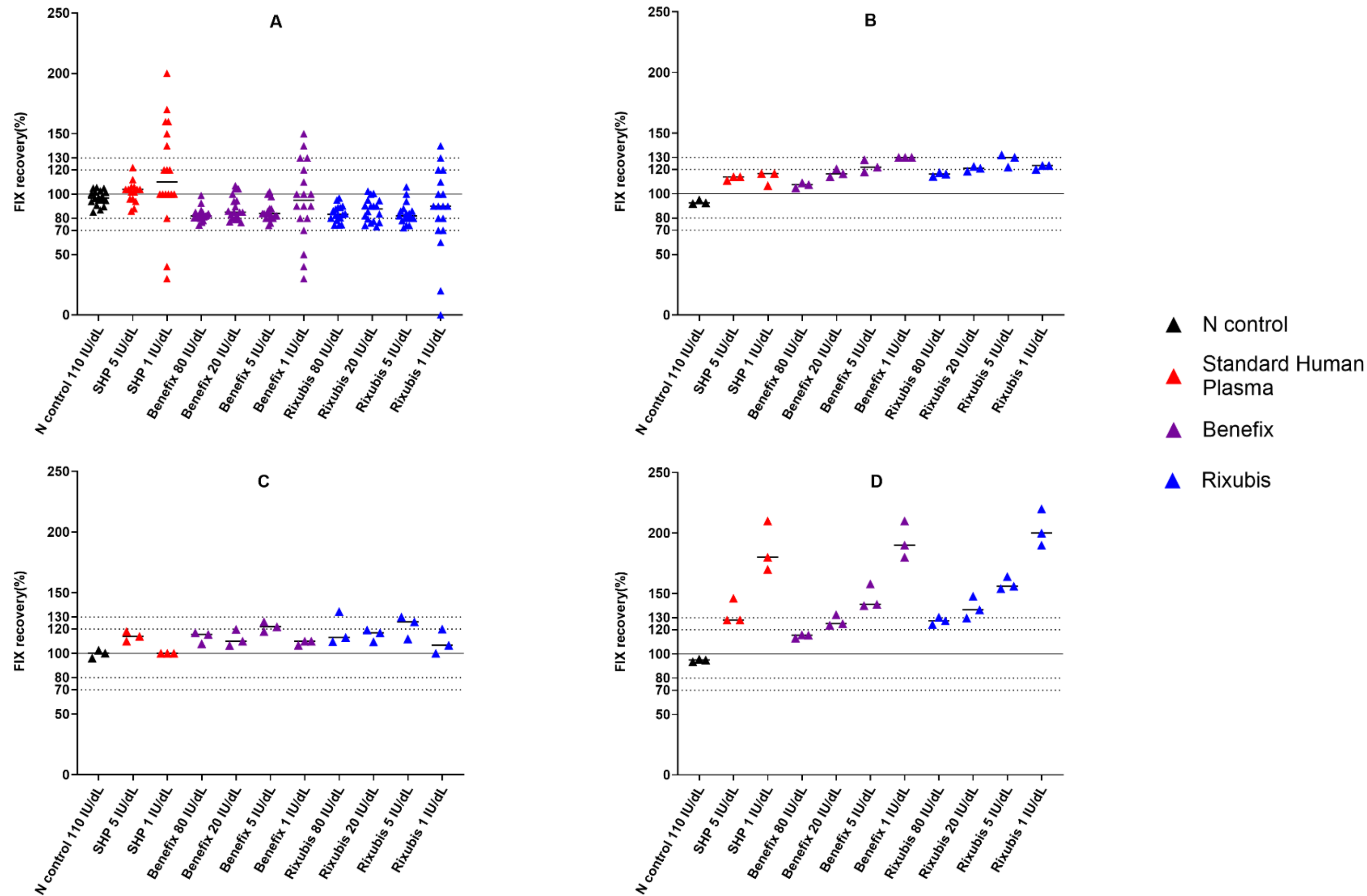

Figure 2. Distribution of individual FIX measurements as a percentage of target values. A: combined chromogenic FIX data with Rossix kit from six test systems showing individual daily runs (n=15-18). B: One-stage assay with Sysmex CS2500 and Synthasil APTT reagent; C: one-stage assay with Sysmex CS2500 and Actin FS APTT reagent. D: One-stage assay with Sta-R and Triniclot aPTT S.

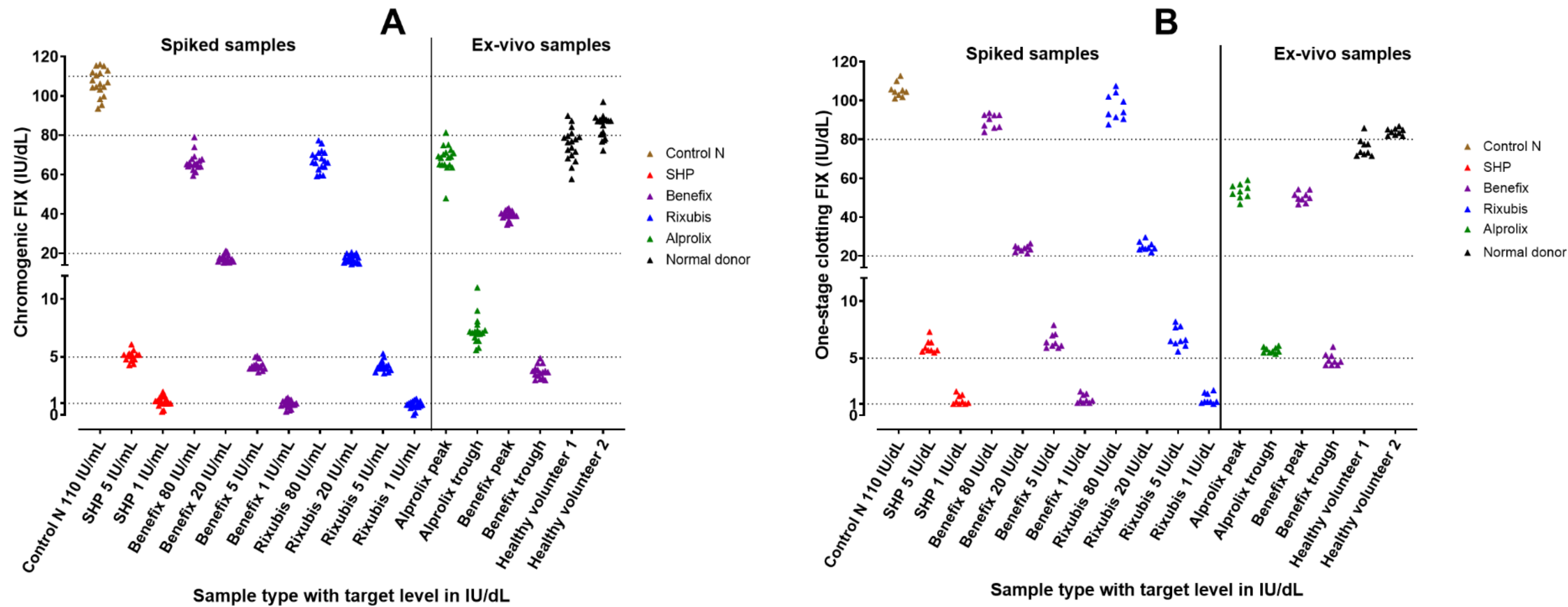

Figure 3. Distribution of individual measurements in absolute values by: **(A)** chromogenic FIX assay using the Rossix kit in five laboratories using six protocols, and **(B)** three one-stage FIX assay protocols at the central laboratory. Three independent measurements were performed for each protocol. The horizontal lines represent the target values for the spiked samples and the N control plasma.

DISCUSSION

The therapeutic options for treating HB have expanded in recent years beyond plasma-derived FIX and SHL rFIX to include three rFIX EHL products Alprolix, Refixia and Idelvion. All may replacement therapies require periodic monitoring of as part of regular surveillance, during surgery, or when there are unexpected bleeding episodes. The OSA has been the main assay for this monitoring, but with some caveats, such as varied restrictions on which APTT reagents can be used to measure each of the EHL products. The FIX CSA is a relatively recently introduced method that can measure some of these post-infusion products with acceptable accuracy where the locally used OSA is not suitable. The current study introduced and validated the Rossix FIX chromogenic assay into several haemophilia reference laboratories by means of a field study, which incorporated multiple FIX plasma samples including wild type, commercial standards, SHL and EHL rFIX. The CSA could then complement the laboratory's OSA in the panel of tests used for diagnosis of HB and monitoring of replacement products. The choice of the Rossix kit for this purpose was based on its previous all round performance on different platforms in a general evaluation (7). The key outcomes from this study were demonstration of adaptability of the Rossix FIX CSA to three different types of commonly used haemostasis analysers in haemophilia reference laboratories without prior exposure to the assay. Only minor only minor teething problems were encountered from some sites.

The CSA interlaboratory reproducibility was acceptable, with CVs between 6.8% and 10.9% for samples with plasma levels were ≥ 20 IU/dL, including from healthy volunteer samples, which was comparable with recent CVs averaging 8% -13% from an external quality assurance program for FIX CSA of 45 result sets testing samples at comparable FIX levels (10). For Alprolix specifically, inter-laboratory CVs for peak and trough levels were 10.0% and 16.6% respectively, comparable to CVs from another field study of CSA for Alprolix of 8.6% and 17.9% for spiked samples of 80 IU/dL and 5 IU/dL, respectively, from 11 participants using

Rossix CSA (11). Fortuitously, all participants in the study used SHP calibrator, which would reduce variability associated with multiple brands of calibrator being used.

Mean CSA recoveries for Alprolix by Rossix CSA (n=3) of 104.5, 115.0 and 108.0% of target for 80, 20, and 5 IU/dL spiked samples, respectively, were found by Sommer et al (11). The current study samples for Alprolix were ex vivo, therefore without specific targets, but our previous evaluation of spiked Alprolix samples measured by Rossix CSA showed similar recoveries of 106, 99 and 125% at the same spike levels (7). It has previously been shown that samples prepared in vitro from Alprolix, Idelvion and Refixia concentrates are commutable with samples taken from patients treated with these products (12, 13).

The CSA/OSA ratios for ex vivo healthy volunteer samples and the N control, were very close to unity. For Benefix and Rixubis the CSA/OSA was in the range 0.70-0.76 in samples of 20 IU/dL or above, and for Alprolix the ratio was 1.28. These differences suggest different behaviour between these SHL and EHL products in FIX chromogenic assays relative to wild type FIX, and how modifications used to extend the half-life change assay results. As all three FIX concentrates have potency assignment by OSA against a FIX concentrate standard, (refs) the differences may reflect different interactions with the FIX CSA components relative to FIX from normal donors. One caveat here is the ratios were based on the means of only three OSA protocols. Nevertheless, the CSA mean percent recoveries of Benefix and Rixubis were within the acceptable limits of 20% of target for levels above 30 IU/dL and 30% of target for lower levels 10-30 IU/dL suggested in published guidelines by Gray et al. (14).

There is potential role for FIX CSA in full characterisation of patients with HB in terms of detecting OSA/CSA assay discrepancies. The classical discrepancy is for a lower CSA and reverse discrepancy is for a higher CSA. Discrepant phenotypes have been well described for non-severe haemophilia A (HA), where OSA and CSA can differ by a factor of two or more,

which has diagnostic and bleeding risk implications for these individuals, with the consensus that clinical phenotype usually correlates better with CSA (15-18). Evidence of such discrepancies in FIX levels is slowly emerging (4, 7, 19), and suggests that The study of Kihlberg et al. reported 15 samples from eight patients having a reverse assay discrepancy with mean FIX by OSA of 2 IU/dL and mean CSA of 6 IU/dL, with six of these patients having the same mutation at the N-terminal cleaving site of the activating peptide (4). Their bleeding frequency was indicative of a mild bleeding phenotype. Schmidt et al. reported FIX:C by CSA with the Hyphen kit was consistently lower than by OSA in individuals with non-severe HB (19). Ten of 17 individuals with defined mutations would have more severe haemophilia sub-type if classified by CSA relative to classification by OSA. Classification by OSA better matched the patient's clinical bleeding phenotype (19). Reclassification of HB severity was also seen in several individuals during the general evaluation of FIX CSAs, with the number of re-classification higher with the Hyphen assay relative to the Rossix assay (7). Current World Federation of Hemophilia guidelines, published in 2020, recommend both FVIII CSA and OSA be used for assessment of patients with suspected HA (20). For suspected HB assessment FIX by OSA only is recommended. This may change when more data on assay discrepancy becomes available.

A limitation of the study is the smaller number of participants in comparison to other field studies (9). This was partly due to the time and funding limits for sending CSA test kits to participants and logistics of preparing and shipping large frozen sample sets. Secondly, the study did not include an assessment of the EHLs Refixia or Idelvion, both due to lack of drug availability for preparing spiked samples. A previous global field study by Nederlof et al. showed Refixia can be satisfactorily assayed by Rossix FIX CSA with a median recovery from 19 participants of 93% of target at 60 IU/dL 100% of target at 6 IU/dL (9). Further, nearly all APTT reagents for OSA are unsatisfactory for measuring Refixia, with large under- or

overestimations noted, unrelated to class of activator, which could pose a clinical risk to patients (9, 21). Tiefenbacher and co-workers found Refixia can measured to within ±30% of target at four levels from 5 IU/dL to 120 IU/dL in a 3-site study (22).

CONCLUSIONS

This study has shown that the chromogenic FIX assay can be adapted to three different haemostasis analyser types, The STA-R series, Sysmex CS, and ACLTOP. There was good agreement between laboratories for measurement of normal FIX, Benefix and Rixubis. Assay precision was acceptable, and in agreement with previous studies. The ex vivo samples for normal donors and patients receiving Benefix or Alprolix did not differ in variability of results obtained when compared to spiked samples containing the same FIX types. The FIX CSA provides an alternate method of post-infusion monitoring of rFIX and some EHL rFIX replacement products.

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CHAPTER 5

Australian comparative field study evaluating the activity of recombinant factor VIII Fc fusion protein (Eloctate®)

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CHAPTER PREFACE

This chapter has been published as:

> Kershaw G, Chen VM, Cai N, Khoo TL. Australian comparative field study evaluating the activity of recombinant factor VIII Fc fusion protein (Eloctate®) Haemophilia 2020:26(5)

The long acting FVIII replacement therapy Eloctate® was introduced selectively in Australia from 2018. This was the first extended half-life FVIII product approved for use. This field study of 16 participants was conducted to allow local laboratories to locally assess both OSA and CSA suitability in measurement of the EHL product Eloctate, coinciding with its introduction in Australia.

Geoffrey Kershaw did most of the work including designing the study, preparing the samples for distribution to the testing laboratories, sample testing, result analysis, and writing the publication. Assistance was received from Liane Khoo for obtaining product and reviewing results. Nancy Cai assisted in sample preparation and testing. Vivien Chen reviewed the results and write-up of the publication.

ARTICLE

Current treatment of individuals with haemophilia A (HA) is predominantly by infusion of replacement (F)actor VIII products. The recent introduction of modified recombinant (r) FVIII products with extended half-life (EHL) adds another potential source of variability in estimates of FVIII recovery dependent on how each EHL product interacts with the components of local factor assays, being one-stage assays (OSA) or chromogenic substrate assays (CSA). Haemostasis laboratories calibrate their assays with plasma standards calibrated to WHO Standard. The EHL product Eloctate (rFVIII-Fc, Bioverativ Therapeutics Inc CA, USA) is a recombinant fusion protein composed of a single molecule of B-domain–deleted rFVIII covalently linked to the human IgG1 Fc domain. There is a single published field study assessing recovery of Eloctate in spiked plasma which concluded Eloctate can generally be measured by OSA or CSA without need of a product-specific calibrator, with slightly higher results expected by CSA. (1). The study also found significant variability in the ratio of CSA to OSA in rFVIII-Fc recovery within individual laboratories performing both assays but did not separate data according to the specific APTT reagent or chromogenic kit used. The Eloctate study employed a recovery level of within 25% of target as being acceptable, and a later review of EHL product measurement has also suggested accuracy of measurement to equate to within 25% of target (2). With the recent introduction Eloctate into Australia, we conducted field study aimed at allowing local haemostasis laboratories to assess the suitability of their OSA and CSA protocols for Eloctate measurement, using the standard half-life product Advate as a comparator. Acceptable recovery was set to an arbitrary level of 25% in line with the previous publications.

Eloctate was supplied by Bioverativ, and Advate (Baxalta, Westlake Village, CA, USA) was purchased. Study samples were prepared in central laboratory (Royal Prince Alfred Hospital)

by spiking reconstituted product into plasma congenitally deficient in FVIII (George King Bio-Medical, Overland Park, KS, USA) to nominal concentrations of 80, 20 and 5 IU/dL, dispensed into coded cryovials and then immediately frozen at −80ºC. The actual spike values as calculated from the lot-specific potency assignments of each product were 84.5, 21.1 and 5.3 IU/dL for Eloctate and 82.6, 20.6 and 5.2 IU/dL for Advate. Sample sets were shipped frozen to 15 specialist haemostasis laboratories with instructions to assay one set for FVIII on each of three days by their usual method(s). Sixteen laboratories submitted OSA results, 12 of which also provided CSA results. Across participants there were six different APTT reagents, two brands of chromogenic assays, three brands of plasma calibrators and four sources of FVIII-deficient plasma. Data for CSA at the lowest spike level of Eloctate and Advate from one participant laboratory were excluded as outliers.

For OSA, mean recoveries for Eloctate spike levels of 84.5, 21.1 and 5.3 IU/dL were 73.1, 21.0 and 6.7 IU/dL, respectively (Table 1). This corresponds to 86.5%, 99.6% and 126.4% recovery of the spike product. For Eloctate measured by OSA, all 16 laboratories had mean recoveries within 25% of target for the 84.5 and 21.1 IU/dL samples, meaning all six APTT reagents showed acceptable performance (Figure 1A). About half the laboratories had over-recovery of Eloctate at the 5.3 IU/dL spike level. For OSA, mean recoveries for Advate spike levels of 82.6, 20.6 and 5.2 IU/dL were 87.1, 25.1 and 7.7 IU/dL, respectively (Table 1), corresponding to 105.4%, 121.8% and 148.1% recovery of spiked product. Though higher than for Eloctate, the majority of individual laboratory Advate means were within 25% of target at 82.6 and 20.6 IU/dL spike levels (Figure 1B). For CSA, mean recoveries for Eloctate levels of 84.5, 21.1 and 5.3 IU/dL were 99.9, 25.3 and 6.4 IU/dL, respectively, representing recovery of 118.2%, 120.0% and 120.8% of spike level (Table 1). The median (range) CSA/OSA ratios for Eloctate at 84.5 and 21.1 IU/dL were 1.44 (0.89-1.59) and 1.25 (0.86-1.65), respectively (Table 1). Inter-

laboratory CVs for OSA across all six spiked samples ranged from 11.6% to 18.7% and for CSA ranged from 10.1% to 23.4%.

Table 1. Mean levels for Eloctate and Advate spiked samples measured by one-stage assays and chromogenic assays

		Eloctate			Advate		
Spike level (IU/dL)		84.5	21.1	5.3	82.6	20.6	5.2
Clotting assays	n	Mean (range) IU/dL	Mean (range) IU/dL	Mean (range) IU/dL	Mean (range) IU/dL	Mean (range) IU/dL	Mean (range) IU/dL
Triniclot aPTT S	6	74.5 (66.9-86.3)	21.5 (20.0-23.0)	6.8 (5.2-7.7)	89.6 (76.7-110.0)	26.4 (24.4-28.3)	8.1 (6.6-9.7)
Triniclot aPTT HS	4	68.0 (64.7-73.0)	19.6 (17.0-22.7)	6.2 (4.7-7.3)	79.4 (69.0-86.0)	24.1 (19.7-30.3)	7.7 (5.6-10.0)
Synthasil	2	69.5 (65.5-73.5)	22.2 (20.1-24.2)	7.1 (6.9-7.3)	78.3 (72.3-84.2)	24.0 (20.5-27.5)	7.9 (7.5-8.3)
PTT A	1	76.7	22.7	6.7	103.7	25.0	7.3
Actin FS	2	72.2 (70.3-74.0)	18.3 (17.0-19.7)	5.7 (4.7-6.7)	81.5 (78.7-84.3)	21.7 (19.3-24.0)	6.0 (4.7-7.3)
Actin FSL	1	90.7	24.0	8.7	114.7	30.0	9.0
All APTT reagents	16	73.1 (64.8-90.7)	21.0 (17.0-24.2)	6.7 (4.7-8.7)	87.1 (69.0-114.7)	26.8 (22.3-29.0)	7.7 (4.7-10.0)

		Eloctate			Advate		
Chromogenic assays	12	99.9 (78.3-115.7)	25.3 (21.3-29.7)	6.4 (4.0-8.1)	102.5 (88.0-119.0)	26.8 (22.3-29.0)	6.9 (4.7-9.0)
Median ratio (range) CSA/OSA	12	1.44 (0.89-1.59)	1.25 (0.86-1.65)	1.00 (0.57-1.31)	1.26 (0.91-1.42)	1.03 (0.77-1.32)	0.95 (0.50-1.12)

Note: Data presented by individual APTT reagent type and for all APTT reagents combined. The median CSA/OSA ratio for the 12 laboratories performing both assay types is shown, with the inter-laboratory range. One laboratory's CSA data at the lowest spike levels were excluded as outliers. For OSA, the analyser types were STA-R series (n = 11), ACLTOP (n = 4) and Sysmex CS5100 (n = 1). For CSA, analyser types were STA-R series (n = 10), ACLTOP (n = 2).

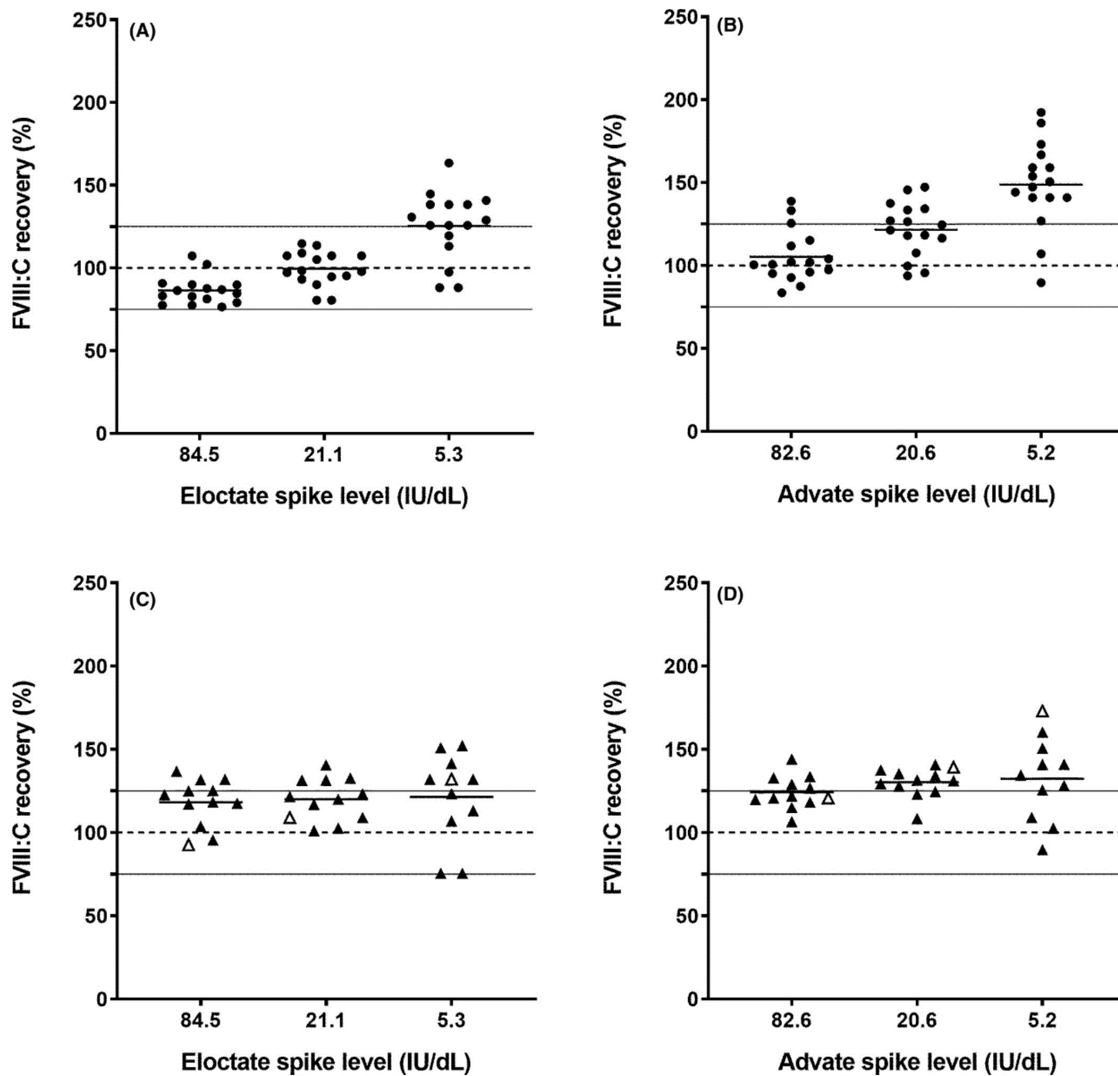

Figure 1: Mean spike recoveries for Eloctate and Advate by one-stage assay tested in 16 laboratories (panels A and B) and by chromogenic assay in 12 laboratories (panels C and D). Chromogenic assays were Hyphen (solid triangles) and Trinity (open triangle). Recoveries were calculated by dividing an individual laboratory's 3-day mean FVIII by the target FVIII and expressed as a percentage. One outlier result excluded from chromogenic data for the lowest spike level. Horizontal bars are the means. The solid horizontal lines are set at 75% and 125% recovery level.

This field study of 16 participants was conducted to allow local laboratories to assess both OSA and CSA suitability in measurement of the EHL product Eloctate, coinciding with its introduction in Australia.

Overall, the data indicate Eloctate can be measured by OSA in participant laboratories using existing protocols. Importantly for the individual participants, data separated by APTT reagent show all reagents behaved similarly for Eloctate assays (Table 1), with no differences between APTT reagents related to type of contact activator, though a limitation here was the small number of users of ellagic acid reagents and absence of kaolin APTT reagents. Overall mean per cent recoveries by OSA for Eloctate spiked to equivalent levels differed slightly in this study compared to the previous field study (2). At the nominal 80 IU/dL spike level, we found a mean recovery of 86.5% compared to 94.6% previously reported (1). Corresponding recoveries for the nominal 20 IU/dL spike level were 99.6% and 106%. There are many potential contributors to these differences, including different lot numbers of product and differences between the two studies in participant profiles of APTT reagent, calibration plasma, factor-deficient plasma and analysers.

The provision of specific recovery data according to specific APTT reagent provided important feedback to the participant laboratories in this field study. While Eloctate recoveries were acceptable by all reagents used in this study, recent studies of other EHL products have shown some much larger differences in recoveries between APTT reagents, enough to regard some APTT reagents as unacceptable for use for assaying EHL levels (3,4). This demonstrates that APTT reagents need to be tested against some EHL on a case-by-case basis to determine validity for recovery estimates. The value of conducting a local field study such as the present one was the feedback supplied regarding suitability individual APTT reagents.

The nonlinearity of OSA with rising mean recoveries as spike levels decrease was seen with both Eloctate and Advate and consistent with previous studies (1,5). At the 5 IU/dL spike level,

a small overestimation of FVIII:C can translate to large per cent over-recovery but may not have an impact on clinical management. Accuracy at low levels may be clinically relevant in the context of determining trough levels in prophylaxis or in pharmacokinetic (PK) analysis. It has been shown that dilution of calibration and test plasma in FVIII-deficient plasma instead of buffer can improve accuracy of FVIII estimates at low levels by removing overestimation (6). A recent study incorporating FVIII-deficient plasma as diluent also eliminated the overestimation seen at low levels of FVIII in samples spiked with Advate or Eloctate (7).

Higher recovery estimates of Eloctate by CSA compared to OSA at the two higher spike levels of around 20% to 30% meant several test sites were getting over-recovery relative to the acceptable limit of within 25% of target. These differences may not always be clinically significant in the context of monitoring the higher levels needed post-surgery. The ratio of CSA/OSA within the same laboratory ranged from 0.89 to 1.59 at the 84.5 IU/dL and 0.86 to 1.65 at 21.1 IU/dL. For laboratories at the high end of this range, consideration may need to be given as to which assay is most appropriate.

In this study, inter-laboratory CVs for Eloctate were similar between OSA and CSA at all spike levels running counter to any perception that CSA are more reproducible than OSA. Both intra- and inter-laboratory variability would be influenced by use of stored versus fresh calibration curves on each day of testing. Recalibration of analysers with each batch of samples run by OSA has been recommended by published guidelines (8), but since data on fresh calibrations for OSA protocols were not gathered in this study, it is not possible to know whether intra-laboratory and inter-laboratory CVs could have been improved by uniform use of fresh calibrations. For CSA, the inter-laboratory CVs in this study of 12.6%, 13.4% and 22.3% were

noticeably lower than in a previous field study measuring Eloctate (19, 23 and 31%, respectively) with a similar number of CSA participants and similar spike levels.1 The lower CVs in this study may have been achieved due to 11 of the 12 chromogenic assays kits being the same (Hyphen), whereas in the previous field study, five different kit types were used among 11 participants. Despite differences in CVs, mean per cent recoveries of Eloctate by CSA at equivalent spike levels were similar. This study was based on spiked samples, so it is not known whether CVs would be different for plasma samples taken from patients receiving Eloctate. However, another multi-laboratory study has shown similar assay variability and good assay correlation when comparing measurement of spiked versus ex vivo samples containing Advate or Eloctate (9).

In conclusion, this field study among 16 Australian specialist haemostasis laboratories in the measurement of the EHL product Eloctate found generally acceptable recoveries at three spike levels by OSA and CSA. There was a trend towards over-recovery at some sites by CSA at all spike levels and by OSA at the lowest FVIII level. Over time, laboratories without access to field studies are encouraged to participate in external QAP programs where EHL product assessments are included, to determine local protocol suitability.

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DISCLOSURES

The authors declare no relevant conflict of interest.

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CHAPTER 6

A field study assessing measurement of Adynovate in specialist haemostasis laboratories in Australia

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CHAPTER PREFACE

Adynovate is an extended half-life FVIII concentrate that is currently licenced for use in Australia. In this work an Australia wide field study of Adynovate measurement was conducted to allow specialist haemostasis laboratories to check accuracy of their reagent/analyser test system. This work provided data on reagent performance by individual APTT reagents which was lacking in a previous international field study. This work complements the previous chapter on measurement of Eloctate, the other extended half-life FVIII replacement product currently used in Australia. Three other field studies of Adynovate have generated contradictory findings to each other to varied extents. The current study is discussed in comparison to these other studies, and is in preparation for submission to the journal Haemophilia as a letter.

SUMMARY

Adynovate is an extended half-life FVIII replacement product, being the pegylated form of the full-length recombinant standards half-life FVIII Advate. This study reports results from an Australia-wide field study of Adynovate measurement by specialist haemostasis laboratories using either clotting (OSA) and/or chromogenic assays (CSA). Ampoules of Adynovate and Advate were reconstituted and spiked into congenital FVIII-deficient plasma to final concentrations of 80, 20 and 5 IU/dL, dispensed into cryovials and kept at -80°C until shipping on dry ice to testing sites. Results contained assayed FVIII values conducted over three different test days from which percent recoveries, intra- and inter-laboratory reproducibility were calculated. For the OSA mean recoveries for Advate at the 80 IU/dL, 20 IU/dL and 5 IU/dL levels were 89.8 IU/dL, 25.6 IU/dL and 7.8 IU/dL, respectively. For the corresponding Adynovate samples, mean recoveries were 86.8 IU/dL, 24.6 IU/dL and 7.4 IU/dL. Adynovate

recovery by chromogenic FVIII assays was 122.9, 31.2 and 8.3 IU/dL. Interlaboratory CVs for Adynovate by OSA for 80, 20 and 5 IU/dL samples were 12.3, 12.6, and 23.0%, respectively, with similar findings by CSA, which were considered acceptable. In conclusion, Adynovate can be measured with sufficient accuracy by one stage clotting assays. By CSA there was a trend to overestimation, which individual laboratories need to consider with respect to their current assay.

ARTICLE

Factor VIII (FVIII) replacement therapy for haemophilia A (HA) requires infusion of FVIII concentrates which are plasma-derived, (r)ecombinant FVIII with standard half-life or rFVIII with extended half-life (EHL). Modifications to FVIII to generate EHL products may alter the FVIII activity measurements by one-stage clotting assays (OSA) and/or chromogenic substrate assays (CSA) used in post-infusion monitoring (1). Demonstration that a laboratory's assay gives recovery estimates close to expected levels is important for correct dosing (2). The EHL product Adynovate (rFVIII-PEG, Shire Lexington, MA, USA) is the PEGylated form of the unmodified rFVIII (Advate) (3). Three field studies of Adynovate measurement have been reported, producing contradictory conclusions. A large international study by Turecek et al concluded OSA or CSA can be used with sufficient accuracy with plasma standards (4). An 8-laboratory study by Bulla et al. found significant overestimation of Adynovate by both OSA and CSA using plasma standards, and more accurate recoveries using an in-house product-specific calibrator (5). An 11-centre study by Ternisien et al. employed the SSC/ISTH Secondary Coagulation Standard Lot #4 (SSCLOT4) as calibrator and found CSA values close to spike target, and OSA values below target in samples with spike levels ≥25 IU/dL, which limited which APTT reagents could be used effectively (6). We present here results of a field

study of Adynovate measurement among Australian specialist haemostasis laboratories and compare the findings with these other studies.

Adynovate and Advate vials were reconstituted using insert instructions then spiked into congenitally FVIII deficient plasma (George King Bio-Medical, Overland Park, KS, USA). Spike concentrations of 80 IU/dL, 20 IU/dL and 5 IU/dL were calculated from lot-specific potency values. Cryovials of spiked plasma were immediately frozen at -80ºC before shipment on dry ice. Fifteen specialist haemostasis laboratories participated. Laboratories received three identical sample sets for testing by OSA and/or CSA on three different days. The central laboratory performed 11 OSA with different reagent/analyser combinations. The 25 OSA protocols tested on Sysmex CS systems (n=9), Diagnostica Stago STA-R (n=13) or Werfen ACLTOP (n=3). Plasma calibrators were Siemens (n=18), Diagnostica Stago (n=5) and Werfen (n=2). APTT reagents are shown in Table 1. FVIII deficient plasmas were Siemens (n=15), Diagnostica Stago (n=4), Werfen (n=3), Helena Laboratories (n=3). CSA reagents were Hyphen Biomed (n=10), Trinity (n=1) and Siemens (n=1). Mean recoveries of triplicate runs were expressed as percent of target. An arbitrary acceptable recovery level of FVIII was set at ±25% of target.

Mean OSA recoveries of Adynovate at the 80 IU/dL and 20 IU/dL levels were 108.5% and 123.0% of target, respectively (Table 2). Most laboratories reported within 25% of target at 80 IU/dL for both Adynovate and Advate. The measured levels were further classified by specific APTT reagent, shown in Table 1. Mean assayed levels for the 80 IU/dL sample of Adynovate ranged from 63.7 IU/dL (APTT SP) to 102.7 IU/dL (Actin FSL). At 20 IU/dL means ranged 16.8 IU/dL (APTT SP) to 27.6 IU/dL (Actin FSL).

Twelve sets of CSA results were returned from 11 laboratories. Two sets of results for Adynovate and one from Advate for the 5 IU/dL level were excluded as outliers. Mean

recoveries of Adynovate at the 80, 20 and 5 IU/dL spike levels were 122.9, 31.2 and 8.3 IU/dL, respectively (Table 2). The Adynovate mean recoveries for 80, 20 and 5 IU/dL were 153.6%, 156.0% and 166.0% of target, respectively. Median intra-laboratory CVs covered the range 2.7% to 5.4% for OSA and 3.1% to 8.6% for CSA. (Table 2). Inter-laboratory CVs across all assays were acceptable and comparable for OSA and CSA, ranging from 8.1% to 21.1%.

The current study supports the use of OSA for measuring Adynovate with a variety of APTT reagents. The higher measured levels by CSA relative to OSA, most noticeably at the 80 IU/dL 20 IU/dL suggest caution may be needed when using CSA, including local validation. The current study needs to be considered in relation to three other field studies of Adynovate measurement. The present study's all-reagent OSA mean percent recovery for 80 and 20 IU/dL spikes of 108.5% and 123.0%, respectively, were only slightly higher than corresponding recoveries of 101.0% and 112.9% reported by Turecek and co-workers (4). The two studies, therefore, both support the conclusion that Adynovate can be measured by OSA using large variety of APTT reagents. The two studies had similar design characteristics: use of the same congenital FVIII deficient plasma for spiking; use of commercial plasma calibration standards; and testing sites using their standard methods without special instructions. Some result differences are inevitable due to different total APTT reagent set being used, and the cumulative effects of all the other test variables associated with OSA (7). For CSA, Turecek and co-workers found average recovery across three spike levels of 114%, versus 158% in this study, giving this study a larger relative difference compared with the OSA. The cause is not clear, but the brands of CSA kits assays may be a component with the Hyphen kit comprising 10 of 12 kits used in this study, but unrepresented in the Turecek study. The 8-centre study of Bulla et al found significant over-recovery of Adynovate by OSA of between 127.7% to 146.6% relative to four spike levels

Table 1. Participant's mean (range) recovery of Adynovate and Advate at three spike levels classified by APTT reagent, plus the all-silica reagent mean and all-ellagic acid reagent means. The upper APTT reagents from Actin to Synthafax have ellagic acid activators. The remaining reagent have silica activators.

	Advate			**Adynovate**		
	80 IU/dL	**20 IU/dL**	**5 IU/dL**	**80 IU/dL**	**20 IU/dL**	**5 IU/dL**
APTT reagent	Mean (range) IU/dL	Mean (range) IU/dL	Mean (range) IU/dL	Mean (range) IU/dL	Mean (range) IU/dL	Mean (range) IU/dL
Actin n=1	89.3 -	25.1 -	7.0 -	99.6 -	27.4 -	7.6 -
Actin FS n=5	83.8 (78.8-88.3)	23.6 (20.5-26.3)	7.2 (5.1-8.7)	90.1 (86.0-95.7)	25.5 (22.4-28.1)	7.6 (5.7-9.2)
Actin FSL n=2	99.9 (96.7-103)	25.5 (25.2-25.7)	7.5 (6.3-8.7)	102.7 (97.6-107.7)	27.6 (26.2-29.0)	7.7 (6.6-8.7)
Cephascreen n=2	89.1 (88.9-89.2)	24.6 (23.6-25.5)	7.8 (6.2-9.4)	88.6 (86.6-90.5)	24.9 (24.2-25.6)	7.5 (6.0-9.0)
Synthafax n=1	86.4 -	23.7 -	6.0 -	90.6 -	25.4 -	6.4 -
Triniclot aPTT S n=5	92.8 (85.8-97.7)	28.1 (25.7-30.9)	9.2 (8.2-10.2)	84.3 (74.0-92.5)	25.0 (23.4-26.7)	8.6 (8.0-9.1)
Triniclot aPTT HS n=2	84.5 (81.6-87.3)	25.3 (24.0-26.6)	8.0 (7.3-8.7)	81.7 (77.4-86.0)	24.7 (24.5-24.8)	7.7 (7.3-8.1)
Synthasil n=3	92.3 (90.9-95.1)	28.2 (22.8-31.8)	8.5 (5.8-10.4)	82.4 (81.5-84.2)	24.5 (19.6-27.2)	7.7 (5.1-9.5)
APTT SP n=2	83.9 (83.6-84.2)	23.0 (22.6-23.3)	6.7 (6.2-7.2)	63.7 (61.0-66.5)	16.8 (16.3-17.3)	4.9 (4.2-5.5)
PTT A n=1	101.6 -	27.3 -	92.1 -	92.1 -	23.1 -	6.3 -
Pathromtin n=1	92.6 -	24.9 -	6.2 -	95.6 -	25.0 -	6.4 -
All ellagic acid reagents n=11	88.4	24.3	7.2	93.0	25.9	7.5
All silica reagents n=14	89.1	27.0	8.4	82.1	23.6	7.6

Table 2. Mean recovery and precision data Adynovate and Advate spike samples tested by one-stage clotting assays and chromogenic substrate assays.

Product	Method	Target FVIII (IU/dL)	Mean recovery (IU/dL)	Mean % recovery of target (95% CI)	Inter-laboratory CV (%)	Intra-laboratory median CV (%) (range)
Advate	OSA	80	89.8	112.2 (109.0-115.4)	8.1	4.2 (1.8-11.6)
		20	25.6	128.1 (122.4-133.8)	11.6	3.9 (0.6-13.1)
		5	7.8	155.3 (142.2-168.3)	21.2	5.3 (1.5-23.8)
Adynovate	OSA	80	86.8	108.5 (103.3-113.8)	12.0	2.7 (0.0-9.3)
		20	24.6	123.0 (116.6-129.4)	13.4	3.7 (0.0-15.1)
		5	7.4	148.1 (136.0-160.2)	21.0	5.1 (0.6-27.9)
Advate	CSA	80	110.0	137.5 (129.2-145.8)	10.8	5.2 (0.5-14.0)
		20	28.9	144.5 (133.8-155.3	12.6	6.4 (2.2-11.1)
		5	7.3	146.4 (129.1-163.7)	19.0	8.6 (1.1-24.7)
Adynovate	CSA	80	122.9	153.6 (141.1-166.2)	13.2	3.1 (0.9-8.9)
		20	31.2	156.0 (147.3-164.7)	11.6	6.6 (0.9-25.2)
		5	8.3	165.8 (145.4-186.2)	19.2	4.8 (0.0-37.7)

OSA, one-stage assay; CSA, chromogenic substrate assay; CI, confidence interval; CV, coefficient of variation

between 80 IU/dL and 10 IU/dL using commercial plasma calibrators, reducing to between 110.0% and 127.2% over-recovery when a locally prepared product-specific standard was used (5). For CSA, they found an even greater overestimation of Adynovate with plasma calibrators, means ranging from ~170-180% of target at the 10, 30, 50 and 80 IU/dL spike levels. These recoveries were greater than seen in the Turecek study and the current study. When the product-specific calibrator was used mean recoveries were 72.7-103.7% of target. The authors concluded the most accurate and precise combination for measuring Adynovate is by CSA with

a product-specific calibrator, and that further evaluation of the use of a product-specific calibrator is warranted (5). The third published field study, from Ternisien et al., compared OSA and CSA measurements of Adynovate in spiked samples at five levels from 2.5 IU/dL to 100 IU/dL with calibration curves created with a plasma standard from the NIBSC (SSC/ISTH Secondary Coagulation Standard Lot #4) (SSCLOT4) (6). CSA recoveries were between 120% (lowest spike levels) and 106% of target at the 100 IU/dL spike level, thus validating the use of CSA to measure Adynovate. This contrasts with the Bulla study (5) and the current study which showed recoveries at 80 IU/dL of 182% and 154% of target, respectively, when using plasma calibrators. Consistent with other studies, the Ternisien study also found OSA vales to be less than CSA values. When expressed as OSA/CSA ratios around half the OSA recoveries in samples with target levels between 25 and 100 IU/dL had ratios below their acceptability cut-off of 0.70. Their conclusion was that CK Prest APTT reagent was acceptable for OSA because of a lower degree of under-recovery, but caution needs to be exercised with Actin FS, Triniclot APTT HS, Synthasil and Synthafax due to the greater degree of under-recovery. This conflicts with OSA results from other studies. Our study, for example, shows acceptable recovery at 80 IU/dL with the latter five APTT reagents (Table 2). The use of SSCLOT4 as calibrator in the Ternisien study was a differentiating feature from the other studies. This raises the question of how can sets of CSA recoveries and sets of OSA recoveries differ so much between studies and especially differ according to use of commercial plasma calibrators in comparison to SSCLOT4. The SSC plasma standards, which are directly traceable to the current WHO international standards (IS) for FVIII, are produced for diagnostic reagent manufacturers to calibrate their commercial plasma calibrators used by diagnostic laboratories (8, 9). A 2020 study by Wilmot et al. found good traceability of commercial plasma calibrators to the 6th IS for FVIII in six of seven brands tested, with recoveries for OSA and CSA were within the acceptance range of 90%-111% of manufacturers' stated values (8). Since both

SSCLOT4 and plasma calibrators are traceable to IS for FVIII, differences in FVIII calibrator assigned values should not be expected to account for the large result differences observed between studies. While the Ternisien study using SSCLOT4 as a calibrator validates the CSA for measuring Adynovate, the over-recovery to varying degrees in the other studies, which used plasma calibrators, remains difficult to explain. The plasma calibrator showing consistent recovery below 90% of its stated value, predicts overestimation of test samples (8). It is possible that some affected batches of this calibrator were used in some of the reported field studies, but not in sufficient numbers to account for all the reported differences on recovery estimates. In routine practice, clinical laboratories must continue to use commercial plasma calibrators for OSA and CSA measurements of FVIII. SSC are not designed for this purpose and are not as readily accessible. Our current practice, based on the study of Turecek et al., and supported by the current study, is to continue using OSA with plasma standards for the measurement of Adynovate.

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CHAPTER 7

In vitro stability characteristics of efanesoctocog alfa and its measurement by one-stage clotting assays using reagent substitutions

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CHAPTER PREFACE

Efanesoctocog alfa (ALTUVIIIO®) is a new class of long acting rFVIII replacement therapy. It is currently not available for use in Australia but is being used in two centres enrolled in clinical trials, including Royal Prince Alfred Hospital. It was licensed for use in the USA in early 2023 and has a high chance of being approved in Australia in 2025 in the current open tender of the National Blood Authority. This chapter reports investigations of laboratory characteristics of efenesoctocog alfa and its measurement, for which there is no published data. This includes in vitro stability studies, plus developmental work on FVIII one-stage assays using reagent substitutions provide a pathway for post-infusion monitoring. The single published international field study on measurement revealed limits on common reagent types used for one stage factor assays. This will need resolving if the drug is to be accurately measured locally using the ACLTOP analysers which used extensively in Australia, should efanesoctocog become available as a therapeutic option for HA in 2025. This is important as very few laboratories with these analysers have access to the reference method used for product potency assignment, and the most used APTT reagents are not suitable for efanesoctocog assays. Furthermore, there is no product-specific calibrator available that could be used to calibrate different one-stage assays. Geoffrey Kershaw designed the studies, performed the assays and analysed the results. Yuet Kee Suki Fu and Tori Tran contributed to sample preparation and testing.

ABSTRACT

Background. Efanesoctocog alfa (Efa) is a new class of extended half-life recombinant FVIII replacement product for treatment of haemophilia A. Additional information is needed on its laboratory characteristics, including measurement in the absence of a product-specific calibrator.

Methods. A reference FVIII assay using Actin FSL reagent on a Sysmex CS2500 analyser formed the basis of the study. Long term storage stability of Efa at -80°C was assessed and short-term stability at room temperature (RT), 4°C and 56°C was measured in timed incubations. Multiple FVIII one-stage assay protocols were performed on the ACLTOP analysers for comparative accuracy against the reference assay in testing ex vivo plasma pools containing Efa at between 12 and 169 IU/dL.

Results. There was no fall in FVIII activity in ex vivo samples containing Efa stored for an average of 48 months at -80°C. Efa levels remained stable for eight hours at RT or 4°C, with very slow loss of activity up to 48 h. For samples with more than ~20 IU/dL Efa heating at 56°C for 90 minutes is required to completely remove Efa activity. Synthafax APTT reagent gave acceptable recoveries on the ACLTOP except at very high levels of Efa. Actin FSL APTT reagent can be successfully adapted to the ACLTOP for Efa assays and shows better linearity than Synthafax.

Conclusion. Efa is stable at RT and 4°C for up to eight hours. Efa can be measured by Werfen Synthafax and Actin FSL APTT reagents on the ACLTOP analysers if specific protocol modifications are made.

INTRODUCTION

Haemophilia A (HA) is an X-linked inherited bleeding disorder resulting from reduced or absent synthesis of the procoagulant protein factor VIII (FVIII). In the severe form of HA baseline factor levels are <1 IU/dL, necessitating regular FVIII infusions to prevent or reduce the incidence of spontaneous bleeding episodes which can result in long term joint damage (1). Monitoring the plasma levels of infused products is essential to prevent under-dosing or over-dosing (2). Haemostasis laboratories need to measure FVIII levels in post-treatment samples to assist monitoring of individuals treated with replacement products, including standard half-life and extended half-life (EHL) (r)ecombinant FVIII products. Guidance from the United Kingdom Haemophilia Centre Doctors' Organisation recommends acceptable results as being within 20% of target based on potency label in samples with >30 IU/dL activity, and within 30% for samples with 10-30 IU/dL (3). For values < 10 IU/dL there is reduced benefit in measuring percent differences. Efanesoctocog alfa (Efa) (ALTUVIIIO®, BIVV001, rFVIII-Fc-VWF-XTEN; Sanofi-SOBI) is a new class of EHL FVIII, designed for once weekly dosing by intravenous injection, composed of single-chain FVIII, the Fc domain of human immunoglobulin G1, the FVIII-binding D′D3 domain of von Willebrand factor (VWF), and 2 XTEN polypeptides (4). It has a fourfold increased half-life relative to other rFVIII products due to these molecular modifications and in phase I clinical trials provided high sustained FVIII activity in the normal-to-near-normal range (>40 IU/dL) for up to 4 days after dosing (5, 6). Importantly, the binding to the D'D3 domain of VWF prevents Efa from binding to recipient's VWF, thus escaping the VWF recycling pathway and the inherent half-life limit of 15-19 hours (5, 7, 8). Plasma levels observed in clinical trials averaged 161 IU/dL for the peak and 17 IU/dL (range 13–23 IU/dL) for the trough in the high dose cohort (9). The manufacturer has assigned vial potency of Efa by FVIII one-stage clotting assay (OSA) using Actin FSL APTT (Siemens, Marburg, Germany) reagent on a Sysmex CA-1500 analyser (Sysmex Corporation, Kobe,

Japan) against the World Health Organization 8th International Standard Factor VIII Concentrate (10). When Efa is used therapeutically there may be a need to check compliance, investigate unexpected bleeding, or manage peri-operative Efa infusions. An international field study of EFA measurement at three spike levels (80, 20 and 5 IU/dL) has been published by Pipe et al. (5). The study included 51 one-stage assay (OSA) protocols with 14 different APTT reagents, and 42 chromogenic substrate assays (CSA) from eight brands of kit, all calibrated with plasma calibrators. Actin FSL was the best performing reagent across all spike levels and showed the best linearity, consistent with its use for Efa potency assignment. There were variable degrees of non-linearity with other APTT reagents, and significant under- or over-estimation of Efa levels with two commonly used APTT reagents, Synthasil (Werfen, Bedford, MA, USA) and Actin FS (Siemens, Marburg, Germany), respectively (5). The field study reagents were run almost exclusively on analysers affiliated with the reagent manufacturer, but it is not uncommon for testing laboratories to utilise third party APTT reagents, FVIII deficient substrate plasma and calibration plasmas. CSAs have been proven useful in measuring other rFVIII extended half-life (EHL) products (11-13), but for Efa, measurement by CSAs led to major overestimation averaging more than twice the target at 80 IU/dL, with differences observed among the seven kits tested (5). Data from recent European-based external quality assessment scheme haemophilia module showed only 3% of submitted results were performed with Actin FSL as the APTT reagent (14). There is no data on the use of Actin FSL on non-affiliated analysers. Data was also limited to a single site for seven of the 14 APTT reagents tested in the field study. Some APTT reagents, including Synthafax (Werfen) (n=1) run on an ACLTOP analyser (Werfen) and CK Prest (Diagnostica Stago, Asnières, France) (n=4) appeared to give satisfactory recoveries, but good laboratory practice requires local validation. With no product-specific calibrator currently available, and limited access to the product

potency assignment method for OSA, many laboratories will face the conundrum of finding an appropriate OSA to measure Efa.

The present study had two aims: first, to further characterise Efa for long- and short-term storage stability of ex vivo samples to give guidance for sample handling; and second, to determine suitability of multiple FVIII assay variations of APTT reagents Actin FSL, Synthafax and Synthasil on the ACLTOP analysers for measurement of Efa, by comparison to a reference assay that uses Actin FSL with a Sysmex CS2500 analyser.

METHODS

Reference assay for efanesoctocog alfa with Actin FSL

The FVIII OSA for measuring Efa levels was performed on the Sysmex CS2500 analyser using Actin FSL APTT reagent, Standard Plasma calibrator (SHP), FVIII deficient substrate plasma and Owren's veronal buffer (OVB), all from Siemens. This assay served as the reference assay for the study. Assay validity had been confirmed by continuous enrolment in the Haemophilia module of an external quality assurance program (ECAT, Leiden, Netherlands).

Long term storage stability at -80°C of ex-vivo plasma containing efanesoctocog alfa

Citrated plasma samples from several patients with severe HA receiving weekly prophylactic intravenous Efa had been periodically monitored with the reference assay during 2020. Surplus plasma was double-centrifuged and stored in 1.5 mL cryovials at -80°C. Plasma collections included trough levels, peak levels and various other timepoints yielding plasma levels from 9 IU/dL to >150 IU/dL. After 47-52 months of storage twelve of these samples from three

individuals, covering a large range of FVIII levels, were rapidly thawed in a 37°C water bath then re-tested for FVIII by the reference assay for comparison to the results obtained at the time of blood collection.

Efanesoctocog alfa 4-sample set preparation and testing

Having established that no loss of FVIII activity had occurred with storage, additional surplus plasma samples from long term storage at -80°C were rapidly thawed and combined into four different pools. Plasma from at least two patients were combined for each pool. Four 10-15 mL pools of differing FVIII levels were created: very high (VH); high (H); medium (M) and low (L). Pools were dispensed into 0.5 mL volumes in pre-labelled cryovials then rapidly re-frozen at -80°C. Sets were later thawed on three different days and tested by the reference assay. Mean FVIII levels in IU/dL were 169.4 (VH), 119.6 (H), 60.0 (M) and 12.0 (L). These levels became the target levels for subsequent method comparisons.

Stability of efanesoctocog alfa at room temperature, 4°C and 56°C

Room temperature (RT) and 4°C stability was determined on VH, H, M and L samples by the reference assay at time 0, 4, 8, 24 and 48-hours incubation. Fresh citrated plasma from two healthy volunteers (Donor 1 and Donor 2) was parallel tested. Tubes were re-capped in-between runs. Heat stability of Efa was assessed on three sets of samples placed in a 56°C water bath 20, 40 and 60 minutes. At each timepoint, the tubes were ultracentrifuged for 1 minute at 13,400 rpm in a mini-Eppendorf centrifuge to remove precipitated fibrinogen and the supernatant immediately tested for FVIII. The 60-minute supernatant tube was re-incubated at 56°C a further 30 minutes for a 90-minute time point. The Donor 1 and 2 samples were tested at 0-, 20- and 40-minutes incubation at 56°C. All tubes were tested in duplicate at one dilution.

FVIII assays on the ACLTOP analysers

The Efa samples VH, H, M and L were assayed for FVIII by 10 different protocols on the ACLTOP750 and/or ACLTOP550 analysers. The test set-up details including calibration sample dilution, reagent volumes, incubation times and other variables are summarized in Table 1. Efa samples were run by six protocols on the ACLTOP750 and five protocols on the ACLTOP550. The Instrument Laboratory (IL)(Werfen) assayed normal control (NC) and low control (LC) were run by all protocols.

ACLTOP's pre-configured FVIII assays (Protocols 1 and 3)

The FVIII assay with Synthasil (Protocol 1) or Synthafax (protocol 3) as the APTT reagent were run using the ACLTOP's pre-configured FVIII assays without amendment.

Actin FSL substitution into pre-configured FVIII assays (Protocols 2, 4 and 8)

A simple APTT reagent substitution of Actin FSL replacing Synthasil (protocol 2), Synthafax (protocol 4) or APTT SP (protocol 8) was made to determine the effects on assayed Efa levels.

Transfer of Siemens FVIII reagents and Sysmex CS2500 test set-up to the ACLTOP (Protocols 5, 6, 7)

Protocols 5, 6 and 7 involved adapting the Sysmex CS2500 reference assay to the ACLTOP including use of Siemens calibrator (SHP), Owren's veronal buffer as diluent (OVB), and FVIII deficient plasma. There were minor variations labelled as V1, V2 or V3 in terms activation time and clot end-point determination detailed in Table 1. Protocol 6 most closely matched the original Sysmex assay by making clot endpoint as time taken to reach 50% of the total absorbance change in the primary clot wave. ACLTOP clot end-point detection of time to 2nd derivative peak was retained in Protocols 5 and 7. Calibration line fit remained as 2nd order

polynomial for protocols 5-7 because the Sysmex point-to-point calibration line fit method was not selectable on the ACLTOP.

Hybrid protocols of sample diluent, calibrator / deficient plasma substitutions (Protocols 9, 10)

This was an exploration to determine effect further changing key assay components on FVIII levels. Protocols 9 and 10 were simple modifications to protocol 8. In protocol 9, IL Factor Diluent was replaced by Siemens OVB. In protocol 10, IL calibrator and FVIII deficient plasma was replaced by Siemens calibrator and Siemens FVIII deficient plasma, while retaining IL Factor Diluent.

Assay accuracy at low levels of Efanesoctocog alfa

The accuracy of Efa measurements at low levels was checked for the unmodified Synthafax assay (protocol 3) and one Actin FSL assay (protocol 6) by diluting the low sample (12.0 IU/dL) in IL FVIII deficient plasma to six levels between 12 to 0.0 IU/dL, then testing in duplicate at the standard dilution.

RESULTS

1. Long term storage stability at -80°C of ex-vivo plasma containing efanesoctocog alfa

The 12 individual patient samples (A to L) re-assayed for FVIII by the reference assay after 47-52 months storage at -80°C showed no loss of FVIII activity in comparison to the original assay (Figure 1). Mean level from original collection was 73.3 IU/dL versus 79.8 IU/dL after storage (P=0.003 by paired t-test). The means of the five samples with <50 IU/dL Efa were 26.2 IU/dL and 27.1 IU/dL (P=0.09).

Table 1. FVIII one stage assay details for protocols used on the ACLTOP. Protocols 1 and 3 are unmodified ACLTOP methods. In protocols 2,4 and 8 Actin FSL substitutes for Synthasil, Synthafax and APTT SP in the analyser's FVIII assays. Protocols 5,6 and 7 are Sysmex Actin FSL protocols adapted to the ACLTOP with minor changes. Protocols 9 and 10 mixed protocols where Siemens OVB or Siemens FVIII deficient plasma/calibrator are substituted IL Factor diluent or IL FVIII deficient plasma/calibrator.

	Numbered protocols for FVIII one-stage assays run on the ACLTOP analysers									
	1. Synthasil ACLTOP protocol	2. Actin FSL substit-uted for Synthasil	3. Synthafax ACLTOP protocol	4. Actin FSL substit-uted for Synthafax	5. Actin FSL Sysmex protocol V1	6. Actin FSL Sysmex protocol V2	7. Actin FSL Sysmex protocol V3	8. Actin FSL substit-uted for APTT SP	9. Actin FSL substit-uted for APTT SP	10. Actin FSL substit-uted for APTT SP
Calibration plasma	IL	IL	IL	IL	Siemens	Siemens	Siemens	IL	IL	Siemens
APTT reagent	Synthasil	Actin FSL	Synthafax	Actin FSL	Actin FSL	Actin FSL	Actin FSL	Actin FSL	Actin FSL	Actin FSL
Sample and calibrator diluent	IL Factor Diluent	IL Factor Diluent	IL Factor Diluent	IL Factor Diluent	Siemens OVB	Siemens OVB	Siemens OVB	IL Factor Diluent	Siemens OVB	IL Factor Diluent
FVIII deficient plasma	IL	IL	IL	IL	Siemens	Siemens	Siemens	IL	IL	Siemens
$CaCl_2$ start reagent	0.020M	0.020M	0.020M	0.020M	0.025M	0.025M	0.025M	0.025M	0.025M	0.025M
Standard dilution	1/10	1/10	1/10	1/10	1/20	1/20	1/20	1/10	1/10	1/10
Calibration points	8	8	8	8	6	7	7	8	8	8
Sample (µL)	25	25	50	50	40	40	40	50	50	50
FVIII deficient (µL)	25	25	50	50	40	40	40	50	50	50
APTT reagent (µL)	50	50	25	25	40	40	40	50	50	50
$CaCl_2$ volume (µL)	50	50	50	50	40	40	40	50	50	50
Activation time (sec)	180-220	180-220	180-220	180-220	180-220	120-130	120-130	180-220	180-220	180-220
Primary algorithm for clot endpoint	2nd deriv-ative peak	2nd deriv-ative peak	2nd deriv-ative peak	2nd deriv-ative peak	2nd deriv-ative peak	50% Threshold	2nd deriv-ative peak	2nd deriv-ative peak	2nd deriv-ative peak	2nd deriv-ative peak
Calibration line fit polynomial	2nd order	2nd order	2nd order	2nd order	2nd order	2nd order	2nd order	2nd order	2nd order	2nd order

IL, Instrument Laboratory; V1-V3, version numbers

2. Stability of efanesoctocog alfa at room temperature, 4°C and 56°C

There was no fall in mean Efa levels after storage at either RT or 4°C for the first 8h, with *P*=0.24 and *P*=0.70, respectively by paired *t*-test (Figure 2, Panels A, C). There were mild falls in Efa levels after 24h at RT and 4°C, with *P*=0.04 and *P*=0.05, respectively. By contrast, the normal donor plasma FVIII levels showed a noticeable decline within 4h which continued logarithmically over 48h at both RT and 4°C (Figure 2, Panels B, D). The heating of Efa at 56°C required 90 minutes to eliminate all FVIII activity in the samples VH, H and M, and 60 minutes for the sample starting at 12 IU/dL (Figure 2, Panel E). The two normal donor samples had FVIII >100 IU/dL initially, declining after 20 minutes at 56°C to <2 IU/dL (Panel F).

3. FVIII assays on the ACLTOP analysers

3.1 Erroneous data point for correction

There were a few erroneous calibration/test sample data points observed mostly at FVIII levels of ≤ 5 IU/dL when Actin FSL was used on the ACLTOP. All were associated with misreads of the clot endpoints when using the 2nd derivative peak algorithm. When this occurred the clot endpoint was manually determined by inspection of the clot wave patterns then edited into the calibration curve data table or used to calculate the test sample results. No errors were seen in the analyser-specific Synthafax or Synthasil FVIII assays for the ACLTOP. No errors were seen in protocol 6, where the 50% threshold was used as the clot endpoint, which had eliminated the need for derivative curves.

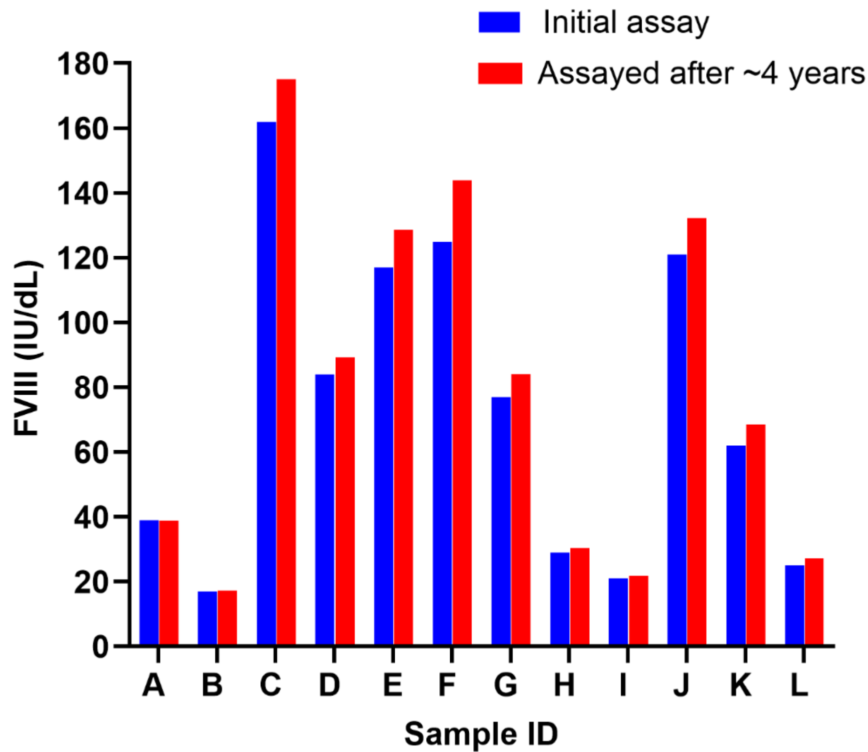


Figure 1. Long term storage stability of efanesoctocog alfa in ex vivo plasma at -80°C. Twelve samples from three different individuals with severe HA receiving efanesoctocog alfa were assayed initially by one-stage assay, then a second aliquot repeat assayed after storage at -80°C for between 47 and 52 months.

3.2 Calibration and percent recovery of efanesoctocog alfa

All protocols run on the ACLTOP750 and ACLTOP550 gave successful calibration curves after correction of occasional clot wave errors as outlined above. Commercial controls, NC and LC, yielded results within the package insert acceptance intervals for all protocols, except protocol 10, where the NC value was on the upper limit of the acceptance range. Recovery estimates of Efa and the two control plasma samples for all protocols are plotted relative to their target values (Figure 3).

3.3 ACLTOP's pre-configured FVIII assays (Protocols 1 and 3)

The Synthasil FVIII assay significantly underestimated Efa at all four levels, percent recoveries of 40-60% of target (Figure 3, Panel A). The Synthafax protocol gave similar and acceptable percent of target estimates on both ACLTOP750 and ACLTOP550 analysers for M and L samples with a tendency towards under-recovery at the highest samples VH and H. The Synthafax assays had mild non-linearity, percent recoveries increasing with decreasing FVIII levels.

3.4 Actin FSL substitution into pre-configured FVIII assays (Protocols 2, 4 and 8)

Figure 3, Panel B shows the effect of the simple substitution of Actin FSL into each of Werfen's three standard FVIII assays. For the Synthasil protocol, there was underestimation of Efa at the two highest samples, VH and H, with acceptable recovery for the M and L samples. For the Synthafax protocol Actin FSL substitution caused Efa levels to fall by more than half to be unacceptably low in all samples. For the APTT SP protocols, reagent substitution with Actin FSL led to unacceptably low recoveries in all four Efa samples, in the range of 30-60% of target.

3.5 Transfer of Siemens FVIII reagents and test set-up to the ACLTOP (Protocols 5, 6, 7)

Figure 3, Panel C shows percent recovery of Efa of all four runs of Sysmex Actin FSL FVIII protocol adapted to the ACLTOP. Each gave acceptable Efa recoveries on all four samples, with close agreement between the two analysers running protocol 6, and good linearity seen for all four data sets. These protocols were very similar, differing only in APTT reagent activation time, or clot endpoint detection algorithm.

3.6 Hybrid protocols of sample diluent, calibrator / deficient plasma substitutions

Figure 3, Panel D shows the effect substituting Werfen's Factor Diluent for Siemens OVB in addition to the existing Actin FSL substitution (Protocol 9). The major underestimation at all Efa levels remained, as seen comparing percent recoveries Protocol 8 in panel B, indicating that the sample diluent alone was not a determinant of the underestimation. In clear contrast, retaining Werfen's Factor Diluent but replacing the IL calibrator and FVIII deficient plasma with Siemens calibrator and deficient plasma caused a near doubling of FVIII levels, making Efa recoveries acceptable at all four levels, range 82-95% of target (Protocol 10). This suggests type of FVIII deficient plasma and/or calibration plasma has a major effect on assayed levels.

3.7 Assay accuracy at low levels of Efanesoctocog alfa

Estimates of Efa at very low levels were close to target (Figure 4) using the Werfen Synthafax protocol and one of the Siemens Actin FSL protocols, both run on the ACLTOP550 (Figure 4).

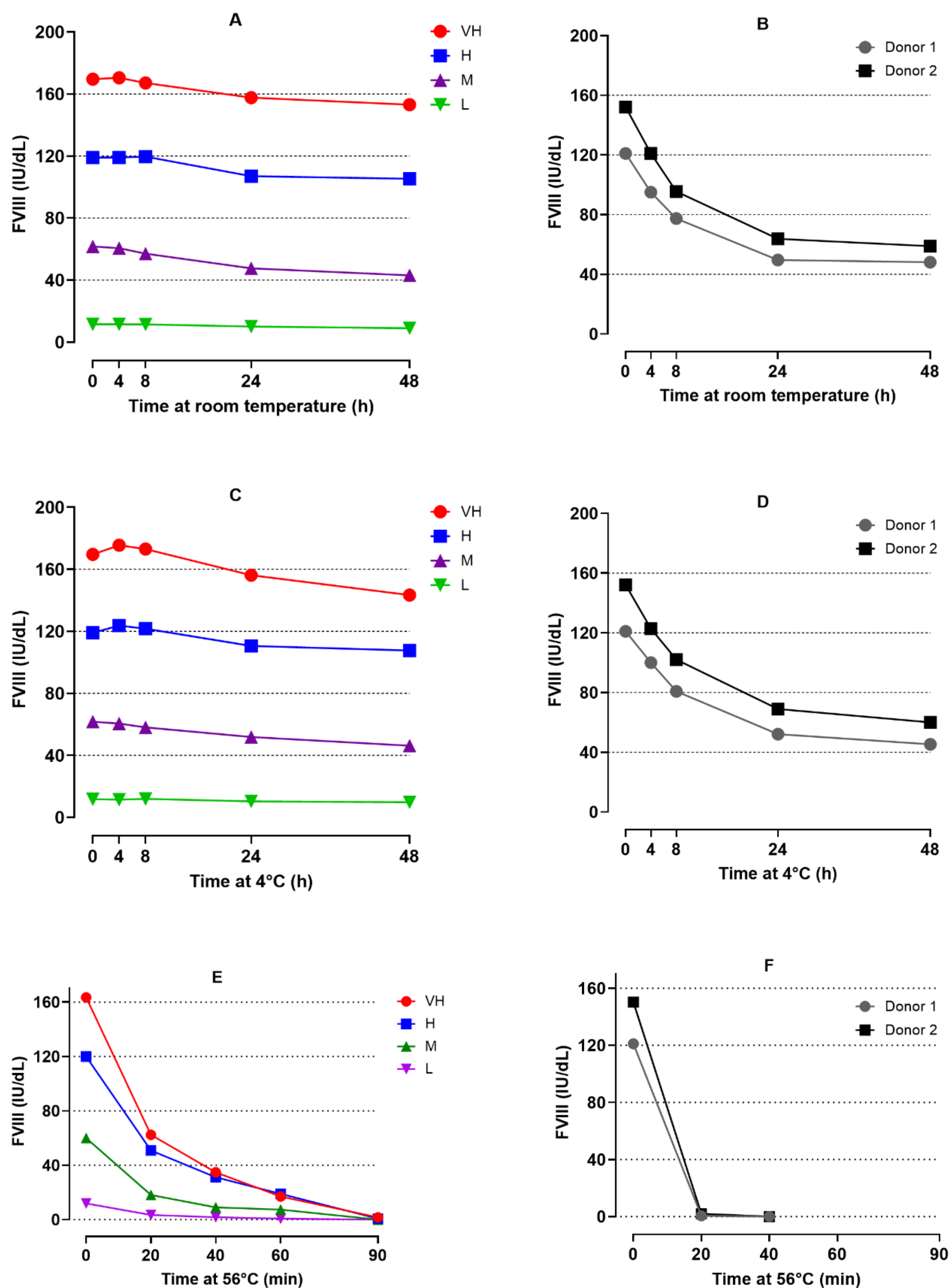
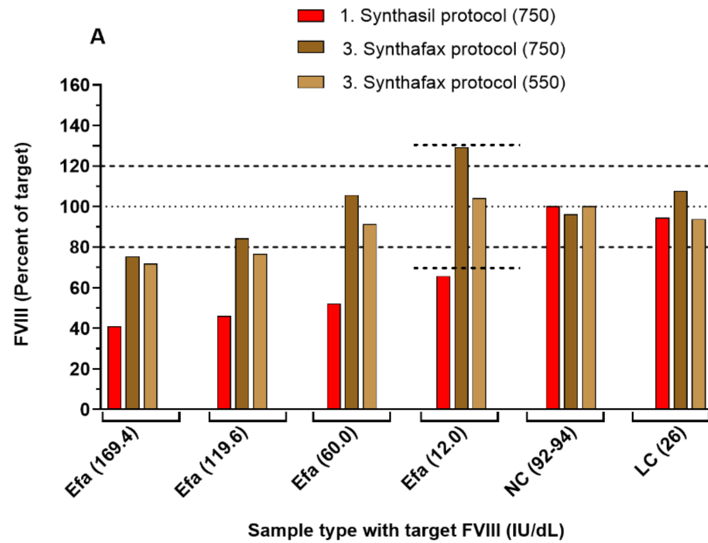
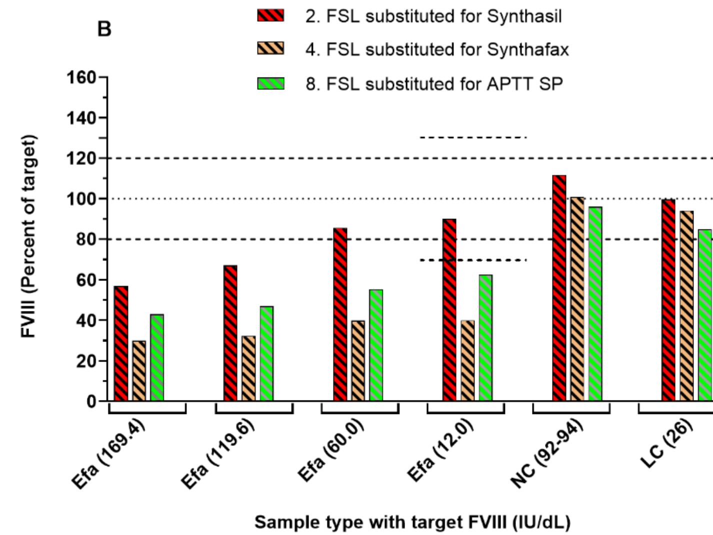
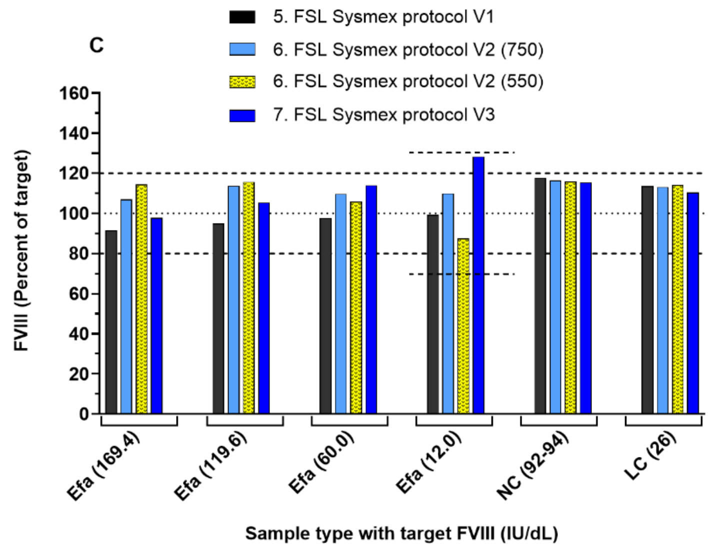


Figure 2. Temperature stability of efanesoctocog alfa samples (VH, H, M, L) in comparison to plasma from two normal donors tested under the same conditions. Samples incubated at room temperature (RT) (A, B); samples incubated at 4°C (C, D); samples incubated at 56°C (E, F). All FVIII assays were performed on the Sysmex CS2500 with Actin FSL APTT reagent calibrated against a plasma standard. All data points are the means of duplicate runs.


A
1. Synthasil protocol (750)
3. Synthafax protocol (750)
3. Synthafax protocol (550)
FVIII (Percent of target)
Efa (169.4)
Efa (119.6)
Efa (60.0)
Efa (12.0)
NC (92-94)
LC (26)
Sample type with target FVIII (IU/dL)


B
2. FSL substituted for Synthasil
4. FSL substituted for Synthafax
8. FSL substituted for APTT SP
FVIII (Percent of target)
Efa (169.4)
Efa (119.6)
Efa (60.0)
Efa (12.0)
NC (92-94)
LC (26)
Sample type with target FVIII (IU/dL)


C
5. FSL Sysmex protocol V1
6. FSL Sysmex protocol V2 (750)
6. FSL Sysmex protocol V2 (550)
7. FSL Sysmex protocol V3
FVIII (Percent of target)
Efa (169.4)
Efa (119.6)
Efa (60.0)
Efa (12.0)
NC (92-94)
LC (26)
Sample type with target FVIII (IU/dL)

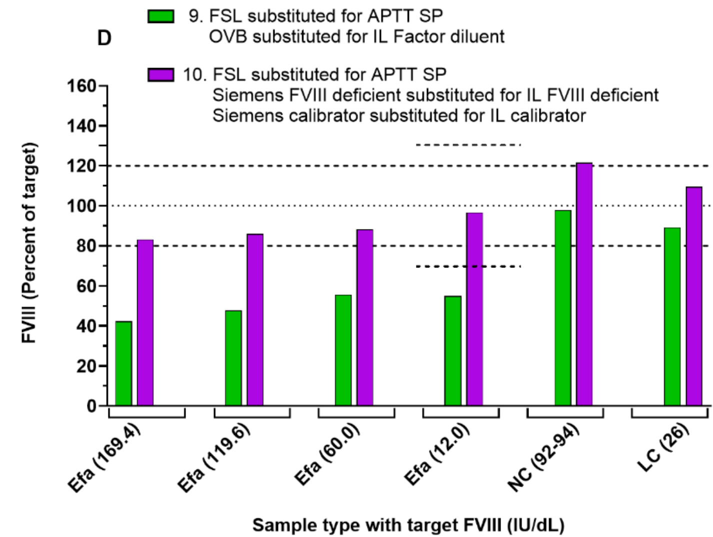

D
9. FSL substituted for APTT SP
OVB substituted for IL Factor diluent
10. FSL substituted for APTT SP
Siemens FVIII deficient substituted for IL FVIII deficient
Siemens calibrator substituted for IL calibrator
FVIII (Percent of target)
Efa (169.4)
Efa (119.6)
Efa (60.0)
Efa (12.0)
NC (92-94)
LC (26)
Sample type with target FVIII (IU/dL)

Figure 3. Percent recovery of efanesoctocog alfa (Efa) in four different pools of ex vivo samples from individuals with severe haemophilia A receiving Efa. Target Efa levels were the means of triplicate runs on the Sysmex CS2500 analyser using Actin FSL APTT reagent. Protocols 1-7 were performed on ACLTOP analysers, with protocols 3 and 6 additionally performed on the ACLTOP550 analyser, and Protocols 8 and 9 on the ACLTOP550, as shown in figure key. Long dashed lines represent target FVIII ± 20%. Shorter dashed lines represent target ±30%. Commercial normal and abnormal plasma controls are included for comparison. Control target values were the relevant package insert values.

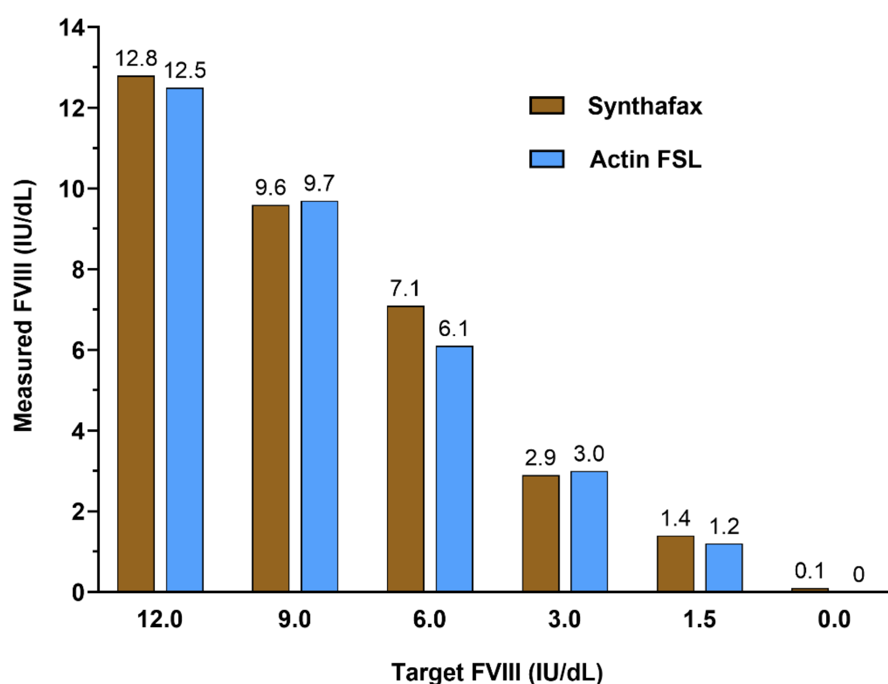


Figure 4. Linearity of efanesoctocog alfa by two assays testing ex vivo samples diluted in IL FVIII deficient plasma. Both assays were run on the ACLTOP550 analyser. Bar height is mean of duplicates run at the standard dilution. Synthafax assay is protocol 3 in Table 1. Actin FSL assay is Protocol 6 in Table 1 with 3rd order polynomial calibration line fit.

DISCUSSION

The introduction of new EHL rFVIII for prophylaxis of HA requires validated methods for measurement of ex vivo samples to assist in patient management. In this work the stability characteristics of Efa have been studied together with an exploration of different OSA protocols

that can be used on the ACLTOP analyser as a guide to measuring Efa levels over a large range of concentrations corresponding to the known peak and trough levels seen in clinical trials.

Efa appears to be stable at -80C storage for at least four years, based on the paired sample testing over this period, using ex vivo samples from three individuals. For samples with <50 IU/dL FVIII activity the post-storage levels were no different to the original assayed levels. For samples >50 IU/dL the slightly higher levels seen post-storage are possibly due to normal assay variation, including use of different calibration curves created from new lots of APTT reagent and FVIII deficient plasma and calibration plasma. This sample stability allowed preparation of four pools subdivided into aliquots for re-freezing at -80°C for use in subsequent studies. An advantage of using ex vivo samples over spiked samples is the taking into account of potential changes to the infused FVIII due to in vivo processing and other interactions (3). It has been demonstrated that Efa samples ex vivo are stable at RT and 4°C for at least eight hours which allows some flexibility of sample handling and processing relative to normal FVIII in the donor plasma. There was a rapid drop on FVIII levels in normal donor plasma within four hours from collection (Figure 2). At 56°C normal donor plasma showed a rapid decline in FVIII levels after 20 minutes as expected, but Efa samples with similar starting FVIII levels required 90 minutes for complete inactivation, and 60 minutes at lower starting levels for complete inactivation. This has significance when performing Bethesda assays for FVIII inhibitors since removal of FVIII from the sample is needed to ensure a 'clean' test system, simplify calculations, and remove the possibility of differential competition between Efa and FVIII contained in the pooled normal plasma for anti-FVIII binding during the incubation phase.

On the critical issue of post-infusion monitoring of Efa level by OSA, potential solutions using an assay that gives results equivalent to the assay used for product potency assignment, or the 'like versus like' principle. For Efa this implies use of Actin FSL APTT reagent calibrated

against a plasma standard and run on a Sysmex coagulation analyser. This was the reference assay used in the current study. If not available, as likely in perhaps the majority of testing facilities, a solution is to switch to a reagent that gave reasonable recoveries in the field study for the analyser type in use in the testing laboratory (5), while accepting this may involve some loss of linearity when measured over a large range of Efa levels. For ACLTOP users that reagent would potentially be Synthafax, which performed reasonably well in the field study, albeit from a single site and a highest FVIII spike of 80 IU/dL The current study adds additional Synthafax data from two ACLTOP analysers located at different sites, using the complete Werfen reagent system. Results were largely acceptable with slight underestimation at very high levels (Figure 3, Panel A). Low level accuracy (0-12 IU/dL) with Synthafax also appears achievable based on dilution studies (Figure 4). These levels are below normal Efa trough levels seen in weekly adult prophylaxis but are nevertheless important in the investigation of unexpected bleeding episodes, inhibitor development or non-compliance. The underestimation of Efa levels with Synthasil was consistent with the published field study results for this reagent. While this study did not test APTT SP reagent, it was shown in the field study to underestimate Efa levels by approximately 50% at both 80 IU/dL and 20 IU/dL spike levels (5).

The possibility of Efa measurement by a simple APTT reagent substitution on the ACLTOP was investigated by replacing each of Werfen's three APTT reagents, Synthasil, Synthafax and APTT SP, with Actin FSL in their respective FVIII OSA protocols. None of the substitutions proved superior to using Synthafax in its original protocol. (Figure 3, Panel B). The three ACLTOP FVIII assay protocols all use specific and different volume ratios of sample, FVIII deficient plasma and APTT reagent, which have presumably been optimised for the use of each Werfen APTT reagents (see Table 1). This makes it unlikely a third-party reagent, including Actin FSL, would perform optimally with any or all of the three protocols.

Complete transfer of the SysmexCS2500/Actin FSL assay set-up ACLTOP in three variations gave good recoveries and the best linearity across the four Efa samples, making this a viable option for measurement of Efa on the ACLTOP. This approach has the advantage of using the APTT reagent used in product potency assignment. Using the 50% threshold clot endpoint detection method (Protocol 6) may be advantageous if it eliminates endpoint detection failures at lower factor levels due to software misinterpretation of 2nd derivative peak locations. In practical terms, reducing the number of ACLTOP protocol and reagent changes without compromising test accuracy would simplify the process of adopting Actin FSL reagent to the ACLTOP. It was shown that OVB was not critical for reagent substitutions, but Siemens FVIII deficient plasma/SHP may be critical to have present in combination with Actin FSL. (Figure 3, Panel D). Conducting these types of studies is in some respects a limitless process, because of the huge number of APTT reagent/deficient plasma/diluent/calibrator combinations that could be created.

Other potential options for measuring Efa include use of correction factors. This has some disadvantages, including the vigilance required to ensure correct application, and sourcing reference plasma sample with known Efa values covering peak to trough levels for deriving the corrector. Also, non-linearity is seen with many reagents, meaning an equation-type correctormay be needed, as opposed to a simple constant.

The use of a product-specific calibrator for Efa would have the advantage of allowing testing on any analyser platform and reagent combination, subject to validation. There are precedents for laboratories relying on product-specific calibrators in OSA to measure therapeutic agents for haemophilia, including the B-domain deleted rFVIII ReFacto AF (15, 16), and the non-factor replacement therapy emicizumab (Hemlibra, Roche) (17, 18). The use of bovine component chromogenic assays (bCSA) for Efa measurement leads to overestimation of by two- to three-fold (5), yet bCSAs remain the only option for measuring any type of FVIII

replacement product in the presence of emicizumab, due to the strong interference of emicizumab in all OSAs for FVIII. A difficulty arises therefore, when Efa is administered to an individual already in the presence of emicizumab, and the level of Efa needs measuring. Use of a product-specific calibrator for Efa has been successfully used to accurately measure Efa in the presence of emicizumab by three different bCSAs in a recent multi-centre international in vitro study comprising seven centres across five countries (19). The main study results showed bCSA can accurately measure Efa values from 5-150 IU/dL using a product-specific calibrator in both the presence and absence of emicizumab. The data thus supported the need for a product-specific calibrator (19). The product-specific calibrator and study samples were prepared by spiking Efa from an ampoule of ALTUVIIIO into FVIII deficient plasma to a level of 100 IU/dL. Interestingly, they assigned a FVIII value of 102 IU/dL to the calibrator based its assayed value on the Werfen ACLTOP750 analyser using ACTIN FSL as the APTT reagent by strictly following a protocol supplied to the authors by Siemens. The protocol included the use of Siemens FVIII deficient plasma and imidazole buffer, but other details including APTT activation times and clot endpoint detection method were not available for comparison to the variations applied in the current study.

This present study has some limitations. Firstly, the EFA target values for the sample pools VH, H, M and L used for calculation of the FVIII recoveries were based on results of the laboratory's validated method with Actin FSL, the same reagent for which the product potency was assigned. We had previously found our results to be close to the clinical trial reference laboratory results for FVIII for a selection of patient samples that were parallel tested (data not shown). More precise target values could be achieved by preparing samples from commercial FVIII deficient plasma spiked with product with known potency, to create sample sets of known values. A strength of the present study was use of stored ex vivo plasma from several patients treated with EFA, meaning the observed differences in FVIII levels between test protocols

reflected differences in testing 'real world' sample types. Another limitation was that not all protocols were tested in duplicate or triplicate, to incorporate day-to-day assay variation. The two protocols with potential use on the ACLTOP analysers, Synthafax and the Actin FSL V2 adaptation, were tested on two different analysers and showed reasonable agreement. Repeat studies on a larger number of analysers would be helpful to confirm these findings.

CONCLUSION

Efa is stable at RT and 4°C for up to eight hours. Efa can be measured by Synthafax on the ACLTOP analysers. Siemens Actin FSL APTT can be used to measure Efa on ACLTOP analysers provided other Siemens test components are transferred at the same time. Further work is needed to fully validate the use of Actin FSL on the ACLTOP by testing spiked samples with known values and including a larger number of analysers.

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CHAPTER 8

Strategies for performing factor assays in the presence of emicizumab or other novel/emerging hemostatic agents

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This single author paper contains an introduction discussing general issues around laboratory testing of recombinant factor replacement products, then discusses more specifically on four novel haemophilia A therapeutics. There is a particular focus on emicizumab, bringing together testing issues around emicizumab including drug measurement (Chapter 9) and FVIII inhibitor measurement in the presence of emicizumab (Chapter 10). In addition, there is some original laboratory data included on the sensitivity of the new Hyphen Biomed Variant chromogenic FVIII assays, effect of emicizumab on intrinsic pathway factor assays and the heat sensitivity of emicizumab. All the experiments were designed and run by the author. There is discussion on laboratory testing two emerging agents for haemophilia treatment not currently available in Australia: Mim8, a bi-specific antibody similar to emicizumab, and efenasoctocog alfa, an ultra-long acting FVIII, which will require adaptations to be made by most testing laboratories due to its unpredictable nature with respect to APTT reagent interactions in clotting factor assays. Laboratory investigation of in vitro temperature stability of efanesoctocog alfa, and its measurement by one-stage factor assay has been performed, to allow Australian laboratories a pathway to measure efanesoctocog alfa should it become available (Chapter 7).

ABSTRACT

For a number of decades, therapeutic options for inherited deficiencies of factor VIII or IX (hemophilia A or B, respectively) have largely been replacement of the missing clotting factor with plasma-derived or recombinant products. Hemostasis laboratories use standard APTT-based clotting or chromogenic assays to monitor plasma factor levels to guide therapy. The emergence in the last 10 years of extended half-life replacement products and other novel therapies for hemophilia has led to a re-appraisal of assay suitability, with studies of product measurement showing some existing assay types or reagents to be unsuitable for some products. The hemostasis laboratory must adapt to the changing landscape by adding new assays or modifying existing assays to ensure accurate results for product measurement. These strategies include switching from a chromogenic assay to a clotting assay, or vice versa, changing an APTT reagent brand, or introducing product specific calibrators. This article evaluates the effects of some of the newer treatment options on the laboratory testing of factor levels and related assays.

INTRODUCTION

Hemophilia A (HA) and hemophilia B (HB) are X-linked hereditary bleeding disorders caused by a deficiency or defect of the plasma proteins factor VIII (FVIII) or factor IX (FIX), respectively. Accurate laboratory measurement of FVIII and FIX is crucial to the diagnosis and ongoing monitoring of therapies in individuals with HA or HB and requires access to a comprehensive laboratory service.[1] Until recently, individuals with severe HA or HB, (<1 IU/dL) of factor, required one to three weekly infusions of factor concentrate to minimize the

occurrence of spontaneous bleeding. These concentrates are either plasma-derived, or recombinant, and can be monitored by standard laboratory APTT-based factor assays. The last 10 years has seen the emergence of newer factor replacement products, including extended half-life (EHL) FVIII and FIX, which have been achieved through modifications to the FVIII or FIX molecules.[2] Due to the heterogenous nature of the modifications, which include glycopegylation, albumin fusion and Fc-fusion, some commonly used APTT reagents may no longer be suitable for measurement of a specific product, giving rise to false low or false high factor levels. Each laboratory, therefore, must assess whether their existing methods are suitable for products in use in their patient population, or whether they give significant over- or under-estimation of infused factor levels. Reagent suitability for different EHL products is often determined by field studies. The two most used tests in hemostasis laboratories for product measurement are the one stage clotting assay (OSA) and the chromogenic substrate assay (CSA). The OSA is based on the degree of correction to the activated partial thromboplastin time (APTT) in a specific factor deficient system. The APTT reagent contains an activator, such as ellagic acid, silica or kaolin, plus a phospholipid component. There are many reagent brands available, and while most laboratories tend to use a single reagent for most purposes, many larger centers would also have additional regents available. The CSA for FVIII or FIX are an alternate to the APTT-based factor assays and provide an option when the OSA methodology available is unsuitable for the particular product being measured. The CSA targets a much narrower range of the clotting factor cascade, centered around the tenase complex of the contact factor (intrinsic) pathway. Ideally, the OSA and CSA methods should be able to accurately measure the peak and trough levels post-infusion, but as seen with the field study reports for various EHL products, there are some notable exceptions. The different types of EHL and their observed effects on different reagent/assay systems have been

summarized in a number of reviews designed to assist laboratories in choosing appropriate methods.[1,3,4]

Other novel therapeutic options for hemophilia that are either currently available or undergoing clinical trials are: the bi-specific antibodies that function as FVIII mimetics; gene therapy; products that quench the activity of naturally occurring anticoagulants antithrombin and tissue factor pathway inhibitor; and the new generation EHL FVIII efanesoctocog alpha (Sobi, Stockholm, Sweden; Sanofi, Paris, France).[2] Other novel therapeutic agents are: recombinant porcine FVIII (rpFVIII, Obizur) (Takeda, Lexington, MA), which has been used in the treatment of acquired hemophilia A (AHA);[5] the bi-specific antibody emicizumab (Roche, Basel, Switzerland); and Mim8 (Novo Nordisk, Bagsvaerd, Denmark), the next generation of bi-specific antibody, which, like emicizumab, mimics the actions of FVIII.[6]

This article reviews the impact on laboratory performance of factor assays in the presence of four of the newer/novel therapeutic agents for hemophilia: the bi-specific antibodies emicizumab and Mim8; rpFVIII; and efanesoctocog alpha. Some of the product characteristics are summarized in Table 1.

Table 1. Characteristics of some novel and emerging treatment products for hemophilia A

Product name	Other name(s)	Characteristics	Laboratory assays	References
Emicizumab	Hemlibra	Bi-specific antibody mimicking FVIII, given sub-cutaneously	Product directly measured by one-stage clotting assay or by human component chromogenic assay with commercial calibrators containing emicizumab. Interferes with APTT-based assays. Administered FVIII, endogenous FVIII such as in moderate HA or in acquired HA, and FVIII in Bethesda assays for FVIII inhibitors can be measured by bovine chromogenic assay.	7,15
Mim8	Denecimig	Next generation bi-specific antibody mimicking FVIII, given sub-cutaneously	Shown to be measurable by one-stage clotting assay with calibrator containing Mim8. Not currently in routine use. Interferes with APTT-based assays. Administered FVIII, endogenous FVIII, and FVIII in Bethesda assays for FVIII inhibitors can be measured by bovine chromogenic assay.	6,27
Recombinant porcine FVIII	Obizur	Porcine FVIII with absent or reduced cross-reactivity to FVIII antibodies; given intravenously	Measured by FVIII one-stage clotting assay with plasma calibrators and a wide range of APTT reagents. Human and bovine chromogenic FVIII assays underestimate levels by around half. Measurement of rpFVIII antibodies requires FVIII deficient plasma spiked with product.	31,33

Efanesoctocog alpha	ALTUVIIIO; BIVV001; rFVIII-Fc-VWF-XTEN	New class of extended half-life rFVIII; given intravenously	Potency assigned by Actin FSL FVIII one-stage assay. Measurable by Actin FSL and some other APTT reagents on affiliated analysers. APTT reagents may not always be transferable to non-related platforms. Validation is needed before implementing any assay. Major overestimation with Actin FS and moderate underestimation with Synthasil APTT reagents. Major overestimation by FVIII chromogenic assays.	35,36

EMICIZUMAB (HEMLIBRA®)

Emicizumab is a humanised engineered bispecific antibody recognising FIX/IXa and (F)actors X/Xa, causing the conversion of FX to FXa by bridging FIXa and FX in a manner similar to FVIIIa.[7] It has been shown to be effective in a non-human primate model of acquired hemophilia A.[7-9] Later studies in humans found emicizumab prophylaxis to be effective in HA patients with or without FVIII inhibitors, reaching steady-state concentration in plasma after four weeks.[10-12] The route of administration for emicizumab is subcutaneous, as opposed to the intravenous route used for factor replacement products. Being an immunoglobulin, emicizumab has a half-life of approximately 30 days,[13] implying a plasma clearance time of about six months after the last dose.

The effects of emicizumab on laboratory assays

There are some functional similarities between FVIII and emicizumab, but key differences influence the results of standard laboratory tests when conducted in the presence of emicizumab, which could result in misleading interpretation of assays in emicizumab-treated patients.[14] The differences described include the 30-day half-life compared to the half-life of FVIII of around 12 hours, the requirement for FVIII to be activated to have a co-factor effect in contrast to emicizumab which is always active and has no inactivation. There is also the insensitivity of emicizumab to FVIII CSA with bovine components (bCSA), which provides an opportunity for FVIII to be measured by bCSA in samples containing both FVIII and emicizumab. A detailed study on the effects of emicizumab on APTT, one-stage and chromogenic assays of FVIII in artificially spiked plasma found normalization of the APTT in severe HA after the first dose of emicizumab.[15] Also found was the markedly raised (and false

high) FVIII levels with multiple APTT reagents when measured by OSA, ranging from 247 IU/dL to over 700 IU/dL. The insensitivity of bCSA to emicizumab was confirmed in a study looking at the effects of emicizumab across a broad spectrum of coagulation tests of differing principles found interference in OSA for FVIII, FIX, FXI and FXII, but no interference in prothrombin time (PT) based assays for (F)actors II, V, VII or X.[16] In contrast, FIX was able to be measured by CSA without any interference. Also noted were two-fold decreases in Protein C and Protein S when measured by APTT-based methods in samples containing clinically relevant levels of emicizumab. There was no interference in assays based on chromogenic principles outside of FVIII, nor on assays based on immunological principles which included Protein C, Protein S and von Willebrand factor assays.[16] The effect of emicizumab on elevating measured FVIII, FIX and FXI was shown locally in my laboratory using surplus plasma pre- and post-commencement of emicizumab in an individual with CHA (Table 2). The largest effect was on FVIII, and no significant change was seen for FV, the latter being a prothrombin time-based assay included as a comparator. Also note the clearly decreasing measured level of FIX and FXI with increasing sample dilution, a pattern consistent with reduced interference of emicizumab as it is progressively diluted to lower concentrations. One-stage FVIII assays may also demonstrate this pattern. Measured FV levels remained unchanged with dilution, consistent with lack of interference of emicizumab in prothrombin time-based factor assays.

Table 2. Effect of emicizumab on one-stage clotting assays of FVIII, FIX, FXII and FV in ex vivo samples taken pre-, and around three weeks post-, commencement of emicizumab prophylaxis. The emicizumab level was 30 mg/mL in the post-sample. Assays performed on

ACLTOP750 calibrated with human plasma calibrator and deficient plasmas from Instrument Laboratory. FVIII, FIX and FXI using Synthasil APTT reagent, and FV assay using Recombiplastin 2G.

	Pre-sample			Post-sample		
	1/10 IU/dL	1/20 IU/dL	1/40 IU/dL	1/10 IU/dL	1/20 IU/dL	1/40 IU/dL
FVIII	3	2		395*	395*	363
FIX	106	109	114	244*	186	137
FXI	111	116	121	267*	219	189
FV	111	107	109	122	127	132

*, factor level obtained by extrapolation from the calibration line

In 2020 the United Kingdom Haemophilia Centre Doctor's Organisation published a guideline containing recommendations for laboratory tests in the presence of emicizumab.[17] Key recommendations were that: APTT-based tests are unsuitable for the measurement of coagulation factors or inhibitors; FVIII assays and FVIII inhibitor levels should be performed using a CSA kit which contains bovine components; an emicizumab assay is to be performed in a patient with suspected inhibitor to emicizumab (anti-drug antibody; ADA).

Emicizumab assay

Emicizumab assays on individuals receiving emicizumab are not normally required, but may be required when unexpected bleeding has occurred, especially to rule out ADA. Another clue to a possible fall in emicizumab concentration is a rise in the APTT. Observation of local patient tests results shows that at steady state concentrations of emicizumab, an individual's APTT may run several seconds below the lower limit of the reference interval. A rise of two to three seconds, or to within the reference interval for the APTT may be a first clue to a lower-than-expected emicizumab level. The incidence of ADA in seven phase 3/3b studies has been reported as 5.1% (n=34) of 668 evaluable persons, with 0.6% (n=4) associated with decreased emicizumab concentration.[18] The plasma emicizumab concentrations in these four individuals

tested by ELISA were reported as: undetectable; <4 mg/mL; stabilising around 15 mg/mL; and reaching a minimum of 10 mg/mL. After excluding these four individuals, the steady-state trough concentrations of emicizumab in ADA-positive individuals generally overlapped that in ADA-negative individuals, with 17/30 (56.7%) of ADA-positive individuals in the first quartile.[18] The lowest steady-state observed trough levels were around 20 mg/mL. This data suggests that it may not be possible to conclude that ADA is present at levels as low as 20 mg/mL. The emicizumab assay as used in clinical diagnostic laboratories is a modified version of the APTT-based FVIII OSA, coupled with the use of a dedicated emicizumab calibration plasma and quality control plasma from r^2 Diagnostics (South Bend, Indiana, United States).[14,19] The main assay modification is the higher standard dilution, typically 1:80, compared to 1:10 standard dilution for FVIII OSA. The dilution series can extend so the lowest calibration point is around 3 mg/mL, depending on the analyzer capacity for high dilutions. Most clinical samples tend not to have emicizumab levels >70 mg/mL so a calibration curve with a top point of ~90 mg/mL is sufficient. In a field study of emicizumab measurement, frozen spiked samples at four levels from 5 mg/mL to 80 mg/mL were distributed to 13 laboratories for 18 sets of results using OSA, which included nine different APTT reagents, three analyzer types, and a mixture of fresh/stored calibration curves.[20] At levels of 20 mg/mL, 50 mg/mL and 80 mg/mL, all laboratories were within 20% of target, with interlaboratory CVs of 6.4% to 8.2%. Additional data on the interlaboratory agreement of emicizumab assays and comparative performance compared to FVIII/FIX OSA and CSA was obtained from the individual participant reports of external quality assurance program run by the ECAT Foundation (Voorschoten,The Netherlands). A total of 11 reports from 2021-2023 for the Emicizumab/Haemophilia modules were reviewed (Figure 1, panels A-C). Overall, the data shows slightly lower CVs for FVIII by OSA versus CSA (Panel A), slightly lower CVs for emicizumab assays versus FVIII OSA (Panel B), and slightly lower CVs for FIX OSA versus

FIX CSA (Panel C). The differences are small and average CVs are around 10% at normal factor levels or steady-state levels for emicizumab. The four most commonly used APTT reagents accounted for 85% of all submitted results (Figure 1, Panel D).

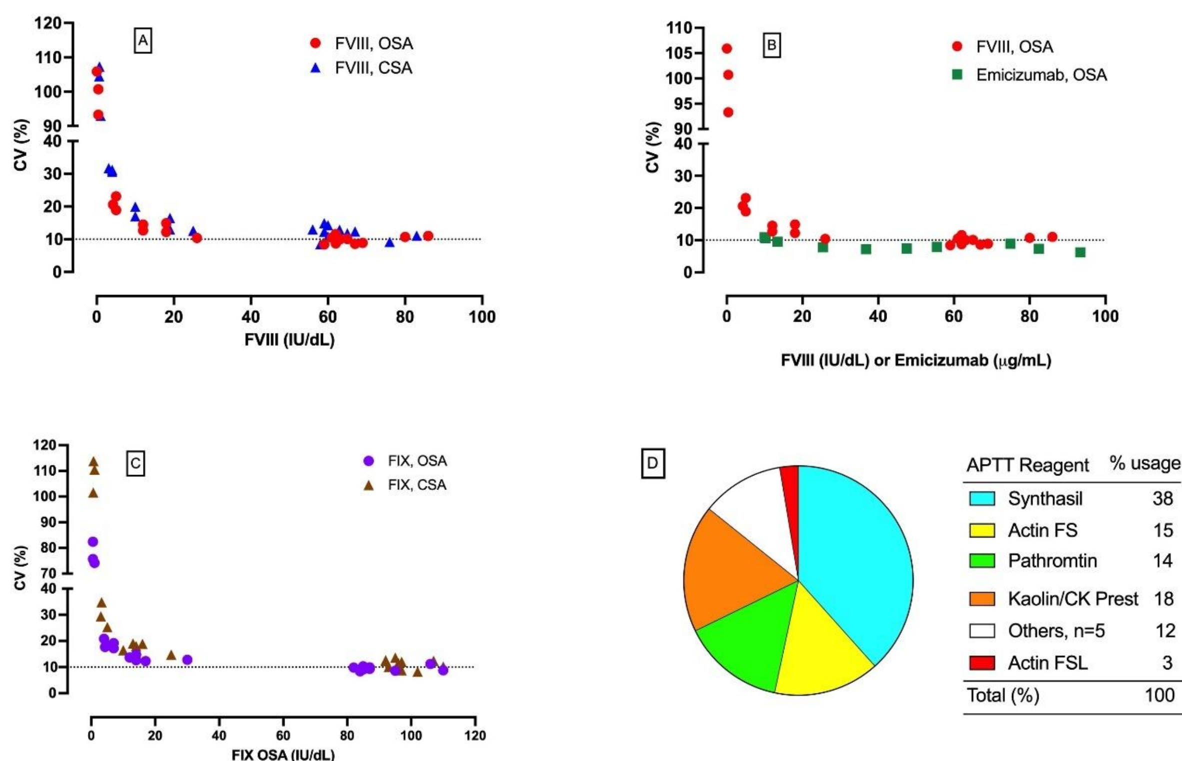

Figure 1. Reproducibility of FVIII, FIX and emicizumab assays as reported in external quality assurance programs. Data was compiled from the reports distributed to participants in the Hemophilia module of the External Control of Assays and Test (ECAT) surveys from 2021-2023. These surveys also showed an average per survey of around 150 reports from one-stage clotting assays, 130 reports from chromogenic FVIII assays, and 45 reports from chromogenic FIX assays. Panel A: The line at 10% coefficient of variation (CV) is for comparative purposes. Factor VIII by one-stage clotting assay (OSA) versus FVIII by chromogenic assays (CSA). Panel B: Factor VIII assay versus emicizumab assays by one-stage clotting. Panel C: FIX assays by OSA versus FIX by CSA. Panel D: The relative usage of APTT reagents for one stage assays averaged from the most recent two available reports periods rounded to percent value.

Measuring FVIII levels and FVIII inhibitors in the presence of emicizumab

Administration of FVIII to an individual with congenital HA who is receiving emicizumab may be needed to control a bleed or in a surgical situation. Measurement of FVIII in the presence

of emicizumab can be accomplished by use of a chromogenic FVIII assay with bovine FIXa and FX, or using a hybrid kit containing human FIXa and bovine FX provided the hybrid kit has first shown to be insensitive to emicizumab.[17] Figure 2 shows the difference between human CSA and bCSA in terms of contribution to FXa generation.

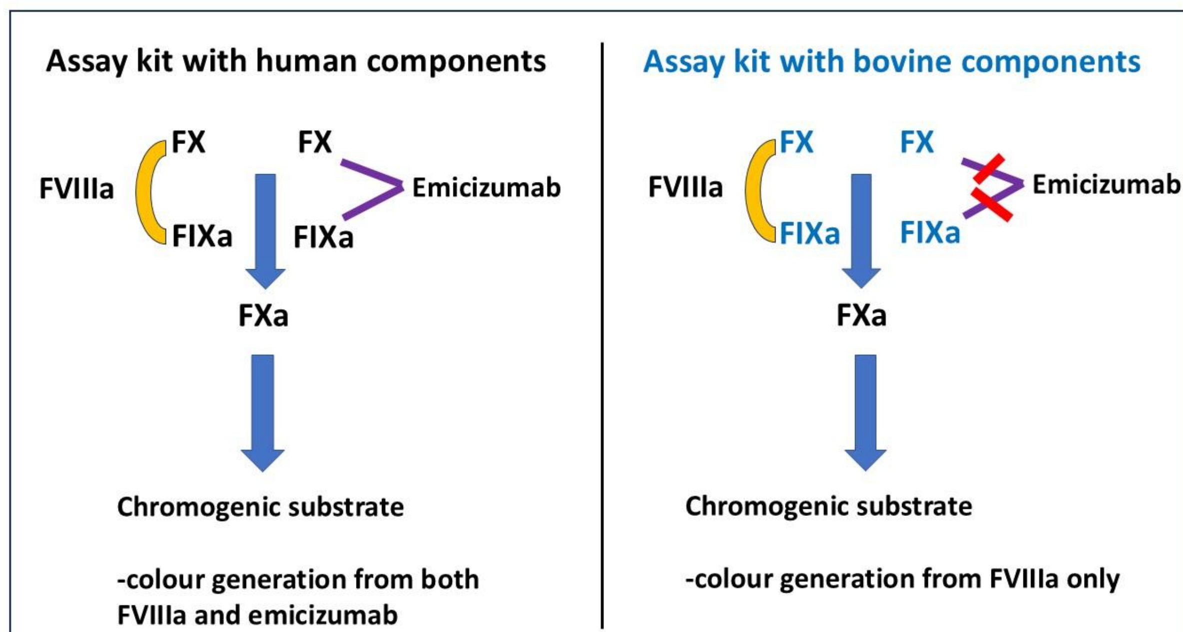

Figure 2. Chromogenic FVIII assays for plasma FVIII in the presence of emicizumab. Assay kits containing human FIXa and human FX components will have FXa generated by the action of both FVIII and emicizumab, leading to falsely high levels of FVIII (left hand panel). Assay kits with both bovine FX and FIXa, or hybrid kits with bovine FX and human FIXa, are insensitive to the presence of emicizumab, allowing accurate measurement of FVIII (right hand panel).

A study of bCSA kits including those with bovine FX and human FIXa found bCSA can be used to accurately determine FVIII activity of rFVIII in plasma which also contains emicizumab.[21] Emicizumab has successfully been used to treat patients with acquired hemophilia A (AHA).[22] Measurement of FVIII by OSA in AHA was achieved by first neutralising the emicizumab with two anti-idiotype monoclonal antibodies. A bCSA FVIII assay was used in parallel to the OSA and FVIII levels correlated well with the FVIII measured by OSA, with the authors concluding bCSA could be used for both FVIII assays and bCSA-

based Bethesda assay in this patient group.[22] In other locations, and in accordance with the published guidelines, laboratories can currently adapt the bCSA to a Bethesda assay for FVIII inhibitors.[17] A relatively new CSA kit for FVIII, the Biomed FVIII:C Variant (Hyphen Biomed, Neuville-sur-Oise, France) referred to here as the Hyphen Variant, contains bovine FX in place of human FX. A local in-house 'low' protocol for the Hyphen Variant FVIII assay, weighted toward accuracy at low levels of FVIII, was evaluated for sensitivity to varied levels of emicizumab spiked into FVIII-deficient plasma with parallel testing of the Siemens bCSA for FVIII and the human Hyphen CSA for FVIII (Figure 3, panel A). The Hyphen bCSA was insensitive to emicizumab up to 100 mg/mL making it suitable to use in patient samples for FVIII assays directly or in the context of a Bethesda assay for FVIII inhibitors. The Siemens bCSA and human Hyphen CSA behaved as expected from previous studies,[15] with the Siemens CSA being insensitive and the Hyphen CSA, with human FX and FIXa, very sensitive to the presence of emicizumab. The effect of co-spiking factor VIII deficient plasma with emicizumab and a commercial normal pooled plasma (Precision Biologic, Dartmouth, Canada) to an expected FVIII level of 45-50 IU/dL showed that the presence of emicizumab did not alter the expected results, further validating the use of either the Siemens or Hyphen Variant bCSA tests for Bethesda assays for FVIII inhibitors (Figure 3, panel B).

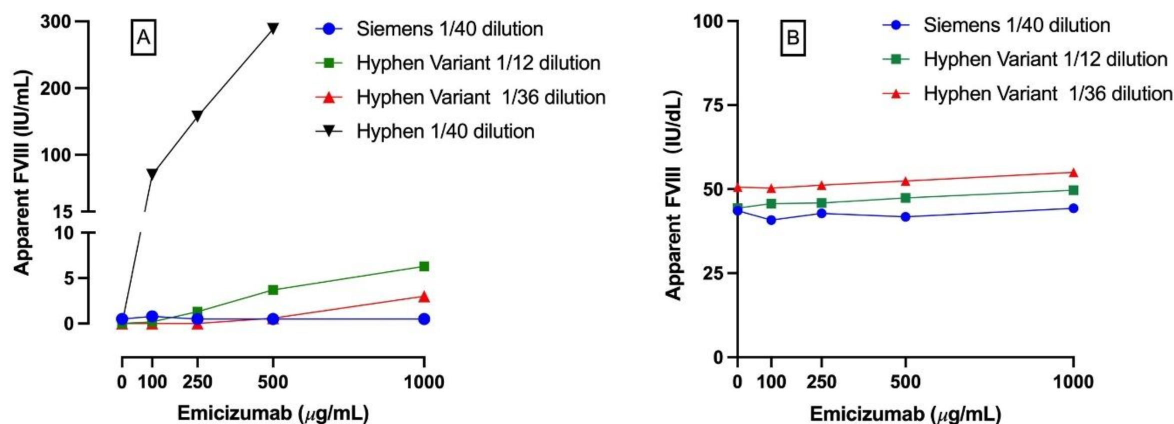

Figure 3. Comparison of three commercial chromogenic FVIII assays for their sensitivity to emicizumab. Panel A: FVIII assays were performed on FVIII deficient plasma spiked with emicizumab to concentrations of up to 1000 mg/mL. The Hyphen assay contains human FX and FIXa, thus generating a large color change in the assay, translating to a false high level of 'apparent FVIII'. The Siemens assay contains bovine FX and FIXa and was insensitive to emicizumab using a 1/40 standard dilution. The Hyphen Variant assay contains bovine FX. The assay shows some sensitivity to emicizumab at levels greater than 100mg/mL at a 1/12 standard dilution. This largely disappears at a 1/36 dilution (Panel A). When FVIII deficient plasma is dual-spiked with emicizumab and pooled normal plasma to a level of about 50 IU/dL FVIII there is minimal interference with increasing levels of emicizumab (Panel B).

Measuring emicizumab levels in the presence of FVIII

When emicizumab and FVIII are present together in plasma, an emicizumab level measured by OSA calibrated against emicizumab can be expected to show some degree of overestimation due to the additive effect of the FVIII in the assay system. In a recent study, Hamedani et al have quantified the FVIII interference in cross-titration experiments where increasing amounts of two types of recombinant FVIII products were spiked into emicizumab-containing patient samples.[23] They found a linear relationship between increasing FVIII spike level and measured emicizumab. They further evaluated three approaches to correcting the overestimation: apply a mathematical formula based on the linear relationship; heat inactivate the plasma for 40m at 56°C to remove the FVIII; or pre-mix the spiked plasma with a high titre commercial FVIII antibody to remove the FVIII. The last approach gave results most in agreement with

emicizumab levels separately assayed by liquid chromotagraphy-tandem mass spectrometry.[23] The heat inactivation of plasma to remove FVIII led to reductions in emicizumab levels of around 20 to 40%. This has been observed in our laboratory also, where emicizumab levels were noted to decrease in a time-dependent manner over 120 min at 56°C in both a patient and a spiked sample, with the relative loss of emicizumab being greater in the patient sample (Figure 4). So, while heat inactivation of plasma remains useful for removing FVIII prior to a Bethesda assay, it is not a tool for allowing accurate measurement of emicizumab. It is not clear how often an emicizumab assay would be required in the presence of FVIII in the laboratory setting, but the method of FVIII removal from plasma by mixing plasma with high titre FVIII antibody appears simple to perform in practice.

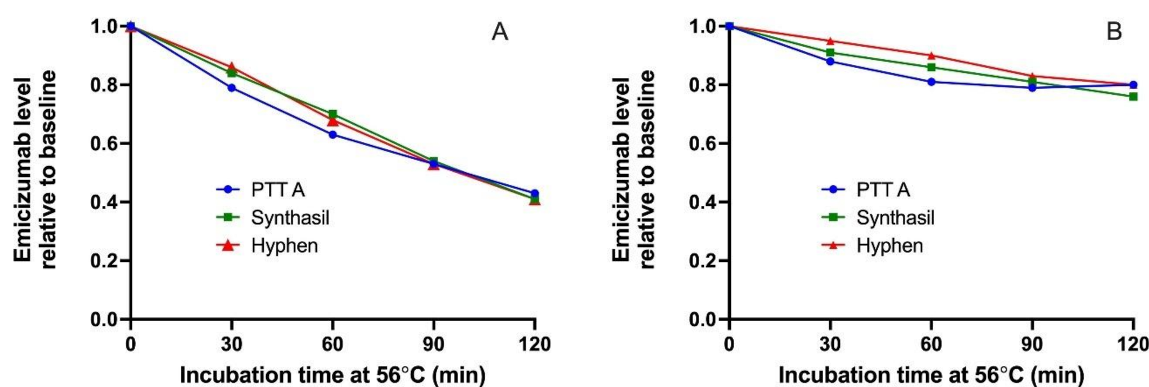

Figure 4. Effect of heating plasma samples at 56°C for 2 hours on measured emicizumab. There is progressive loss of emicizumab in both a patient sample containing 70 µg/mL emicizumab (Panel A), and a commercial FVIII deficient plasma spiked to a level of 80 µg/mL emicizumab (Panel B). Levels were measured by one stage assay with PTT A on a Sta-R analyser, by one stage assay with Synthasil on the ACLTOP750, and by the Hyphen chromogenic assay on Sysmex CS2500 analyser.

MIM8 (DENECIMIG)

Mim8 is a next-generation FVIIIa mimetic designed for the subcutaneous prophylactic treatment of patients with HA with and without inhibitors.[6] Like emicizumab, Mim8 is a humanized, bispecific antibody that mimics FVIIIa function by bridging FIXa and FX on the

phospholipid surface of activated platelets, enhancing the proteolytic activity of FIXa, and thus facilitating FX activation.[6] Mim8 has shown favorable safety and pharmacokinetics in both cynomolgus monkeys and healthy adult humans.[24] Clinical trials in adults and children with or without inhibitors are ongoing.[25] While the mode of action of Mim8 is similar to emicizumab, there are differences, including Mim8 having equivalent thrombin generating activity to emicizumab at around a 15-fold lower plasma concentrations of drug.[26]

Two laboratory-based studies have been published assessing the effects of Mim8 on FVIII-related and non-FVIII related hemostasis assays in samples spiked with Mim8 over the range 0 to 20 μg/mL.[27,28] These concentrations covered the targeted plasma drug concentrations in the five cohorts in the FRONTIER1 clinical trial of Mim8 in persons with HA, which ranged from 1 μg/mL to 20 μg/mL.[26] For the APTT-based assays it was shown that as little as 1 μg/mL spiking was enough to shorten APTTs to within the reference interval for all nine APTT reagents studied, and 2 μg/mL produced FVIII levels of >250 IU/dL for all reagents measured by OSA with a human plasma calibrator.[27] When using a Mim8-containing calibrator, all APTT reagents were able to measure Mim8 concentrations with acceptable accuracy in a set of spiked plasma samples in a modified OSA. The modification required use of 1/40 or 1/80 standard dilution, where the usual OSA FVIII with a plasma calibrator has a standard dilution of 1/10. When measuring FVIII mimetic activity of Mim8 by CSA, only the kits containing both bovine FX and bovine FIXa were insensitive to all concentrations of Mim8. The hybrid CSA with bovine FX and human FIXa showed variable sensitivity to Mim8. This suggests that the bovine-only CSA method would be the preferred option to assay FVIII in samples containing Mim8. For non-FVIII related assays, APTT-based assays of FIX, FXI and FXII are significantly elevated in the presence of Mim8, but a chromogenic FIX assay could be used to accurately measure FIX activity.[28] Overall, the pattern of test interferences is similar between emicizumab

and Mim8 over a broad range of special hemostasis assays. It appears an assay for Mim8 will best be achieved through use of a product-specific calibrator as is the current situation with emicizumab, but when FVIII is to be assayed in the presence of Mim8, the bovine FX/bovine FIXa CSA kits may be a better option. By extension, these same CSA kits could be used for Bethesda assays of FVIII antibodies in the presence of Mim8.

RECOMBINANT PORCINE FVIII (OBIZUR®)

The recombinant porcine FVIII (Obizur®) (rpFVIII), has been shown to have a favorable safely profile and been suggested to be a valuable treatment alternative to recombinant activated FVII or activated prothrombin complex concentrate as a FVIII inhibitor by-passing agent in AHA.[29,30] Clinical trials and case series reports of patients with AHA show rpFVIII to be safe and effective in treating bleeding episodes in subjects with AHA.[5,31] The lack of complete homology of rpFVIII to human FVIII allows rpFVIII to have efficacy in the presence of human allo- and auto-antibodies against human FVIII, and be useful for the management of bleeding episodes in patients with AHA of 20 BU/mL or less.[32] From the laboratory perspective, a suitable FVIII assay is required to measure rpFVIII levels during rpFVIII therapy. An international field study of assay variability in rpFVIII measurement recommended rpFVIII be measured by OSA, after finding mean recoveries at the 80 and 20 IU/dL spike levels of 114.4% and 119.6% of target value, respectively, with little difference between APTT reagents containing either ellagic acid or silica as the activator.[33] The same OSA for FVIII could therefore be used for both diagnosis and dose monitoring of rpFVIII during the infusion therapy for AHA. To predict the effectiveness of rpFVIII in the treatment of AHA it is essential to measure the baseline titre of anti-porcine FVIII antibodies.[30] This requires spiking of rpFVIII into a commercial vial of FVIII deficient plasma to a level close to 100 IU/dL for use as the

FVIII source in the Bethesda assay, replacing the pooled normal human plasma used in a standard Bethesda assay. Parallel determination of the antibody titre in a standard Bethesda assay gives an estimate of the degree of cross-reactivity of the antibody to rpFVIII. There is considerable inter-individual variability in titre of rpFVIII antibodies.[32] In contrast to the acceptable measurement of rpFVIII by OSA, the field study of rpFVIII measurement found FVIII CSA recovery was around half the target value, making CSA not the preferred option.[33] The CSA geometric mean recovery of 11 laboratories at the 80 IU/dL and 20 IU/dL levels was 60.9% and 54.9% of target value, respectively, and corresponding interlaboratory CVs of 13.2% and 28.8%. This underestimation by CSA was demonstrated in a case report where ex-vivo samples in a patient with AHA treated with rpFVIII were measured for FVIII by both assays for several days.[34] The FVIII OSA levels were considerably higher than the CSA FVIII levels until the rpFVIII infusions were ceased, where upon the two assay types gave concordant results within 48 hours as only endogenous FVIII was being measured at that stage.

There is a potential scenario where an individual with FVIII inhibitors being treated with emicizumab requires additional treatment for a bleeding episode or in a surgical situation. An advantage of using rpFVIII over an alternate by-passing agent, such as recombinant FVIIa, is the longer half-life of rpFVIII and the availability of laboratory measurement.[30] Since the presence of emicizumab in plasma renders FVIII OSA unusable, and bCSA underestimates rpFVIII by about 50%, this underestimation if assayed by bCSA needs to be considered in result interpretation. Ideally, the degree of underestimation of rpFVIII by bCSA with the local kit and analyzer combination should be assessed if rpFVIII is available for spiking into FVIII deficient plasma to create different levels for bCSA testing. Alternatively, ex vivo samples containing rpFVIII, but not emicizumab, could be parallel tested by OSA and bCSA.

EFANESOCTOCOG ALFA (ALTUVIIIO™, formerly BIVV001, rFVIIIFc-VWF-XTEN)

Efanesoctocog alfa is a new class of long acting FVIII replacement therapy that circulates in blood independently of endogenous von Willebrand Factor (VWF).[35] The half-life of currently available EHL recombinant FVIII replacement products is limited to 15-19 hours because of the VWF chaperone effect.[36] Efanesoctocog alpha is a FVIII-Fc fusion protein covalently linked to the D'D3 fragment of VWF, and fused to 2 XTEN polypeptides.[37] In a small, early-phase study involving men with severe hemophilia A, a single intravenous injection of efanesoctocog alfa resulted in high sustained factor VIII activity levels, with a half-life that was up to four times the half-life associated with recombinant factor VIII.[36] Phase 3 clinical trials of efanesoctocog alpha found once-weekly prophylaxis provided superior bleeding protection compared with pre-study care FVIII prophylaxis.[38] Laboratory measurement of efanesoctocog alpha was assessed in an international field study in 35 laboratories in 13 countries in which data from 51 OSAs and 42 CSAs were analaysed.[35] For OSA, several APTT reagents provided reliable results, defined as ±25% of target. Results for these reagents were closer to target at the 80 IU/dL spike level. Most reagents give higher relative recoveries with progressively lower levels of spiking, namely 20 IU/dL and 5 IU/dL. The best reagent overall was Actin FSL (Siemens, Marburg, Germany), which might be expected as the product potency was assigned with Actin FSL.[35] The all-reagent interlaboratory CVs for efanesoctocog alpha were 58.5% at 80 IU/dL and 52.3% at 20 IU/dL. For the comparator drug, octocog alpha (Advate, Takeda Pharmaceutical, Lexington, MA), the corresponding CVs were 8.0% and 12.1%. This large discrepancy in inter-laboratory CVs between the two products stemmed from large differences between measured levels of efanesoctocog alpha by different APTT reagents. Actin FS (Siemens, Marburg, Germany) (n=10) gave over-estimates of between 225%-285% across all activity target levels. Synthasil (Werfen, Bedford, MA, USA) (n=15) gave

underestimation by 30%-40% at the 80 IU/dL and 20 IU/dL levels. The authors concluded that Actin FS and Synthasil should not be used for measuring efanesoctocog alpha. This study highlights some important issues facing testing laboratories. Firstly, the large number of modifications made to the FVIII molecule to extend its half-life corresponds to significant assay discrepancies between measured values and target values for some APTT reagents. Secondly, in the absence of a product-specific calibrator for efanesoctocog alpha, the strategy for individual laboratories will in many cases require choosing an alternate APTT reagent to their usual reagent. As seen in Figure 1, panel D, slightly more than half the data sets submitted by laboratories in external quality assurance surveys for the hemophilia module of ECAT were using Synthasil or Actin FS as the APTT reagent for OSAs, the same two reagents giving the largest discrepancies in the field study. Thirdly, field study participants reported almost all the APTT reagents used were paired with analyzers affiliated with the APTT reagent supplier. It is not necessarily the case that a simple reagent substitution of, say, Actin FSL for Synthasil on an analyzer affiliated with Synthasil would resolve the measurement issue, as other test components such as end-point detection system, FVIII deficient plasma brand, sample diluent, calibrator, calibration line fit algorithm all influence results of OSAs. Initial testing in my laboratory showed that APTT reagent substitutions to non-affiliated analysers generates large discrepancies between measured and expected levels of efanesoctocog alpha, but only minor insignificant differences when simultaneously testing quality control plasma. Any APTT reagent change would need validation studies of accuracy against efanesoctocog alpha-containing samples with known values. Provision of such samples to laboratories needing to validate a FVIII assay would be very beneficial. Perhaps the safest initial approach for laboratories setting up an assay is to select an APTT reagent that gave acceptable recoveries in the field study and is affiliated to the coagulation analyser type in use. In particular, the linearity of recovery estimates over the range of expected values of peak to trough in once weekly dosing

needs checking. The peak levels averaged 161 IU/dL and trough levels 17 IU/dL (range 13-23 IU/dL) in the high dose cohort in a clinical trial.[36] On the other hand, laboratories switching from Actin FS to Actin FSL will likely be doing so on an affiliated analyzer and so achieve acceptable measurements of efanesoctocog alpha based on the field study data for Actin FSL. Finally, the field study found a two to three-fold overestimate of efanesoctocog alpha with CSA, meaning OSAs are the better option once the reagent used is validated. CSA could potentially be used with a correction factor, provided the right corrector is chosen, as there are differences among CSAs in measured levels of efanesoctocog alpha. Again, this requires laboratories to have access to samples with known values of product. A protocol would be needed to identify samples containing efanesoctocog alpha, to ensure an assay is used with a built-in corrector, or a corrector is applied to data entered into the laboratory information system. For OSA, a corrector could also be applied, with the same caveats.

CONCLUSION

The significant advances in the number of therapeutic options available for hemophilia care in the past 10 years has impacted how hemostasis laboratories monitor replacement therapies using existing OSAs and CSAs. The non-factor replacement product emicizumab has necessitated adaptations of the OSA for its accurate measurement, namely the use of product-specific calibrators and higher sample dilutions. Measurement of FVIII and FVIII inhibitors in the presence of emicizumab requires use of bCSA. Equivalent modifications to standard laboratory methods will also be required for measurement of the next generation of bi-specific antibody Mim8, with possibly a tighter restriction on which types of bCSA can be employed. Use of rpFVIII remains an option in individuals with AHA with FVIII inhibitors, and rpFVIII can be easily measured with standard OSA. Efanesoctocog alpha is measurable by some but

not all APTT reagents by OSA. The large number of laboratories not using one of the reagent analyzer systems shown to give acceptable results will need switch APTT reagents to achieve accurate measurements. This may require other OSA protocol changes as part of the validation process. As new products emerge that necessitate changes to laboratory measurement process, having updated published guidelines on laboratory measurement will be important to ensure appropriate tests are chosen by the laboratory to measure factor levels and perform related hemostasis assays. Each new treatment product adds to the overall complexity of testing, including knowing which drug each patient is receiving, which can be a major challenge for reference laboratories.

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CHAPTER 9

Emicizumab assay evaluations and results of an Australian field study of emicizumab measurement

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CHAPTER PREFACE

This work was conducted prior to the licencing of emicuzumab as therapeutic for haemophilia A in Australia. There was a need for Australian haemophilia reference laboratories to be introduced to the assay by means of issuing testing instructions for their coagulation analysers, and by issuing samples containing emicizumab to each site for testing. This study is the first published field study of emicizumab measurement. This study also includes detailed new data on the reproducibility at multiple drug levels on multiple platforms, quality control freeze-thaw stability, and assay linearity within the therapeutic window.

Geoffrey Kershaw designed the study, performed the research, analysed the results, and wrote the manuscript. Caroline Dix designed the study, performed the research, analysed the results, and wrote the manuscript. Vivien Chen designed the study, analysed the results, and wrote the manuscript. Nancy Cai performed the research and approved the final manuscript. Liane Khoo designed the study, analysed the results, and wrote the manuscript.

SUMMARY

Emicizumab is a recombinant, humanised bispecific antibody which acts as a FVIII mimetic and is a therapeutic option for Haemophilia A. Plasma emicizumab levels may sometimes be required. Multiple one-stage clotting assays (OSA) and one human-component chromogenic assay (CSA) were used to measure emicizumab both centrally and by a field study. Study samples drug concentrations range included within therapy range of 35-70 µg/mL. All assays

were modified from traditional FVIII assays to enable replacement of plasma calibrators with emicizumab calibrators. Central laboratory OSA mean recovery levels (target) for six spike levels of emicizumab were close to target at 120.5 (120), 81.6 (80), 40.9 (40), 21.4 (20), 10.7 (10) and 5.5 (5) µg/mL. Field study OSA mean recoveries were similarly close to target. Between method CVs were <9% in both the central laboratory and field study assays, except for the 5 µg/mL sample which was 12.3%. CSA mean recoveries were within 10% of target at 80, 50 and 20 µg/mL levels. This study affirms that Emicizumab can be measured by OSA using many types of APTT reagents and is also measurable by the human CSA. The assays showed good precision, accuracy and linearity both locally and in a field study setting.

INTRODUCTION

Haemophilia A (HA) is an X-linked bleeding disorder caused by a deficiency of factor VIII (FVIII), a key factor in the coagulation pathway.[1] Treatment of HA currently involves intravenous infusions of recombinant FVIII (rFVIII) or plasma derived FVIII, however around 25-30% of severe HA patients will develop anti-FVIII antibodies/inhibitors, which renders treatment with FVIII infusions ineffective and resulting in an increased bleeding risk.[1] Treatment of those with HA with inhibitors have traditionally involved use of bypassing agents (recombinant activated factor VII (rFVII) and/or activated prothrombin complex concentrate (aPCC)), and elimination of the inhibitor with immune tolerance induction (ITI) using high-dose FVIII products.[2] These treatments place a huge burden on the patient due to the number of intravenous infusions required, are of considerable cost and have variable success rates.

Emicizumab (HEMLIBRA®), a bispecific, humanized monoclonal antibody, bridges activated factor IX (FIXa) and factor X (FX), replacing the function of missing activated FVIII, with resultant downstream thrombin generation and activation of the coagulation cascade.[3] It has been shown to be safe and effective in phase 3 trials in patients with HA with and without inhibitors.[4-6] It has substantial benefits including being subcutaneous rather than given intravenously; it has a long half –life (28.3-34.4 days), thus decreasing the injection frequency; and does not induce anti-FVIII antibodies, though perhaps does induce anti-emcizumab antibodies.[7] The use of emicizumab is increasing worldwide, hence a need for haemostasis laboratories to be familiar with the effect of emicizumab on routine assays. Unlike native FVIII, emicizumab does not require activation by thrombin and therefore FXa is generated more rapidly than usual.[8] The result is that the APTT is oversensitive to emicizumab with a marked shortening of the APTT to within low-normal levels even at low plasma concentrations of emicuzimab and cannot be used a guide to estimate the level of emicizumab. Studies outlining the effect of emicizumab on routine coagulation tests demonstrated interference in APTT-based clotting assays including one-stage factor assays (OSA), protein C, protein S and lupus anticoagulant tests, but no effect on various immunoassays and chromogenic tests.[9,10]

As emicizumab has a long half-life, its peak-to-trough variations are small.[11] Plasma emicizumab levels in the original Phase 1 study were determined by a validated sandwich enzyme-linked immunosorbent assay (ELISA).[11] The mean emicizumab concentrations at steady state in the HAVEN 1 trial were found to be 50 µg/mL, with a range of 35-70 µg/mL.[5] While it is given as weight-based dose without the need for laboratory-based monitoring, there are certain situations where measurement of emicizumab levels may be desired, for example when there is a suspicion of the development of neutralising anti-drug antibodies (ADA), possibly to check patient compliance or when there is unusual or unexpected bleeding.[12]

Recently, emicizumab calibrator and controls have become commercially available for use in OSAs modified from FVIII OSAs. Chromogenic substrate assays (CSA) for FVIII that use human factors can also be modified to measure emicizumab. A recent report on modified OSAs showed a wide range of APTT reagents and analyser types can be used to measure emicizumab both in plasma from patients receiving emicizumab, and in samples created by in vitro spiking of FVIII-deficient plasma to concentrations up to 400 µg/mL.[8] The study also confirmed the sensitivity of CSA with human components to the presence of emicizumab and the insensitivity of CSA with bovine components to emicizumab.

The current study was prompted by the recent introduction of emicizumab into Australia for the treatment of individuals with severe and moderate HA, and the need for local specialist haemostasis laboratories to establish emicizumab assays. The aims were two-fold. Firstly, to evaluate the performance of OSA- and CSA-based emicizumab assays with multiple reagents systems and analyser platforms in a single reference laboratory, with an emphasis on assay precision and spike recovery estimates of a range of concentrations likely to be encountered in patient samples. Secondly, to perform a field study of emicizumab measurement among specialist haemostasis laboratories to ascertain the level of inter-laboratory agreement in measuring emicizumab by OSA and CSA methods.

METHODS

Emicizumab assay protocols at central laboratory

The study was approved by the Research Ethics and Governance Office of Royal Prince Alfred Hospital (X18-0535/0536). In the central laboratory (Royal Prince Alfred Hospital) emicizumab levels were assayed on three analyser platforms with 6 OSAs and 1 CSA evaluated. The APTT reagent and analyser combinations were: Triniclot aPTT S and PTT A on

the STA-R (Diagnostica Stago); Actin FS and Actin FSL on the Sysmex CS2500 (Sysmex Corporation); Synthasil and APTT SP on the ACLTOP750 (Werfen). All emicizumab assays were modifications of existing FVIII OSA present in the test menus of the analysers, with protocol features summarised in **Table 1**. The modifications were: substituting commercial plasma calibration plasma with commercial emicizumab calibration plasma (r^2 Diagnostics); use of two levels of emicizumab controls (r^2 Diagnostics); and increasing the standard test dilutions from 1/10 to 1/40 (STA-R), from 1/10 to 1/80 (ACLTOP750), and from 1/20 to 1/80 (CS2500). The PTT A protocol for the STA-R was supplied by the manufacturer. The Hyphen CSA (Hyphen Biomed) for emicizumab was run on CS2500 from an adaptation of a FVIII protocol, retaining the 1/40 standard dilution used for FVIII assays, and use of emicizumab calibrator. Calibration line fits used for the CS2500, STA-R and ACLTOP750 analysers were point-to-point, linear, and 3rd order polynomial, respectively. All assays report results as μg/mL of emicizumab. Commercial calibrators and controls contained FVIII deficient plasma spiked with emicizumab, presented in lyophilised form for reconstitution with deionised water. Stored calibration curves were used for the duration of the study. Control plasma had target values of approximately 25 μg/mL (QC1) and 75 μg/mL (QC2) of emicizumab, levels just either side of the range of steady state levels observed in the clinical trials.[5]

Precision studies and control stability

Replicate precision was assessed from 10 runs each of control plasma, plus a very low sample of ~5 μg/mL emicizumab (VL), prepared by diluting control plasma in FVIII deficient plasma with cryovials stored at -80ºC until tested. For between run precision freshly reconstituted QC1, QC2 and thawed VL were assayed one to four times per day to reach a minimum of 20 data points per control. Freeze-thaw stability of controls was assessed by simultaneous assays of

fresh and thawed control material by the Actin FS and Actin FSL emicizumab protocols. The frozen/thawed controls were stored at -35ºC in between testing and rapidly thawed at 37ºC for testing through each of three freeze/thaw cycles over eight days.

Multiple dilution analysis

To assess assay linearity three spike levels of emicizumab (20, 40, 80 µg/mL) were assayed at three doubling dilutions starting with the standard dilution. The six OSA and one CSA were evaluated.

Spike recovery study

Study samples were prepared by spiking emicizumab (30 mg/mL ampoule, Lot B2002B19, supplied by Roche) into immunodepleted FVIII deficient plasma (Precision Biologics, Dartmouth, Canada) to create sample sets with final concentrations of 5, 10, 20, 40, 80 and 120 µg/mL, with aliquots frozen without delay at -80ºC. Spike recovery was assessed on three different days by all protocols at the standard dilution of each assay. Individual protocol

Table 1. Test set-up features for the six one-stage assay protocols (OSA) and the Hyphen chromogenic assay protocol (CSA) used for emicizumab measurement. The activation time of 450s for the chromogenic assay refers to first stage incubation time, namely FXa production. All tests used the same commercial emicizumab calibration plasma.

	OSA	OSA	OSA	OSA	OSA	OSA	CSA
Analyser:	CS2500	CS2500	STA-R	STA-R	ACLTOP 750	ACLTOP 750	CS2500
Assay diluent	Siemens OVB	Siemens OVB	Stago Owren-Koller	Stago Owren-Koller	IL Factor Diluent	IL Factor Diluent	Hyphen Tris- BSA
APTT reagent	Actin FS	Actin FSL	Triniclot aPTT S	PTT A	Synthasil	APTT SP	
APTT activator	Ellagic acid	Ellagic acid	Silica	Silica	Silica	Silica	N/A
Activation time	120	120	240	240	180	300	450
FVIII deficient plasma	Siemens	Siemens	Siemens	Stago	IL	IL	N/A
Calcium Chloride	0.025M	0.025M	0.020M	0.025M	0.020M	0.025M	N/A
Calibration points	6	6	6	6	6	6	7
Calibration measures	Duplicate	Duplicate	Duplicate	Duplicate	Duplicate	Duplicate	Duplicate
Calibrator dilution range	1/80 - 1/2560	1/80 - 1/2560	1/30 - 1/400	1/30 - 1/400	1/80 - 1/2560	1/80 - 1/2560	1/32 - 1/1280
Standard dilution	1/80	1/80	1/40	1/40	1/80	1/80	1/40
Calibration line fit	Point-to-point	Point-to-point	Linear	Linear	3rd order polynomial	3rd order polynomial	Point-to-point

X/Y axes	Log_{10}/Log_{10}	Log_{10}/Log_{10}	Log_{10}/Log_{10}	Log_{10}/Log_{10}	$1/x^{1/2}$ / $y^{1/2}$	$1/x^{1/2}$ / $y^{1/2}$	Log_{10}/Log_{10}
Calibration line range (μg/mL)	92.0 - 2.9	92.0 - 2.9	122.7 - 9.2	122.7 - 9.2	92.0 - 2.9	92.0 - 2.9	115.0 - 2.9

precision was assessed from the three runs, plus total reproducibility across all protocols by calculation of CVs.

Patient samples

Five samples from three patients with HA with FVIII inhibitors, being treated with emicizumab, were tested at the standard dilution by all protocols on three different days to compare assay variability between ex vivo samples and spiked samples. All patients were treated with loading doses of 3 mg/kg weekly for four weeks to reach steady state, followed by weekly maintenance doses of 1.5 mg/kg [5]. Patient samples were collected into 0.109M citrate vacuum tubes (Becton Dickinson). Samples were double centrifuged at 2500 x g with plasma frozen at -80ºC until tested. Patients A and C were sampled ~24 months post-commencement of emicizumab. Patient B was sampled at ~9 days (B1), 8 months (B2) and 12 months (B3) from commencement of emicizumab, representing a pre-steady state and two steady state timepoints, respectively. Pre-emicizumab FVIII inhibitor titres of these patients covered the range 0.7-4.3 BU/mL.

Emicizumab measurement field study participating centres and sample preparation

Thirteen laboratories Australia-wide, including the central laboratory (Royal Prince Alfred Hospital, Sydney), participated in the study. All laboratories were affiliated with Haemophilia Treatment Centres and/or had expertise in specialized haemostasis testing. Samples sets for the field study were prepared separately from the spike recovery samples used in the central laboratory spike recovery study. Each set contained four levels of emicizumab, from spiking

emicizumab (30 mg/mL ampoule, Lot B2002B19, supplied by Roche) into immunodepleted FVIII deficient plasma (Precision Biologics) to final concentrations of 80 µg/mL, 50 µg/mL, 20 µg/mL and 5 µg/mL. These represent high, steady-state, low and very low levels of emicizumab, respectively, based on steady-state levels observed in the HAVEN 1 study.[5] These dilutions were dispensed into cryovials pre-labelled with a de-identifying code, then frozen without delay at -80ºC until shipping. Participants were sent three sample sets on dry ice with instructions to test each set on different days.

Field study sample testing

Analyser-specific protocols for emicizumab OSA were provided to each participant as per central laboratory evaluation; however, participants were free to modify the protocols to include their local APTT reagent/FVIII deficient plasma combinations. The Hyphen CSA was performed by the central laboratory on the CS2500 and by two other participants, one each on STA-R and ACLTOP analysers. The central laboratory supplied all participants with lyophilized emicizumab calibrators and control plasma (r^2 Diagnostics) for assay set-up. Each participant was given a short questionnaire to complete describing their test methodology.

Statistical analysis

Precision of multiple dilution analysis was assessed as CVs, control stability data assessed by paired t-test, and agreement of spiked recovery with spike levels assessed by both percent recovery of target and linear regression analysis. Otherwise, data is represented as means, medians, and CVs.

RESULTS

Central laboratory evaluation assay precision and control stability

The CVs for replicate precision for the six OSA and one CSA protocols ranged from 1.3% to 4.4% for commercial controls and from 0.9% to 8.7% for the 5 µg/mL sample (**Table 2**).

Table 2. Replicate and between run precision of emicizumab assays using six different APTT reagents and the Hyphen chromogenic method. Emicizumab sample types were two levels of controls of approximately 75 µg/mL (QC2) and 25 µg/mL (QC1), and control material diluted to a very low level of around 5 µg/mL emicizumab (VL).

Reagent / Analyser		Replicate precision			Between run precision		
		QC2	QC1	VL	QC2	QC1	VL
Actin FS	n	10	10	10	28	28	21
CS2500	Mean (µg/mL)	73	24	5.7	74	28	5.5
	CV (%)	3.8	2.6	8.1	6.9	4.8	12.6
Actin FSL	n	10	10	10	25	25	20
CS2500	Mean (µg/mL)	73	24	5.7	71	24	5.5
	CV (%)	4.4	3.0	2.6	5.7	7.4	4.1
Triniclot aPTT S	n	10	10	10	28	28	23
STA-R	Mean (µg/mL)	77	25	6.2	74	25	5.5
	CV (%)	3.2	2.7	3.6	5.7	6.7	10.2
PTT A	n	10	10	10	20	20	24
STA-R	Mean (µg/mL)	77	33	5.7	69	24	5.7
	CV (%)	2.3	2.3	3.9	5.5	4.6	8.8
Synthasil	n	10	10	10	32	32	25
ACLTOP750	Mean (µg/mL)	73	25	5.3	73	25	5.4
	CV (%)	3.3	3.2	8.7	3.0	5.7	5.0
APTT SP	n	10	10	10	28	28	23
ACLTOP750	Mean (µg/mL)	78	27	6.2	73	24	5.0
	CV (%)	2.3	1.6	2.9	4.6	5.6	7.8
Hyphen	n	10	10	10	21	21	20
CS2500	Mean (µg/mL)	80	24	4.6	79	24	4.6
	CV (%)	1.5	1.3	0.9	3.4	3.6	3.5

Between-run CVs (n=21-32) ranged from 3.0% to 6.9% for commercial controls and 3.5% to 12.6% for the 5 µg/mL sample. The chromogenic method had lower CVs overall than the OSAs. It was noted that the Hyphen chromogenic method performed better with reagents prepared freshly on the day of testing, as compared with using previously frozen and thawed reagents, with quality control levels drifting downwards with the frozen reagents. Control samples gave similar assayed values of emicizumab, using Actin FS and Actin FSL, regardless of whether tested freshly prepared or after freeze/thawing three times over an 8-day period. The 3-day mean emicizumab levels for Actin FS for fresh/thawed material were 69.3/70.1 µg/mL for QC2 (*P*=0.53) and 26.1/24.9 µg/mL for QC1 (*P*=0.10). Corresponding values for Actin FSL were 68.4/66.8 µg/mL (*P*=0.36) and 23.6/21.9 µg/mL (*P*=0.09) (Fig. 1).

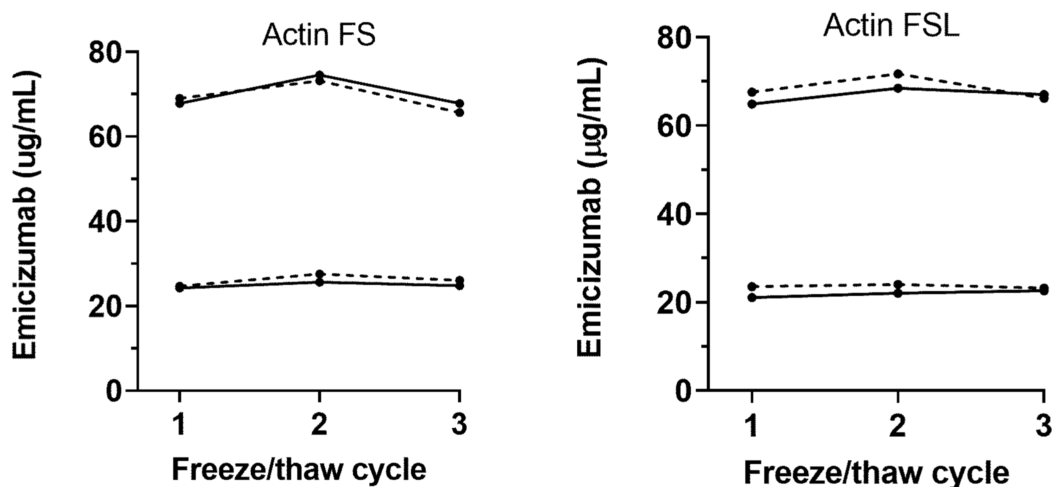

Figure 1. Freeze thaw stability of two levels of commercial control over three freeze/thaw cycles. Fresh (solid line) and frozen/thawed (dashed line) controls were run simultaneously by two emicizumab assay protocols on the Sysmex CS2500 analyser.

Multiple dilution analysis

Three spike levels of emicizumab (20, 40, 80 µg/mL) were tested at three doubling dilutions starting with the standard dilution by protocols. There was good agreement between the three dilutions with CVs ranging between 1.1% and 10.4% (median 4.9%). The highest CV's in five of the seven protocols occurring at the lowest spike level sample (20 µg/mL).

Spike recovery estimates

The accuracy and linearity of the seven protocols was assessed by recovery estimates of emicizumab spiked into FVIII deficient plasma to six different levels from 120 µg/mL to 5 µg/mL. Mean and median emicizumab levels with CVs of triplicate runs with each APTT reagent are shown in Table 3. The CVs at all spike levels were ≤9.6%, except for Actin FS at the 5 µg/mL level (CV=14%). Between method CVs for the seven protocols ranged from 4.9% to 8.0%. Close agreement between target values and measured values was observed at all spike levels by each of the seven protocols. At the 40 µg/mL spike level, mean recoveries ranged from 38.7 µg/mL (Actin FSL) to 44.8 µg/mL (PTT A). At the low spike level of 10 µg/mL mean recoveries ranged from 9.4 µg/mL to 11.7 µg/mL. At the high 120 µg/mL spike level, mean recoveries for OSA ranged from 115.1 µg/mL to 129.8 µg/mL. The CSA at 120 µg/mL gave slight over-recovery with a mean of 138.2 µg/mL. The all-method combined mean recoveries for the 120, 80, 40, 20, 10 and 5 µg/mL spiked samples close to the target values at 123.0, 82.7 40.9, 21.1, 10.5 and 5.4 µg/mL, respectively (Table 3). Linear regression of emicizumab verses spike level found the OSA slopes ranging between 0.959 to 1.076, with a slope of 1.001 obtained on all OSA data combined. The OSA R^2 values ranged from 0.991 to 0.999 (Table 4). For the Hyphen CSA the slope was 1.165 with R^2 of 0.996.

Patient samples

The all-methods mean emicizumab levels for samples A, B1, B2, B3 and C were 33.7, 14.2, 60.3, 63.3 and 66.8 μg/mL, respectively (Table 3). Samples A, B2, B3 and C had levels of emicizumab broadly consistent with levels reported in a clinical trial.[5] Sample B1 had a relatively low level, consistent with the pre-steady state time of sampling. The all-method CVs for four of the five patient samples were in the range 6.0% to 7.9%, in good agreement with the all-method CVs of the spiked samples (Table 3). Sample A had a slightly higher all-method CV of 11.0%.

Table 3. Central laboratory emicizumab measurements of spiked samples and patient samples by six OSA and one CSA. Means and CVs calculated from triplicate runs. The combined OSA values were calculated from the 18 individual runs of the six protocols.

APTT Reagent/ analyser		Emicizumab-spiked samples (µg/mL)						Patient samples				
		120	80	40	20	10	5	A	B1	B2	B3	C
Actin FS CS2500	Mean (µg/mL)	118.8	82.7	40.7	20.3	10.3	5.6	32.8	13.5	61.4	63.2	65.7
	CV (%)	6.7	3.2	4.0	1.2	4.2	14.0	1.5	4.1	2.0	3.0	3.0
Actin FSL CS2500	Mean (µg/mL)	120.7	81.3	38.7	20.9	10.5	5.4	31.5	13.3	55.6	59.3	64.8
	CV (%)	1.2	3.6	2.7	2.4	2.5	4.3	1.6	2.0	7.9	1.1	4.8
Trinicot aPTT S STA-R	Mean (µg/mL)	129.8	85.3	43.6	21.9	10.7	5.7	31.7	14.7	63.1	60.6	64.1
	CV (%)	1.3	1.4	8.0	1.4	3.3	5.6	2.6	5.9	5.0	5.6	0.7
PTT A STA-R	Mean (µg/mL)	118.9	80.6	44.8	23.2	11.7	5.4	38.1	15.7	65.3	69.1	73.5
	CV (%)	7.1	6.1	4.9	9.0	3.6	2.4	4.0	3.0	3.5	7.3	2.6
Synthasil ACLTOP750	Mean (µg/mL)	115.1	79.4	38.8	20.8	10.2	5.3	33.1	15.0	57.5	64.7	64.6
	CV (%)	1.2	0.8	4.8	6.4	5.4	2.9	1.5	6.4	5.0	2.2	5.4
APTT SP ACLTOP750	Mean (µg/mL)	119.7	80.6	39.1	21.3	10.5	5.5	29.6	14.3	58.5	61.1	64.0
	CV (%)	2.8	2.2	6.0	0.9	4.9	9.6	3.7	3.5	1.6	1.9	2.2
Combined OSA	Mean (µg/mL)	120.5	81.6	40.9	21.4	10.7	5.5	32.8	14.4	60.2	63.0	66.1
	CV (%)	5.2	3.7	7.6	6.0	5.8	7.0	8.6	7.1	6.9	6.4	5.9

Hyphen CSA	Mean (µg/mL)	138.2	88.8	40.8	19.2	9.4	4.8	39.6	12.8	60.9	63.3	71.0
	CV (%)	4.6	5.0	3.7	4.3	4.3	3.6	8.5	5.1	3.9	3.7	3.3
All methods	Mean (µg/mL)	123.0	82.7	40.9	21.1	10.5	5.4	33.7	14.2	60.3	63.0	68.6
	CV (%)	7.2	4.9	7.1	7.0	7.0	8.0	11.0	7.9	6.5	6.0	6.1

CV, coefficient of variation

Table 4. Linear regression analysis of emicizumab spike recovery in µg/mL versus spike level. Six one stage assays and the Hyphen chromogenic assay are included, plus values of all OSA data combined. Figures calculated from the triplicate measures of each of the six spike levels of 5, 10, 20, 40, 80 and 120 µg/mL emicizumab.

	Actin FS	Actin FSL	Triniclot aPTT S	PTT A	Synth -asil	APTT SP	All OSA combined	Hyphen
R^2	0.995	0.999	0.999	0.991	0.999	0.998	0.994	0.996
Slope	0.996	1.005	1.076	0.976	0.959	0.993	1.001	1.165
Y-intercept (µg/mL)	0.7	0.2	0.2	2.7	1.0	0.6	0.9	-3.2

Field study methodology questionnaire results

All study participants reported receiving the study samples in frozen condition. A total of 18 OSAs were performed by 13 laboratories, including four assays by the central laboratory, three by one other centre, and a single OSA by each of the remaining centres. Silica activator APTT reagents were Trinclot aPTT S (n=5), Synthasil (n=4), Pathromtin (n=1), Trinclot aPTT HS (n=1) and PTT A (n=1). Ellagic acid activator reagents were Actin FS (n=2), Actin FSL (n=2). Other reagents had polyphenol activator reagent (Cephascreen, n=1) and kaolin activator reagent (CK Prest, n=1). Factor VIII deficient plasma was from Siemens (n=7), Diagnostica Stago (n=7) and Instrument Laboratory (n=4). Emicizumab calibrator lot numbers were ECO250 (n=15), ECO190 (n=2) and ECO200 (n=1). Protocols were performed on three analyser types: Diagnostica Stago STA-R series (n=11), Werfen ACLTOP series (n=4), Sysmex CS2500 (n=3). The Hyphen CSA was additionally performed at three sites, each with a different analyser type. Laboratories performing CSA used freshly prepared reagents on each test day.

Of the total 21 assays run (18 OSA plus 3 CSA) four had daily fresh calibrations for the triplicate runs, four used stored calibration curves daily. The other 13 assays were run with a mixture of fresh and stored calibration curves. Participants also varied in their choice of sample dilutions, electing to either report from a single dilution for each level (usually the standard dilution of 1/30 for the Stago analysers and 1/80 for the ACLTOP and CS2500 analysers) or report the average of two dilutions. Several users of Stago analysers reduced the dilution used for the 5 μg/mL sample to 1/20 to ensure the clotting time fell within the range of the calibration line.

Field study emicizumab measurements demonstrated accuracy and precision

The overall mean assayed values by OSA for the 80, 50, 20 and 5 μg/mL spiked samples were very close to the target at 81.8, 52.4, 20.8 and 5.6 μg/mL, respectively, or to within 5% of target value for the three highest spiked samples and 12.9% of target for the 5 μg/mL sample (Table 5). For CSA, overall mean assayed values were 79.5, 47.6, 18.1 and 4.2 μg/mL, also close to the target values.

The mean assay emicizumab levels obtained of each spike level for each individual 18 OSAs and three CSAs are shown in Fig. 2. There were no clear trends according to APTT reagent and activator type, which is consistent with the low inter-assay CVs. When individual laboratory mean recoveries were expressed as a percent of target all laboratories fell within an arbitrary limit of 80-120% of target for the 80, 50, and 20 μg/mL samples, with the majority within 10% of target. Intra-assay precision was acceptable with the median CV at ≤ 4.7% for all levels, and inter-assay precision for OSA showed CVs between 6.4% and 8.2% for the three highest spike samples and 12.3% for the 5 μg/mL spiked sample (Table 5). Four of the seven OSA or CSA intra-laboratory CVs of >10% occurred at the lowest spike level of 5 μg/mL. Inter-assay

coefficients of variation (CVs) for CSA ranged from 8.2% to 8.9% across the four spike levels (Table 5).

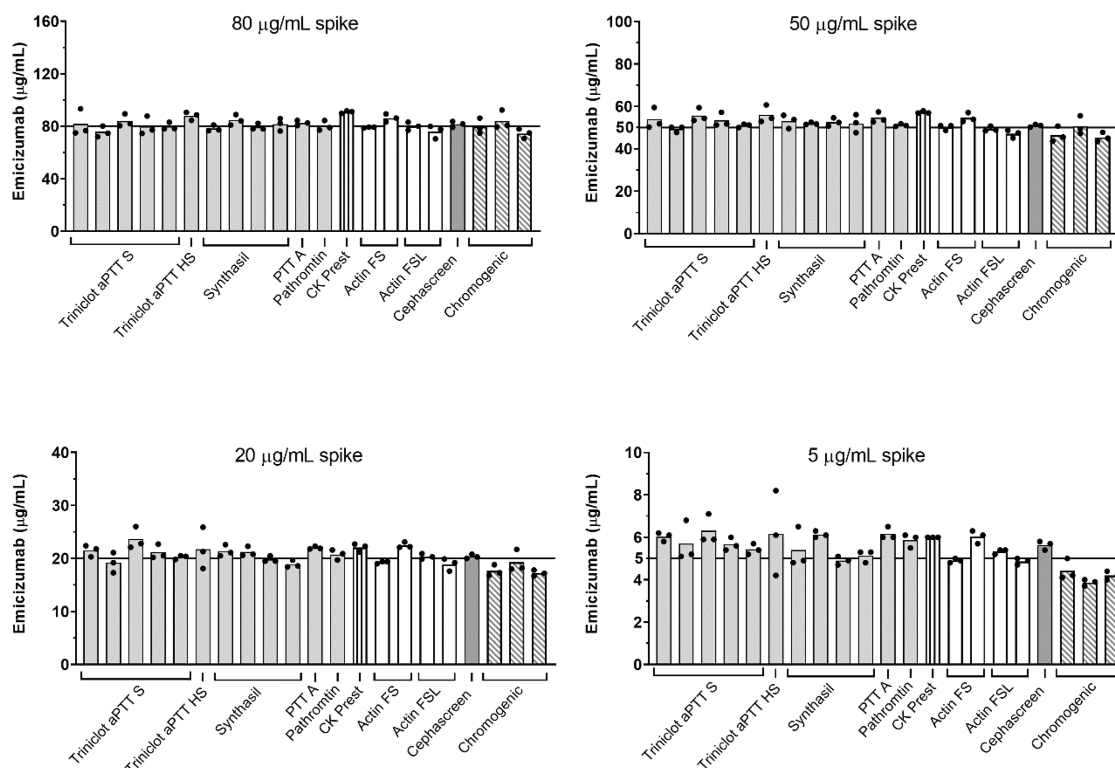

Figure 2. Emicizumab recovery levels by 18 one-stage assay protocols performed by 13 field study participants, plus three chromogenic protocols additionally performed by three participants. Horizontal lines are at the four spike levels indicated in each panel. Bar heights are mean emicizumab level. Dots are the three individual results for each method. The APTT reagent activators were silica (light grey bars), kaolin (vertical stripes), ellagic acid (white bars) and polyphenols (dark grey bar). The Hyphen chromogenic assay is represented by diagonal dashed bars.

Table 5. Field study emicizumab measurements expressed as all-assay means, medians, means as a percent of spike, intra-assay precision and inter-assay precision.

	Emicizumab target level (μg/mL)	All assays mean (range) (μg/mL)	All assays median (range) (μg/mL)	All assays mean % of spike (range)	Median intra-assay CV (range) (%)	Inter-assay CV (%)
Clotting assays, n=18	80	81.8 (75.6-90.0)	81.0 (74.7-91.1)	102.3 (94.5-113.7)	4.3 (0.5-12.4)	6.4
	50	52.4 (47.1-57.2)	51.7 (47.5-56.9)	104.9 (94.2-114.5)	3.8 (1.0-9.2)	6.4
	20	20.8 (18.9-23.6)	20.6 (18.6-22.8)	104.2 (94.5-118.2)	4.0 (1.2-18.0)	8.2
	5	5.6 (4.9-6.3)	5.7 (4.9-6.1)	112.9 (97.3-126.0)	4.7 (0.4-32.4)	12.3
Chromogenic assays, n=3	80	79.5 (74.5-84.2)	78.2 (75.0-80.7)	99.3 (93.2-105.2)	7.4 (4.9-8.5)	8.2
	50	47.6 (45.4-50.7)	47.2 (45.0-49.4)	95.2 (90.8-101.5)	7.9 (4.9-8.6)	8.2
	20	18.1 (17.2-19.3)	17.8 (17.2-18.3)	90.3 (86.2-96.5)	5.9 (3.2-10.8)	8.5
	5	4.2 (3.9-4.4)	4.1 (3.9-4.2)	77.3 (77.3-88.7)	4.8 (4.0-11.1)	8.9

CV, coefficient of variation

DISCUSSION

The increasing use of emicizumab, a FVIII mimetic, as an alternative option to factor replacement for prophylaxis is likely to revolutionise care of patients with HA, and usher in a requirement for some laboratories to measure plasma emicizumab levels. The current laboratory study was undertaken to evaluate multiple emicizumab assays using commercial emicizumab calibrators and controls in the setting of a Haemophilia Treatment Centre associated with a large teaching hospital laboratory. Secondly, a field study was performed to validate the central laboratory findings and evaluate the variability between haemostasis laboratories in the measurement of varied concentrations of emicizumab spiked into FVIII-deficient plasma using their routine analysers and reagents. To our knowledge this is the first reported field study of emicizumab measurement. The protocols chosen for the initial validation study were six OSA, which aligns with recent guidance that emicizumab assays be performed by OSA[12]. The Hyphen CSA for FVIII contains human FIX and FX components so was included in the study as an alternative method for measuring emicizumab, as has recently been demonstrated.[8]

The key strengths of this study are the provision of detailed replicate and between-run reproducibility data for emicizumab assays, and detailed recovery estimates of emicizumab-spiked samples covering below, therapeutic and slightly above therapeutic levels. Further, the study demonstrated equivalent precision and spike recovery accuracy in the context of a multi-laboratory field study. Both the replicate precision and between run precision studies indicate good reproducibility for each assay evaluated, with minimal variation observed between the different APTT reagents or analysers. The spike recovery study found agreement of measured emicizumab to spike concentration across the full range of levels, to within 20% of target, and

in most cases to within 10% of target; this indicates the assays show acceptable accuracy in the clinically useful range of emicizumab concentrations, including a low level of 5 μg/mL.

We are not aware of any other emicizumab field study to compare our results to, however the inter-laboratory variability of emicizumab measurement was lower than that seen in equivalent field studies of FVIII (recombinant and EHL) measurement[13-18]. These FVIII field studies which found higher interlaboratory CVs of 11-22% (Advate, Adynovate and Obizur)[13], 14-35% (Advate and AFSTYLA)[16], 10-34% (Advate and Eloctate)[18], 11.6-18.7% (Advate and Eloctate)[15], 12.4-35.1% (N8 and Advate)[14], and 12.4-33.7% (Advate and BAY 81-8973)[17]. Possible reasons for the better precision relative to equivalent field studies with FVIII, may relate to the nature of emicizumab itself in relation to the test methodologies. Unlike FVIII, emicizumab, being an antibody, does not require conversion to an active form during the test incubations, which for FVIII occurs through thrombin generation in the testing process. Emicizumab also does not bind directly to phospholipid surfaces, thus removing assay variability associated with the different phospholipid types and concentrations present in different APTT reagents. In addition, the standard assay dilution for emicizumab assays is four to eight times greater than for the equivalent FVIII assays, possibly improving assay specificity. Finally, the emicizumab assay calibrator is the drug itself, diluted in FVIII deficient plasma, making it the equivalent to a 'product-specific' calibrator, with all calibration material used in this study sourced from the one manufacturer.

A number of laboratory and clinical issues need consideration in relation to measuring plasma emicizumab levels and related assays required for patient management. Firstly, emicizumab levels may be requested in patients who had FVIII inhibitors at the time of commencement of emicizumab treatment. The ongoing presence of inhibitors, even at high titres, will in principle

have no impact on assayed levels of emicizumab as demonstrated in the Haven 1 study[5]. This is because neither OSA nor CSA systems contain FVIII as a target for the inhibitor. The OSA employs FVIII-deficient substrate plasma as a source of coagulation factors in amounts to allow clot formation following the action of emicizumab in generating FXa from FIXa and FX. The CSA first stage is also generation of FXa from FIXa and FX, which occurs provided a kit using human factor components is employed.

Secondly, when patients receiving emicizumab prophylaxis have additional treatment with FVIII replacement products, for example during surgery, those FVIII products may have an additive effect to FXa production, possibly leading to an overestimation of emicizumab levels as measured by OSA. This has been demonstrated in preliminary spiking studies in our laboratory (data not shown) and needs further evaluation. The infused FVIII products can be adequately assayed using a CSA that contains bovine components, which are insensitive to emicizumab. A number of such kits are commercially available and readily adaptable to coagulation analysers. This limitation of the emicizumab assay need to be recognised; and a delay in the measurement of emicizumab levels be considered until the infused FVIII has been cleared.

Thirdly, measurement of FVIII inhibitor titres in the presence of emicizumab may be required to check if titre is decreasing over time, for example during ITI therapy, or to predict the efficacy of FVIII replacement therapy in a bleeding scenario or prior to elective surgery. Bethesda assays for FVIII inhibitors, that use a chromogenic FVIII assays containing bovine components, have been validated for samples containing emicizumab[19]. Our current practice is

to measure FVIII inhibitors by Bethesda assay, measuring residual FVIII levels with a CSA containing bovine components.

Fourthly, there may be occasions where by-passing agents such as rFVIIa are required to control bleeding in a patient receiving emicizumab, but possibly have no effect on measured emicizumab levels due to the high sample pre-dilution of either 1/40 or 1/80 needed to perform emicizumab assays.

Finally, there have been case reports[20, 21] of a rare development of anti-drug antibodies (ADA) to emicizumab in HA patients, leading in each case to a drop in emicizumab levels to <1 μg/mL. Demonstrating a decrease from steady state emicizumab levels by a plasma emicizumab assay may be useful in aiding the diagnosis when there is a clinical suspicion of ADAs. However, due to the rare occurrence of ADA, the heterogeneity of ADA and the lack of validated assays for ADA, confirming the laboratory diagnosis of an ADA remains a challenge and beyond the scope of this field study.

CONCLUSION

We conclude that plasma emicizumab levels can be easily measured on different platforms using modified OSAs or a CSA with human factor components. The assay performs well with a variety of commonly used APTT reagents and different coagulation analysers, with minimal variation in the performance characteristics between them. As per good laboratory practice each laboratory involved in the care of emicizumab-treated HA patients should validate their own method of measurement based on local reagent and analyser combination.

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CONFLICT OF INTEREST

LK has received honoraria from BioMarin, Takeda, and Sanofi, is on the speaker bureau of Takeda, F. Hoffmann-La Roche Ltd and Sanofi, and has received reimbursement for travel/accommodation/expenses from F. Hoffmann-La Roche Ltd;

GK, CD, NC and VC have no competing interests.

CHAPTER 10

Measuring FVIII inhibitors in samples containing emicizumab using chromogenic assays: results of a multi-laboratory field study

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CHAPTER PREFACE

This chapter is a study validating the use of bovine chromogenic FVIII assays for quantitating FVIII inhibitors by Bethesda assay in the presence of emicizumab. The use of bovine chromogenic FVIII assays for this purpose is required because of interference of emicizumab in clotting-based FVIII assays and human-component chromogenic FVIII assays. It contains Bethesda assay data from five invited laboratories, plus two data sets from the author's laboratory. This work builds on the previous work on emicizumab assays because Bethesda assays for FVIII inhibitors are an on-going requirement for management of patients treated with emicizumab. This paper also brings into focus the use of emicizumab in treating acquired haemophilia A, and the requirement for bovine-component FVIII assays.

Geoffrey Kershaw designed the study, prepared the samples, distributed the samples to interstate sites, performed assays locally, analysed the results and wrote the manuscript. Assistance was received from Yuet Kee Suki Fu in sample preparation, local Bethesda assays, and data collection. This study received funding from Roche.

The study is a published meeting abstract from the ISTH2024 Congress:

> Kershaw GW, Fu YKS, Chen VM, Khoo TL. Measuring FVIII inhibitors in samples containing emicizumab: results of a multi-laboratory field study. Research and Practice in Thrombosis and Haemostasis. 2024;8:341

SUMMARY

Individuals with severe haemophilia A are at risk of inhibitor development as a response to FVIII replacement therapy. Treatment with the monoclonal antibody emicizumab, a FVIII

mimetic, prevents laboratories from using the traditional one stage clotting method to monitor for presence FVIII inhibitors. The use of bovine component chromogenic assays allows measurement of FVIII in Bethesda assays without emicizumab interference. A field study was conducted to measure FVIII inhibitors titres in four samples with different FVIII inhibitors by bovine component chromogenic and clotting-based Bethesda assays, then Bethesda assays were repeated for the chromogenic assay with a matched set of samples spiked with emicizumab. There was no statistically significant difference in FVIII inhibitor titres between clotting assays and chromogenic assays in samples without emicizumab, and no statistically significant difference in titres between titres measured chromogenically in samples in the presence or absence of emicizumab. Interlaboratory variability was acceptable for the inhibitors with simple kinetics, but high with inhibitors with complex kinetics.

INTRODUCTION

Treating individuals with congenital haemophilia A (CHA) with (F)actor VIII concentrates carries a risk of the recipient developing an immune response to FVIII, which renders the therapy ineffective and is considered a major complication of such therapy (1). These inhibitors can make prophylaxis impossible in some situation, which increases the risk of life-threatening bleeds (2). Inhibitors develop in approximately 30% of severe CHA with inhibitor development risk equivalent between plasma-derived and recombinant FVIII products (3). More recently, non-factor replacement therapy with the monoclonal antibody and FVIII-mimetic emicizumab (Hemlibra®, Roche Chugai, Zurich, Switzerland) has been successfully used to treat CHA patients both with and without inhibitors (4, 5). Emicizumab binds (F)actor X and (F)actor IXa in the correct spatial orientation for the generation of FXa, by-passing the need for FVIII (6). Being constantly present in plasma during prophylaxis, emicizumab has profound effects on

laboratory assays based on the activated partial thromboplastin time (APTT) method, including marked shortening of the APTT itself into or below the laboratory's reference interval, and major overestimation of plasma FVIII levels measured directly or as part of a Bethesda assay for FVIII inhibitors (7, 8). Factor VIII inhibitors may also arise as an autoantibody to FVIII in a person with no prior bleeding tendency, leading to the condition of acquired haemophilia A (AHA). This condition is rare and can arise spontaneously or in association with other conditions including autoimmune disorders (9). Patients with AHA are also being successfully treated with emicizumab (10). Detecting and quantifying FVIII inhibitors in AHA or CHA during immune tolerance induction, or for routine surveillance in CHA, requires the laboratory to perform a Bethesda assays (11). Performance of a Bethesda assay requires mixing equal volumes of test plasma either neat or in a dilution series, with a source of FVIII approximating 100%, such as pooled normal plasma. After a two-hour incubation at 37°C the FVIII is measured by an APTT-based one stage clotting assay (OSA) and compared to the FVIII in a control tube for calculation of final titres. Bethesda assays can no longer be performed by APTT-based FVIII assays and must be performed by a FVIII chromogenic assay that contains bovine FX and either human or bovine FIXa (bCSA) components. Emicizumab fails to bind bovine FX allowing for FVIII measurement without interference. The bCSA for Bethesda assays of FVIII inhibitors has been validated in a large single centre study (12). Many specialist laboratories may be using bCSA Bethesda assays for the first time in the context of Bethesda assays. To assist in these laboratories in method validation and characterisation a multi-laboratory field study was conducted. Because emicizumab is being used to treat both AHA and individuals with CHA who have inhibitors, inclusion of both inhibitor types in a field study would be beneficial.

This study had three aims: first, to compare inhibitor titres performed by OSA verses bCSA on samples free of emicizumab; second, to compare inhibitor titres obtained by CSA in the

presence verses absence of emicizumab; and third, to assess between laboratory assay variability of OSA and CSA.

METHODS

Study participants

Five specialist haemophilia testing laboratories in four Australian states were invited to participate. The central laboratory (Royal Prince Alfred Hospital) was responsible for preparation and distribution of all samples and collation of results. Participants were asked to complete a methods questionnaire.

Emicizumab for spiking

The emicizumab was donated by Roche Australia as vials clinical of Hemlibra®. The material was pre-diluted in FVIII-deficient plasma to a strength that could be used for final spiking into study sample pools.

Study samples

Two identical sets of four samples, Set 1 and Set 2, were made from FVIII inhibitor-containing citrated plasma (Table 1). Inhibitor 1, Inhibitor 3 and Inhibitor 4 were prepared by diluting by high titre inhibitor plasma samples into congenital FVIII deficient plasma (George King Bio-Medical, Kansas, USA) to final titres expected to be less than 10 Bethesda units (BU). Inhibitor 1 contained a FVIII inhibitor from an individual with CHA spiked to approximately 1 BU. Inhibitors 3 and 4 were from individuals with AHA, spiked to final titres of approximately 5 and 6 BU, respectively. Inhibitor 2 was a FVIII-inhibitor positive commercial control

purchased from George King Bio-Medical in frozen form, stated as from and individual with CHA with a titre of 6.2 BU. These control samples were pooled for later spiking, but otherwise undiluted. A negative control sample comprising congenital FVIII deficient plasma was included in each set. Set 2 inhibitor pools were spiked with emicizumab to final concentrations of 100-600 µg/mL (Table 1). Set 1 pools were spiked with matching volumes of FVIII deficient plasma to maintain identical final inhibitor titres with Set 2 pools. Once prepared, the test plasma pools were dispensed into coded cryovials, rapidly frozen at -80°C until shipping to participants.

Shipping and testing

Samples sets were shipped overnight on dry ice to participants. Set 1 was for Bethesda assay by both OSA and bCSA. Set 2 was for FVIII testing by bCSA only (Table 1). Participants were told the samples were free of FVIII and asked not to heat inactivate before testing. They were also told to expect titres of around 10 BU or less.

Table 1. Characteristics of the field study samples issued for Bethesda assays.

Test Sample	Sample source	Set 1 No emicizumab Tests required	Set 2 Contains emicizumab Tests required	Set 2 Emicizumab spike level (µg/mL)
Inhibitor 1	Congenital HA	OSA & bCSA	bCSA	100
Inhibitor 2	Congenital HA	OSA & bCSA	bCSA	600
Inhibitor 3	Acquired HA	OSA & bCSA	bCSA	500
Inhibitor 4	Acquired HA	OSA & bCSA	bCSA	600
Negative control	Congenital HA	OSA	bCSA	0

HA, haemophilia A; OSA, one-stage clotting assay; bCSA, bovine chromogenic assay

Statistical analysis

All statistics analysis was performed using GraphPad Prism 10 (GraphPad Software, Bostonm MA, USA). Significance between OSA and CSA mean titres for each inhibitor were performed by the Wilcoxon matched pairs test.

RESULTS

Test methodology

Seven sets of results were returned from the six participant laboratories, including two sets from the central laboratory. One set of results from each of Set 1 and Set 2 were excluded from analysis due to unsatisfactory results in the negative control sample, leaving six sets for analysis. All other sites returned negative control inhibitor titres of between 0.0 and 0.4 BU by OSA and CSA. Table 2 summarises the test components used by each site. For OSA, there were three analyser types, four sources of pooled normal plasma, four different APTT reagents, three brands of FVIII-deficient plasma, and four different sample diluents. For CSA three different bCSA kits were used, with other needed Bethesda assay components the same as for the OSA. No two sites had identical test components for all aspects of the assay. Two sites reported delays in post-incubation FVIII testing of 30-60 min in a few samples due to technical issues. All sites made their own decisions on sample dilution(s) used to calculate the final titres for reporting.

Table 2. Test details for setting up sample incubations and FVIII measurements during Bethesda assays for FVIII inhibitors for six different protocols from five testing laboratories. For the chromogenic FVIII assay the coagulation analyser, pooled normal plasma, sample and control tube diluents were identical to those used in one-stage assays.

	One stage clotting assays					
	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6
Coagulation analyser	Sysmex CS2500	STAR-Max	STAR Max 3	STAR-Max	ACLTOP 550	ACLTOP 750
Source of pooled normal	Precision Cryocheck	Siemens SHP	Precision Cryocheck	Helena Norm-trol	In-house	Precision Cryocheck
Pooled normal lot	A1325	563115	A1329	1215186	N/A	A1325
APTT reagent	Siemens Actin FS	Stago Triniclot aPTT S	PTT Automate	Stago Triniclot aPTT S	Synthasil	Synthasil
Source of FVIII deficient	Siemens	Stago	Stago	Stago	IL	IL
Control and sample tube diluent	4% bovine albumin	FVIII deficient plasma, immunodepleted	Stago Owren-Koller buffer	Helena Factor VIII deficient, congenital	4% bovine albumin	4% bovine albumin
Lower limit of detection (BU)	0.5	0.5	0.5	0.5	0.5	0.5
	Chromogenic substrate assays					
Bovine chromogenic FVIII kit	Siemens FVIII	Siemens FVIII	TriniCHR-OM FVIII	TriniCHR-OM FVIII	Hyphen FVIII Variant	Hyphen FVIII Variant

Bethesda assay titres

For samples free of emicizumab (Set 1), mean OSA titres were 1.1, 2.5, 3.5 and 4.8 Bethesda units (BU), and for bCSA, titres were 1.2, 2.1, 3.4 and 4.4 BU (Table 3). There was no statistically significant difference in inhibitor titres between OSA and bCSA for each the four samples in this group (Figure 1). Similarly, there was no significant difference in mean inhibitor

titres for all inhibitors between bCSA assays performed in the absence (Set 1) or presence of emicizumab (Set 2). Assay variability by all methods for Inhibitor 1 and Inhibitor 3 was moderate to high, with CVs ranging from 12.9% by OSA for Inhibitor 1, up to 27.9% by CSA in the samples without emicizumab (Table 3). In contrast, the CVs for Inhibitor 2 and Inhibitor 4 were very high, ranging from 40.1% to 69.3%. These high CVs reflect the high inter-laboratory range of final titres in these samples. For example, Inhibitor 2 final titre by OSA ranged from 1.0 to 5.1 BU. When the residual percent FVIII for each dilution was plotted against sample dilution factors, two relatively distinct patterns were visible (Figure 2). Inhibitors 1 and 3 showed 'simple' kinetics, with the FVIII residuals rising steeply through the 50% mid-point. Inhibitors 2 and 4 showed FVIII residuals rising much more slowly with sample dilution or having 'complex' kinetics, which correlated with the high variability in final titres of these two samples.

Table 3. Summary of FVIII inhibitor titres from six test sites

		Inhibitor 1	Inhibitor 2	Inhibitor 3	Inhibitor 4
OSA	Mean titre (BU)	1.1	2.5	3.5	4.8
-no	(range)	(0.9-1.3)	(1.0-5.1)	(2.7-4.7)	(1.4-9.2)
emicizumab	CV (%)	12.9	65.7	23.6	69.3
bCSA	Mean titre (BU)	1.1	2.1	3.4	4.5
-no	(range)	(0.7-1.5)	(0.9-4.1)	(2.5-4.8)	(2.1-7.4)
emicizumab	CV (%)	27.4	55.4	27.9	54.8
bCSA	Mean titre (BU)	1.2	2.2	2.9	6.0
-contains	(range)	(0.8-1.5)	(0.8-3.4)	(2.4-3.5)	(3.6-9.2)
emicizumab	CV (%)	22.1	40.1	17.2	41.9

OSA, one-stage clotting assay; bCSA, bovine chromogenic assay; BU, Bethesda unit; CV, coefficient of variation

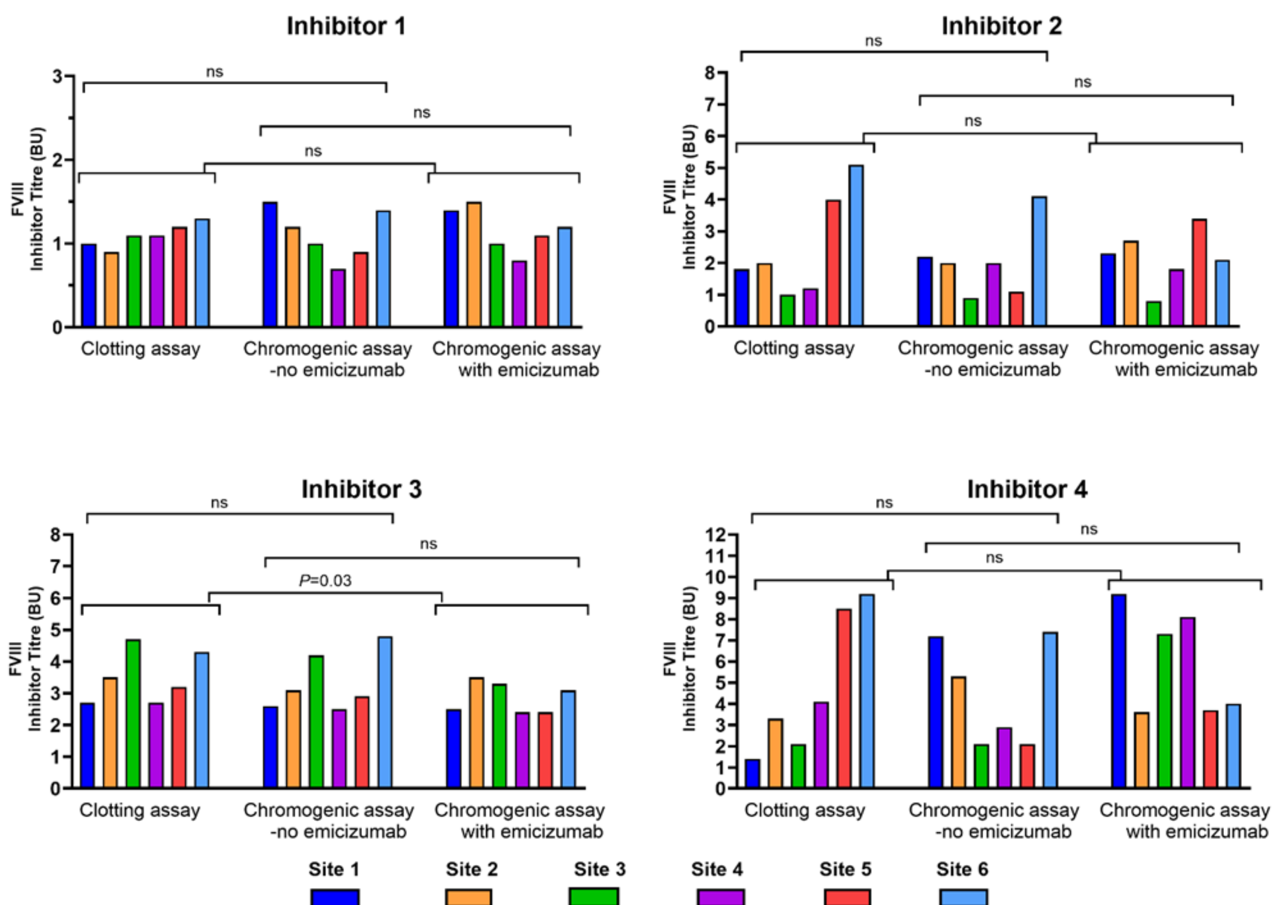

Figure 1. Bethesda assay titres of four inhibitors from six sites by FVIII clotting assays and FVIII chromogenic assays in samples without emicizumab, and FVIII chromogenic assays in samples containing emicizumab. ns=not significant.

DISCUSSION

The recent use of emicizumab as a therapeutic agent for CHA and AHA has highlighted the need for laboratories to introduce CSA with bovine components to measure FVIII levels in the presence of emicizumab either for a FVIII level per se, or in the context of a Bethesda assay for FVIII inhibitors. In this study six different reagent / analyser combinations were used for Bethesda assays if four samples with different FVIII inhibitors by means of a field study of five specialist haemophilia reference laboratories. It was shown that in the absence of emicizumab mean inhibitor titres did not differ between OSA and bCSA, nor did mean titres

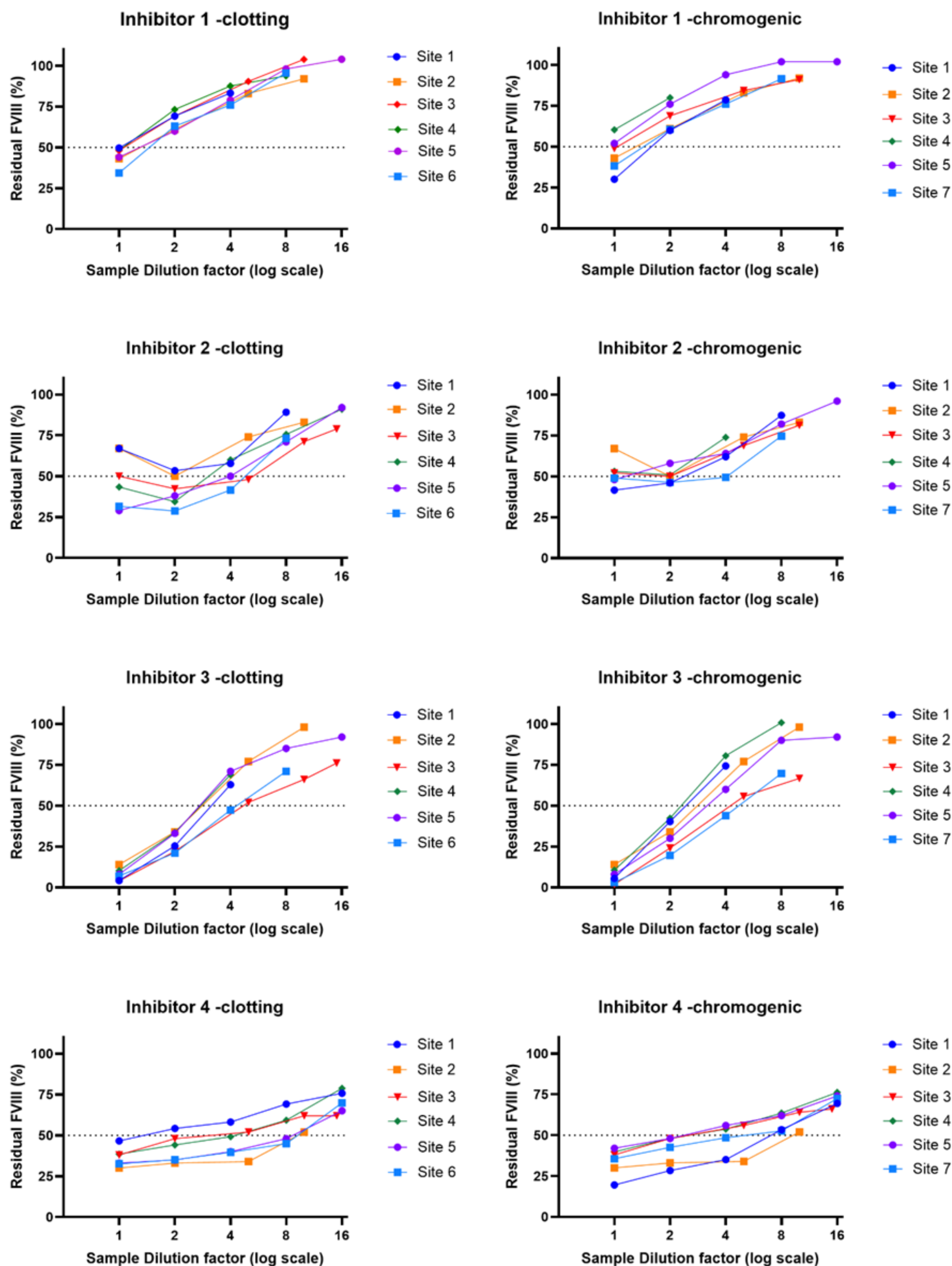

Figure 2. Residual FVIII versus sample dilution on Bethesda assays for Set 1 samples tested by both one-stage FVIII assay and bovine chromogenic FVIII assay.

differ when bCSA was used on matched samples where emicizumab was present or absent. The use of bCSA for performance of Bethesda assays for FVIII inhibitor titres in the presence of emicizumab has been endorsed by different published guidelines, including the suggestion that CSA may be less subject to interference than OSA from other inhibitors such as lupus anticoagulants (13, 14). Available bCSA kits vary in that some contain both bovine FX and FIXa, whereas others, known as 'hybrids' contain bovine FX and human FIXa. In the present study three different bCSA kits were used of which two were the hybrid type. A previous single centre study by Bowyer et al. found no difference in measured inhibitor titres between two different bovine/bovine CSAs and three different bovine/human CSAs when assaying samples from 10 CHA with inhibitors who were receiving emicizumab therapy (7). In the same study between method CVs were <10% in seven of the 10 samples and 19-24% in the remaining three samples. All titres were weak averaging less than 2.2 BU. The present study had CVs by bCSA of 22.1% and 17.2% for Inhibitor 1 and Inhibitor 3, respectively in the samples containing emicizumab. This is reasonable by comparison because this study contained six different reagent combinations incubation tube preparation, whereas the 10-sample study of Bowyer et al employed a single set of dilutions, tested by all CSAs. In general, between laboratory variability for Bethesda assays is very high ranging from 20-80%, due to the large number of variables affecting the assay, including source of pooled normal plasma, use of buffering, type of diluent and other reagents making up the assay (13). An design feature of the present study was the inclusion of two samples from individuals with AHA (Inhibitors 3 and 4), done because of increasing use in Australia of emicizumab to treat AHA, which necessitates regular use of bCSA to monitor inhibitor clearance over time. It was somewhat paradoxical that one of these has simple kinetics (Inhibitor 3) while an inhibitor from a CHA individual had complex or Type kinetics (Inhibitor 2). The two inhibitors with complex kinetics had inter-

method CVs of 40-69% with bCSAs being lower than OSAs. In both inhibitors the lower dilutions from 1/1 to 1/4 mostly yielded residuals close to 50% (Figure 2), which causes large variability in final titre because the tube taken for final inhibitor titre calculation will vary. It is important with this type of pattern, where multiple sample dilutions have residuals close to 50%, to be consistent over time in choice of dilution from which to calculate the final titre, such as using the highest dilution before the inhibitor rapidly weakens (11). In the present study the high CV for Inhibitor 2 reflected the inhibitor's kinetics, and not the choice of OSA or CSA. In fact, the CVs for Inhibitor 2 were lower with CSA than with OSA (Table 3).

In this study the degree of emicizumab spiking was aimed at achieving therapeutic levels of emicizumab in the tubes where dilutions predicted to have residual FVIII of around 50% based on preliminary testing, This inevitably involved some guesswork. For antibodies #2, #3 and #4 this was 500-600 µg/mL, levels of emicizumab far exceeding those normally observed in clinical practice, and this can be a seen as study limitation. As an alternative, consideration was given to spiking pooled normal plasma with emicizumab to a uniform 100 µg/mL and distributing this to participants for use as the FVIII source in the Bethesda assays. This would have achieved a consistent therapeutic level of 50 µg/mL of emicizumab in all sample dilution used by all participants. This was decided against because it would have altered each laboratory's routine protocol by standardizing on the same pooled normal, partially defeating the aims of evaluating inter-laboratory variability, and allowing each site to assess their own assay performance.

A future consideration with the use of bCSA for FVIII measurement concerns the potential introduction of next generation bi-specific antibodies that mimic FVIII, including Mim8 (Novo Nordisk, Bagsvaerd, Denmark) and NXT007, developed by Chugai Pharmaceuticals (Tokyo, Japan) (15). Hybrid CSAs, which have bovine FX/human FIXa components have been shown to have varying degrees of sensitivity to Mim8, but bovine/bovine FVIII CSAs were insensitive

at all Mim8 concentrations (16). If confirmed, in future studies FVIII measurements in the presence of Mim8, and potentially NXT007, will require bovine/bovine CSAs.

CONCLUSION

The measurement of FVIII inhibitors in samples containing emicizumab has been successfully carried out in several haemophilia reference laboratories by use of bCSAs. There was no statistical difference in FVIII inhibitor titres when comparing OSA and bCSA in samples without emicizumab, nor with bCSA with or without emicizumab present. Chromogenic FVIII assays can be successfully used to measure FVIII inhibitor titres in the presence of emicizumab.

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CHAPTER 11

DISCUSSION AND CONCLUSIONS

The ongoing development of new replacement therapies for HA and HB has created some challenges for specialist haemostasis laboratories due to the nature of the modifications to the FIX and FVIII that are required to extend their half-life and improve patient quality of life. Molecular modifications are known in some cases to cause large inaccuracies in measured clotting factor levels requiring the implementation of alternate strategies to ensure closeness of results to true levels is achieved. The primary focus of this work has been to address the laboratory testing issues relating to monitoring of current replacement therapies for haemophilia. Emphasis has been placed on validating newer assays including the chromogenic FIX assays and emicizumab assays and exploring the suitability of current clotting and chromogenic assays for the measurement of new products. This was achieved through extensive testing in the central laboratory, combined with the use of field studies where other haemostasis reference laboratories participated in measurement exercises. This had the twofold benefit of determining which reagents were acceptable for ongoing testing, plus allowing the participant laboratories to have validation data for their own methods. The main replacement products studied were those currently licensed for use in Australia by the National Blood Authority, and which therefore have the most relevance for specialist haemostasis testing laboratories nationwide.

The work on the two commercial kits for chromogenic FIX assays resulted in the first published general evaluation of these assays. The findings covered multiple aspects of these assays, including the establishment of reference intervals derived from plasma calibration standards testing 128 healthy volunteer donors, precision studies at multiple FIX plasma levels, spike

recovery estimates of samples containing SHL replacement rFIX Benefix, Rixubis and the EHL product Alprolix. The diagnostic accuracy of the CSA was compared to current FIX OSA on a series plasma samples from untreated donors with HB. There were some lower results with the CSA compared to OSA in untreated individuals with HB particularly for the Hyphen kit, which led to re-classification from mild to moderate Hb, or from moderate to severe HB. An interesting finding was the apparent over-assignment of FIX in one of two commercial standards used to calibrate the Rossix test, which led to a higher lower limit for the reference interval. Following the method validation studies, several Australian testing laboratories accepted invitations to participate in a study of FIX CSA for the measurement of a range of plasma derived FIX samples, rFIX and the EHL Alprolix using the Rossix kit. The findings showed good agreement between laboratories over a wide range of FIX levels in all sample types. The FIX CSA remains available for assessing FIX levels in newly diagnosed HB, though this is not yet a specific recommendation of the World Federation of Haemophilia.

The field study of Eloctate measurement (Chapter 5) yielded results in line with the one previously published study. This field study of 16 participants was conducted to allow local laboratories to assess both OSA and CSA suitability in measurement of the EHL product Eloctate, coinciding with its introduction in Australia. This study found that Eloctate can be measured by either OSA or CSA with CSA giving slightly higher results. The provision of specific recovery data according to specific APTT reagent provided important feedback to the participant laboratories in this field study.

The Adynovate field study (Chapter 6) provided reagent specific recovery information for participants that was not previously available for this EHL rFVIII. The present study's all-reagent OSA mean percent recovery for 80 and 20 IU/dL spikes of 108.5% and 123.0%, respectively, were only slightly higher than corresponding recoveries of 101.0% and 112.9% reported by Turecek and co-workers (1). The study confirmed that OSA is suitable for

Adynovate measurement, but that CSA tended to overestimate FVIII levels in some sites. By contrast, the study of of Bulla et al found significant over-recovery of Adynovate by OSA of between 127.7% to 146.6% relative to four spike levels between 80 IU/dL and 10 IU/dL using commercial plasma calibrators (2). A third study by Ternisien et al found significant under-recovery by many OSA APTT reagents, some of which were found acceptable in the current study, the main difference being the use of SSC/ISTH Secondary Coagulation Standard Lot #4 (3). As a group these studies highlight some discrepancies on OSA and CSA that are not expected when all calibrator types are traceable to International Standards.

In chapter 7, the in vitro characteristics of efanesoctocog alfa were studied at a single site. It was able to be established that two models of ACLTOP analysers could measure efanesoctocog alfa with the affiliated manufacturer's SynthaFax APTT reagent using a plasma calibrator. This was important as the previously published field study only contained data from a single site with this reagent. The reference assay for this drug was to use Actin FSL APTT reagent on a Sysmex analyser. The ability to adapt this reagent to the ACLTOP analyser was successfully explored through multiple protocol variations. FSL was found to be suitable provided the same sample/deficient/ plasma/reagent ratios as used on the Sysmex analyser were maintained, and that the Siemens FVIII deficient plasma was used instead of Werfen FVIII deficient plasma. The reason for this is not clear. The advantage of using Actin FSL is that this was the reagent used to assign product potency and therefore retains the like- versus-like principle. The in vitro stability of efenesoctocog alfa was studied at four different temperatures. There is no previously published data on drug stability. A key finding was the requirement for 90 minutes at 56° to remove all FVIII activity, as opposed to 20 minutes for normal plasma FVIII. This is important to know prior to performing Bethesda assays on sample where efenesoctocog alfa needs removal.

In Chapter 9 the laboratory characteristics of emicizumab measurement were studied in detail. Original data published in the work included the reproducibility of emicizumab assays at multiple drug levels in both spiked and ex vivo samples, the linearity of recovery estimates in the clinically important range 5-120 µg/mL, and a freeze/thaw stability of control materials. The first field study of emicizumab measurement among specialist haemostasis laboratories was also conducted and published. The product was funded for use towards the completion of these studies meaning the Australian haemophilia reference laboratories have validated assays available for emicizumab measurement. The emicizumab work was extended in Chapter 10 to study interlaboratory agreement of Bethesda assays for FVIII inhibitors in samples containing emicizumab between several reference centres. This presence of emicizumab in the test samples necessitated the use of CSA for the FVIII measurements, which was relatively new to most participants. Interlaboratory agreement was found to be similar to levels currently seen in external quality assurance programs for sample tested by OSA. CV's of around 20% were found, partly due to the many known subtle variations in reagent use between laboratories.

FUTURE DIRECTIONS

Recombinant factor replacement products, non-factor replacement products and gene therapies for the treatment of HA and HB continue to be developed. Each of these will need assessment in terms of best methods of laboratory measurement and their impact on other coagulation assays if or when they are funded for use in Australia. Examples of recently developed products not currently licensed in Australia include dalcinonocog alfa, fitusiran, Mim8 (denecimig), and NXT007. Dalcinonacog alfa (DA) has been developed by Catalyst Biosciences and is full length rFIX molecule with amino acid substitutions in the protease domain that confer the product with increased potency, enhanced FVIIIa binding and reduced inhibition by

antithrombin (4). Importantly, the enhancements give DA a 22-fold greater potency than Benefix in vitro, and DA can be administered subcutaneously (5). Laboratory monitoring of dalcinocog alfa by OSA showed 3-fold differences between APTT reagents, and valid results could not be obtained with FIX CSA, which means careful consideration will be needed in reagent choice when monitoring this product (4).

Fitusiran is an interfering RNA agent that reduces antithrombin synthesis in the hepatocytes, ultimately rebalancing haemostasis (6). Fitusiran effective in bleed control in severe HA or HB patients with inhibitors in phase 3 clinical trials with once-a-month subcutaneous administration (7). The reduction in plasma antithrombin levels has no impact on standard laboratory assay of APTT, PT, and FVIII levels measured by either OSA or CSA (8). It is likely that validation of a suitable antithrombin assay will be required to measure plasma antithrombin levels.

Mim8 and NXT007 are both next-generation bi-specific antibodies having a similar mechanism to emicizumab, namely the generation of endogenous FXa from binding to FIXa and FX. Mim8 has been demonstrated to have similar impact of laboratory assays to emicizumab, namely strong interference with any APTT-based tests including APTT-based factor assays and lupus anticoagulant assays (9). The increased FVIIIa-mimetic activity of NXT007 translated to significantly increased potency in thrombin generation, clotting, fibrinolysis, and bleeding assays in mouse models (10). Detailed analysis of the effect of NXT007 on a range of assays routinely performed in clinical laboratories is not currently available but may be like emicizumab and Mim8 in the effects on APTT-based assays, and in requiring a product-specific calibrator to measure plasma levels.

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