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Verification and implementation of a chromogenic Factor VIII assay to monitor Factor VIII levels in patients with Haemophilia A on emicizumab therapy.



Tlangelani V Masia

A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of requirements for the degree of Master of Medicine (Haematology)

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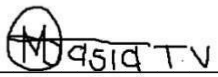
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DECLARATION

I, Tlangelani V Masia, declare that this Research Report is my own, unaided work. It is being submitted for the degree of Master of Medicine (Haematology) at the University of the Witwatersrand, Johannesburg. It has not been submitted for any degree of



_____ (Signature)

3rd of June 2024, Johannesburg, South Africa.

CERTIFICATE OF SUBMISSION

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG | FACULTY OF HEALTH SCIENCES

CERTIFICATE OF SUBMISSION FOR EXAMINATION SIGNED BY SUPERVISORS OF HIGHER DEGREES CANDIDATES

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The names of the examiners should be confidential during the examination process and may only be revealed to the candidate with the acquiescence of the examiner once the final version of the thesis has been submitted to the Faculty and the process has been completed.

ACKNOWLEDGEMENTS

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Finally, to my loving husband, mother, brothers and son thank you for your encouragement, support and all the sacrifices.

PUBLICATIONS FROM THIS STUDY

This research is presented as a submissable article for publication in the peer reviewed Clinical Laboratory journal.

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ABBREVIATIONS

AARMS - Academic Affairs and Research Management System

aPTT - Activated Partial Thromboplastin Time

ACE910 - Emicizumab

BUs - Bethesda units

CMJAH - Charlotte Maxeke Johannesburg Academic Hospital

CLSI - Clinical and Laboratory Standards Institute

CV - Coefficient of variation

EQA - External quality assurance

FVIIIa - Activated factor VIII

FIXa - Activated factor IX

FXa - Activated factor X

FVIII - Factor VIII

FIX - Factor IX

FX - Factor X

ICSH - International Council for Standardization in Haematology

ISTH - International Society on Thrombosis and Haemostasis

NHLS - National Health Laboratory Service

N - Normal

OSA - One-stage assay

P - Pathological

RCPA - Royal College of Pathologists of Australasia

SD - Standard deviation

SOP - Standard operating procedure

SUBMISSABLE ARTICLE

TITLE PAGE

Verification and implementation of a chromogenic Factor VIII assay to monitor Factor VIII levels in patients with haemophilia A on emicizumab therapy.

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TITLE: Verification and implementation of a chromogenic Factor VIII assay to monitor Factor VIII levels in patients with haemophilia A on emicizumab therapy.

ABSTRACT

INTRODUCTION: Hemophilia A is an X-linked inherited bleeding disorder traditionally managed with replacement of the deficient clotting factor. A subset of patients however, develops inhibitors to infused FVIII rendering this treatment ineffective. Emicizumab is a novel, non-factor based therapy for patients suffering from hemophilia A with efficacy in the presence of inhibitors. Assessment of FVIII activity in patients on emicizumab treatment however, requires analysis with a chromogenic FVIII reagent such as the TriniCHROM FVIII:C (Stago) assay.

METHODS: STAGO® engineers set-up the TriniChrome FVIII:C assay followed by 20 consecutive analyses of normal and pathological TriniChrome FVIII:C controls on both analyzers to assess precision.
FVIII levels were measured in 50 samples from patients with (n 15) and without (n 35) hemophilia with a time-to-clot and the TriniCHROM FVIII:C assays to determine agreement between the 2. Samples from
10 patients with hemophilia A known to have FVIII inhibitors who were not exposed to emicizumab were also analyzed for FVIII inhibitor activity with both assays. FVIII levels in 10 patients with hemophilia A on emicizumab therapy were also determined with both assays. These were all for the accuracy study. Results were analyzed with standard statistical methods.

RESULTS: Acceptable SD <8 according to the manufacturer and International Council of Standardization in Hematology and % CV <8.4 according to the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) were obtained on both analyzers. Acceptable agreement of FVIII levels between the 2 assays was obtained on the 50 random plasma samples with a linearity coefficient of determination (R^2) of 0.92. FVIII inhibitor levels on the 10 samples from patients with known inhibitors were also within the significance category of more than 0.6 Bethesda Units (BUs) with both assays. FVIII levels of patients with severe hemophilia A on emicizumab therapy analyzed with the time-to-clot assay produced measurable results reflecting the endogenous activity of the drug but FVIII results with the chromogenic assay were similar to the patient's baseline pre-treatment FVIII levels.

CONCLUSION: The TriniCHROM FVIII:C assay is compatible with routine automated coagulation analyzers and can be utilised to assess endogenous FVIII and FVIII inhibitor levels in hemophilia A patients on emicizumab therapy.

Key words: Hemophilia A, Factor VIII inhibitors, Emicizumab, Time-to-clot FVIII assay, Chromogenic FVIII assay.

INTRODUCTION

Congenital hemophilia A is an X-linked inherited bleeding disorder characterized by deficient coagulation factor VIII (FVIII) with an incidence of 1:5,000 live male births [2]. This disorder most commonly results from a large inversion in the long arm of the X-chromosome at position 28 (Xq28). Consequently, patients suffer from a bleeding diathesis which presents at various ages [1, 2]. Hemophilia A is classified according to the FVIII plasma level as per the International Society of Thrombosis and Haemostasis (ISTH) guideline which defines 3 severity groups: mild (FVIII >5 - 40 IU/dL), moderate (FVIII 1-5 IU/dL) and severe (FVIII <1 IU/dL) [3]. Patients with hemophilia suffer from recurrent, often unprovoked deep seated bleeding episodes into joints, muscles, soft tissue and internal organs. These episodes lead to, amongst other manifestations, chronic joint and muscle damage with significant morbidity and even mortality [2].

The management of patients with hemophilia A includes regular replacement therapy with FVIII concentrates, also known as prophylaxis, however, this does not allow for levels to achieve hemostasis but rather prevent bleeding. FVIII replacement is either administered episodic when bleeding occurs or at a regular interval as prophylaxis e.g. 3 to 4 times per week with either human or recombinant factor concentrates [2, 3]. A major complication of factor replacement therapy is the development of neutralizing alloantibodies (inhibitors) against the replacement FVIII rendering therapy ineffective. These auto-antibodies develop in 20-30% of patients with severe and 5-10% with mild to moderate hemophilia A [2, 4].

Therapeutic options to overcome the neutralizing effect of inhibitors include non-factor replacement therapies including pro-coagulants such as emicizumab [2, 5, 6]. This agent is a recombinant, bispecific monoclonal antibody that recognizes and simultaneously bind to 2 distinct target antigens namely the active sites of activated factor IX (FIXa) and factor X (FX) [5]. Emicizumab therefore simulates the action of endogenous FVIIIa in bringing FIXa and FX in close proximity with resultant proteolytic activation of FX. This agent binds with similar affinity to both inactive and active forms of FIX and FX without requiring pre-activation by thrombin resulting in more rapid activation of FX versus endogenous FVIII. In addition, the natural anticoagulants, proteins C and S, do not have any proteolytic action on emicizumab which contributes to its prolonged hemostatic activity [5, 7].

One of the challenges with emicizumab is however the laboratory monitoring of FVIII and FVIII inhibitor levels in patients on therapy which is usually required prior to invasive surgery and with breakthrough bleeding on therapy. The commonly used time-to-clot FVIII activity assay based on the activated Partial Thromboplastin Time (aPTT) produces falsely elevated FVIII activity results in patients on emicizumab therapy [8-10]. In addition, due to its prolonged halflife of approximately 28 days, the results of additional coagulation assays based on the aPTT including the Bethesda inhibitor assay will be affected for up to 5-6 months [7, 11]. Chromogenic FVIII assays based on bovine coagulation factors, such as the TriniCHROM FVIII:C ® (Diagnostica STAGO®, Asnières-sur-Seine, France) reagent, accurately assesses endogenous FVIII as well as FVIII inhibitor levels in patients on emicizumab therapy [6]. Chromogenic FVIII assays containing human coagulation factors are however sensitive to emicizumab and will result in the same overestimation of endogenous FVIII levels as the aPTT based FVIII assay in patients on this novel therapy [11, 12].

The aim for the current study was to verify the performance of the TriniCHROM FVIII:C® reagent in the determination of FVIII and FVIII inhibitor levels in patients with hemophilia A on emicizumab therapy.

MATERIALS AND METHODS

This verification study was performed at the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH), a tertiary care medical facility in South Africa, in the onsite National Health Laboratory Service (NHLS) laboratory over a period of 4 weeks in November 2021. The study was performed in accordance with the International Committee on Standardization in Hematology (ICSH) guidelines [13] and the NHLS validation standard operating procedure (SOP). In order to minimize inter-operator bias, the analyses were performed by the same dedicated NHLS technologist. Ethics approval for the study was obtained from the University of the Witwatersrand (Wits) Human Research Ethics Committee (HREC) (Medical) (M210830).

Study samples and analysis

All analyzes were performed on the previously validated STAGO® MAX automated coagulation analyzers in use for routine analyses in the NHLS laboratory. The TriniCHROM FVIII:C assay test set-up was performed by STAGO® instrument engineers in accordance with the reagent package insert with the following modifications to improve assay performance which were also incorporated in the TriniCHROM FVIII:C® SOP:

- Reconstitution of the FIXa and FX reagents was performed with 2 mL of distilled water instead of 1 mL.
- The reagents were incubated at room temperature (18-25ºC) for 30 minutes instead of 5 minutes.

Reagents, calibrators and controls

The FVIII and FVIII inhibitor analyses were performed utilizing the one-stage time-to-clot STA® - deficient VIII plasma with STA®-PTT Automate 5 reagents and the chromogenic TriniCHROM FVIII:C® reagents. The test principals of the 2 assays are as follows:

1) STA® FVIII deficient with Automate aPTT®: measurement of time-to-clot formation in seconds after agonist introduction with subsequent determination of the FVIII activity level from a calibration curve.
2) TriniCHROM FVIII:C®: measurement of change in color detected upon enzymatic FXa action on a chromogenic substrate with subsequent determination of the FVIII activity level from a calibration curve. Calibrations were performed with the respective commercial calibrators and study samples were only analyzed after satisfactory internal quality control (IQC) results were obtained.

Precision (repeatability) study

Precision was assessed on normal (N) and pathological (P) commercial controls of the TriniCHROM FVIII:C® reagent which were both analyzed 20 consecutive times.

Accuracy study

The accuracy study was performed on the FVIII activity results obtained with the one-stage time-to-clot STA® - deficient VIII plasma with STA®-PTT Automate and the TriniCHROM FVIII:C® chromogenic reagents on the following 3 groups of samples:

1) Fifty (50) residual patient Citrate plasma samples submitted to the laboratory for routine coagulation tests including 15 from patients with hemophilia A.
2) Ten (10) residual Citrate plasma samples from patients with hemophilia A with documented inhibitors of more than 0.6 Bethesda units (BUs).
3) Ten (10) Citrate plasma samples from patients with hemophilia A on emicizumab therapy.

DATA ANALYSIS

For the precision analysis, the collected data was presented in tabular form considering mean, standard deviation (SD) and coefficient of variation (%CV) in Excel® 2016. The results were compared to known acceptable precision standards from the manufacturer, the International Committee on Standardization in Haematology (ICSH) and European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) variation database. The data collected for the accuracy analysis were captured in Excel® and linear regression and Bland-Altman analyses were performed. The y-intercept and the coefficient of determination (R^2) were determined with linear regression and the SD and 95% limits of agreement with the Bland-Altman analyses.

Statistical terms

Mean - the average of data set.

Interquartile range (IQR) - spread of the middle half of the data.

SD - the average amount of variability within a data set.

%CV - the ratio of the SD to the mean.

Y-intercept - the value where the regression line crosses the y-axis, representing the value of y when x=0 (zero). R^2 - the proportion of the variation in the dependent variable that is predicted from the independent variable; determines whether a statistical model predicts an outcome.

95% limits of agreement - an interval within which 95% of differences between 2 methods of measurement lies.

RESULTS

Precision study

The results of the precision study on normal (N) (within reference range) and pathological (P) (below reference range) TriniCHROM FVIII:C® commercial controls are depicted in Table 1 with the Manufacturer (STAGO®) acceptable performance, ICSH recommendation and EFLM biological variation also shown.

Table 1. Precision of TriniCHROM FVIII:C® controls.

	STAGO® MAX 1 analyzer (Serial no CJ 06030478)		STAGO® MAX 2 analyzer (Serial no CJ 06030483)		Manufacturer (STAGO®) acceptable performance	ICSH recommendation	EFLM biological variation database
	Control N	Control P	Control N	Control P			
Standard Deviation (SD)	3.88	1.28	3.53	1.18	<8	<8	
Coefficient of Variation (%CV)	4.53	2.94	2.78	2.83			8.4

ICSH, International Council for Standardization in Hematology; EFLM, European Federation of Clinical Chemistry and Laboratory Medicine; N, normal; P, pathological.

Accuracy study

The FVIII results of the 50 patient samples analyzed with the STA® FVIII deficient with Automate aPTT® reagent had a range of 1-285 IU/dL (median 107 IU/dL (IQR 28-134 IU/dL) compared to the range with the TriniCHROM FVIII:C® reagent of 1-260 IU/dL (median 102 IU/dL (IQR 40-154 IU/dL). Thirty-five (70%) of the 50 plasma samples were from patients without hemophilia A and normal FVIII levels of greater than 50 IU/dL were obtained with both reagents. 15 (30%) of the 50 plasma samples were from patients with hemophilia A with FVIII levels below the normal reference index (RI) of 50-150 IU/dL. Eight of the 15 samples (53%) indicated moderately reduced FVIII levels of 2-10 IU/dL and 4 (26%) mildly reduced levels of 10-40 IU/dL with both reagents. The remaining 3 (20%) samples from patients with hemophilia had discrepant severity levels of FVIII reduction which was moderately reduced with the STAGO® time-to-clot reagent and mildly reduced with the TriniCHROM FVIII:C® reagent (Figure 1). No samples from patients with severe hemophilia A were included in the accuracy analysis. The R^2 between the 2 assays was 0.92 (Figure 2) with a bias of 9.08 (Figure 3).

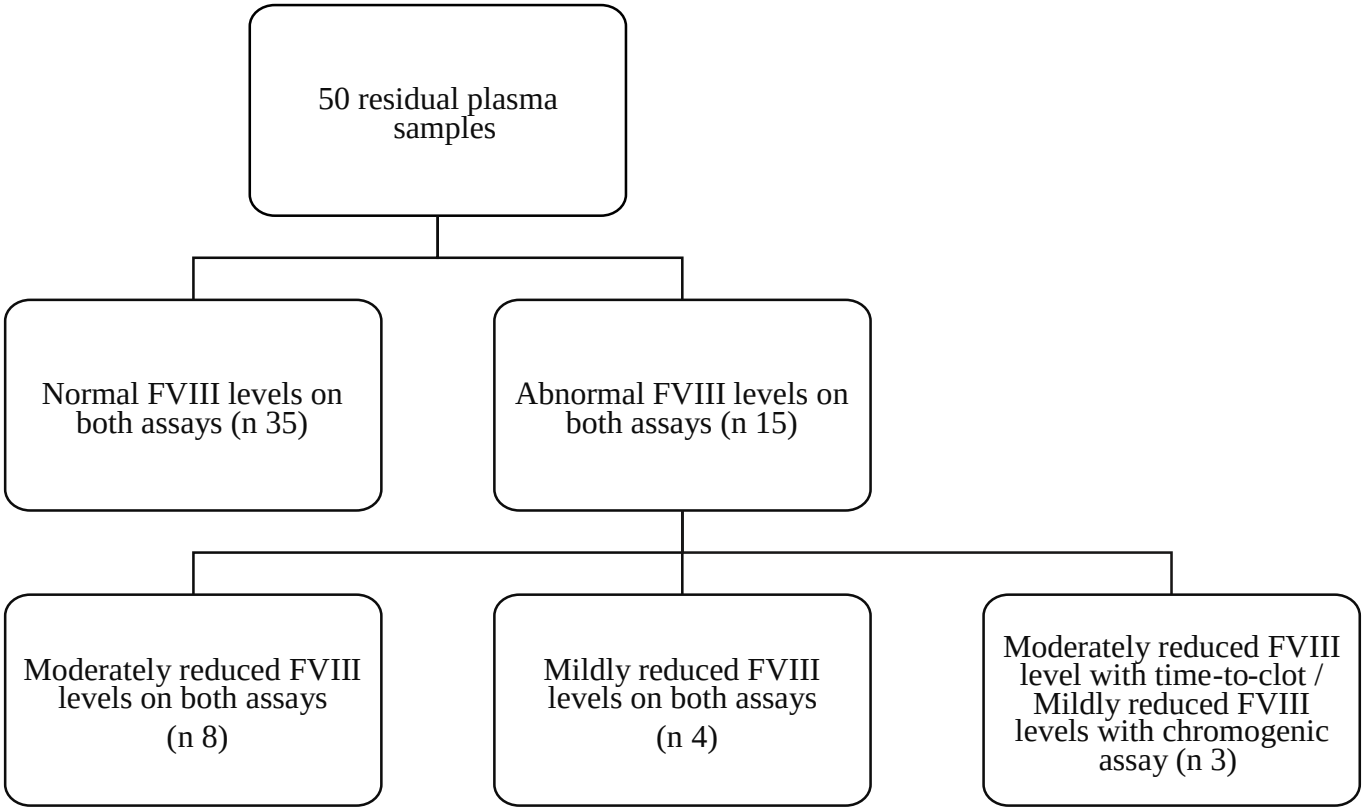


Figure 1: Flow diagram of FVIII results of patient plasma samples (n 50) analyzed with a one-stage time-to-clot and the chromogenic TriniCHROM FVIII:C® assays including 15 samples from patients with hemophilia A. FVIII, Factor VIII

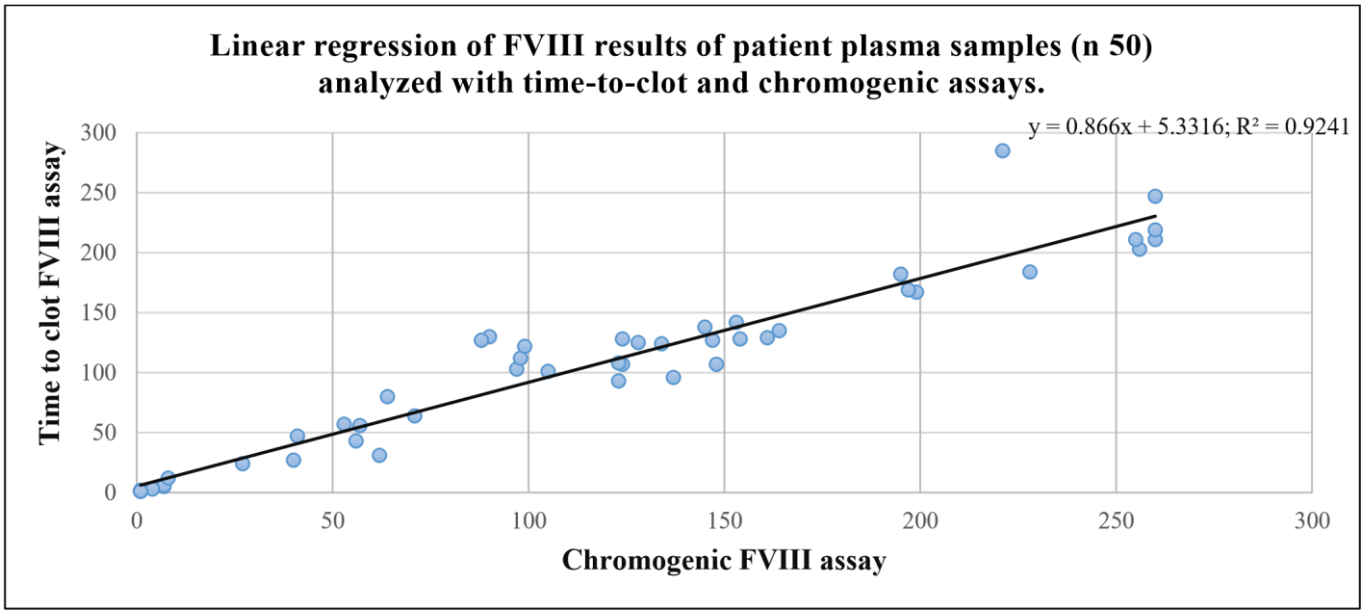


Figure 2: Linear regression of FVIII results of patient plasma samples (n 50) analyzed with a one-stage time-to-clot and the chromogenic TriniCHROM FVIII:C® assays including 15 samples from patients with hemophilia A. FVIII, Factor VIII

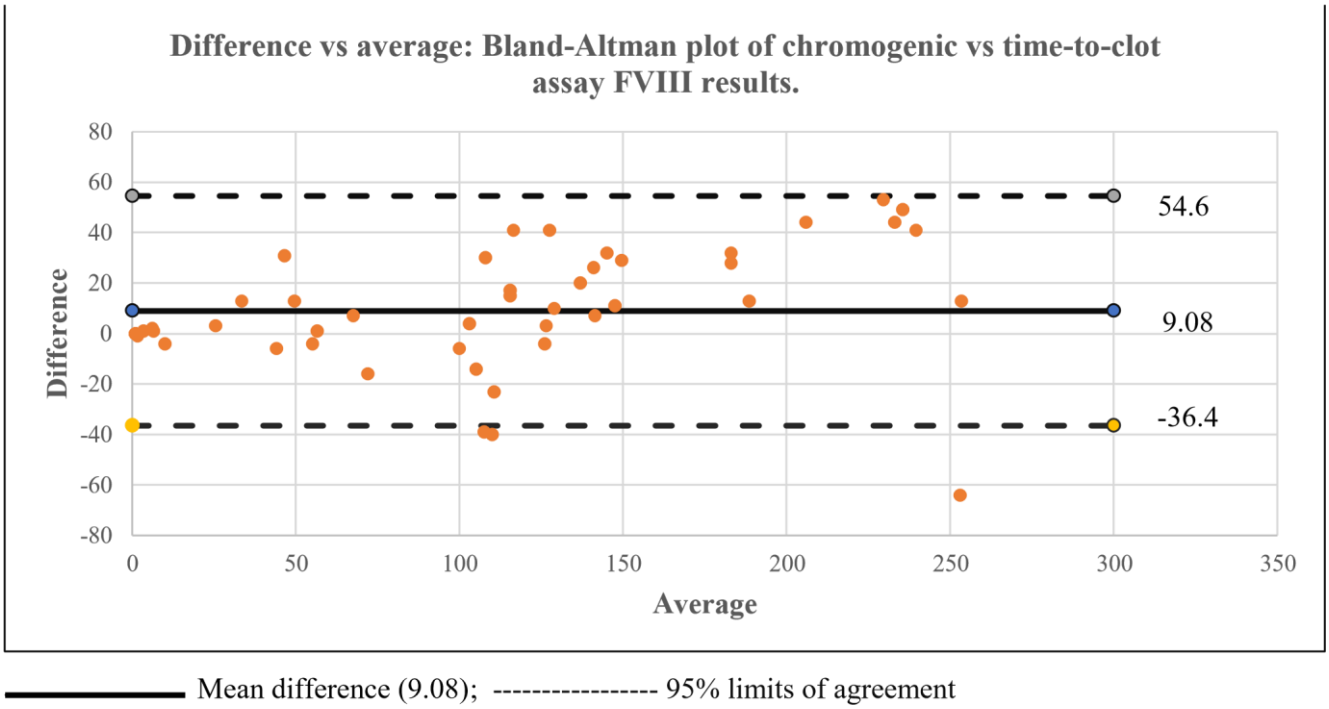


Figure 3: Bland-Altman plot of FVIII results of patient plasma samples (n 50) analyzed with a one-stage time-to-clot and the chromogenic TriniCHROM FVIII:C® assays including 15 samples from patients with hemophilia A. FVIII, Factor VIII

The results of all 10 plasma samples analyzed for FVIII inhibitor levels with a Bethesda assay based on FVIII levels obtained on both reagents were within the clinical significance category of inhibitors of more than 0.6 BUs with a R^2 of 0.96 (Figure 4) and a bias of 13.08 (Figure 5).

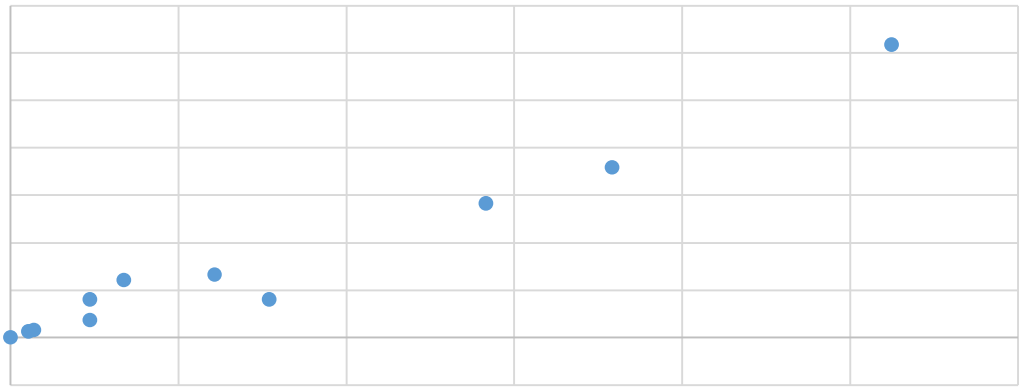
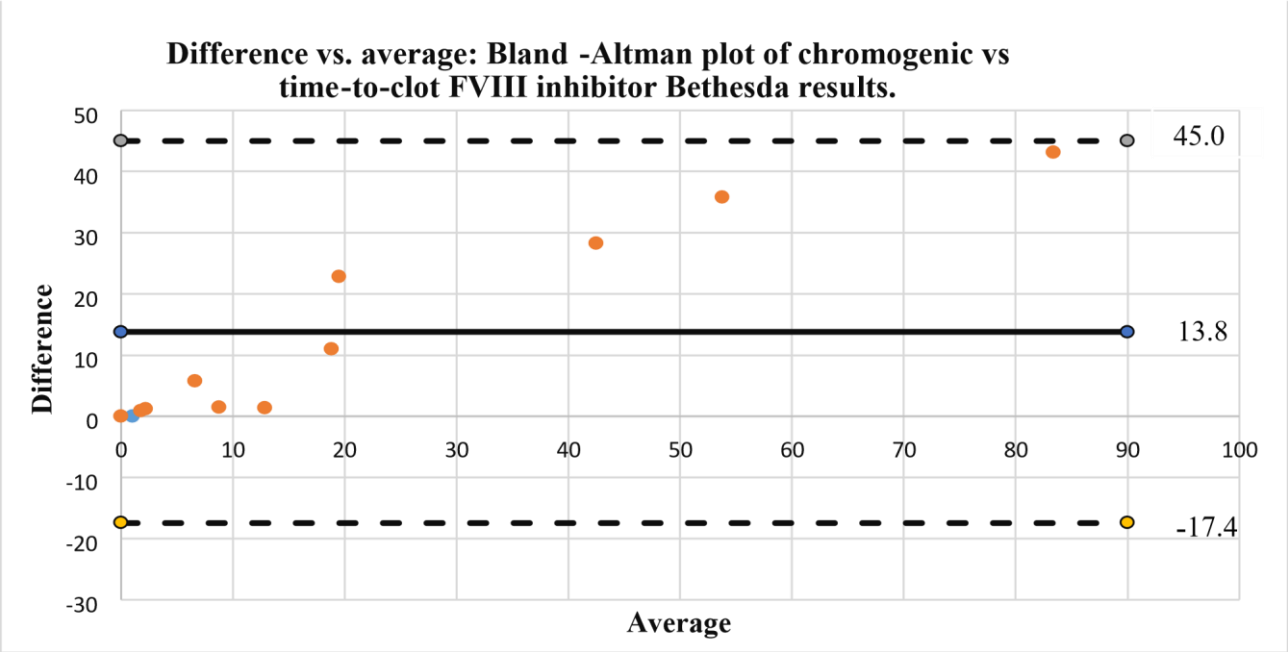


Figure 4: Linear regression of FVIII inhibitor results of patient samples (n 10) with known FVIII inhibitors analyzed with a one-stage time-to-clot and the chromogenic TriniCHROM FVIII:C® assays. FVIII, Factor VIII



_____ Mean difference (13.8); ---------- 95% limits of agreement

Figure 5: Bland-Altman plot of FVIII inhibitor results of patient samples (n 10) (Series 1) with known FVIII inhibitors analyzed with a one-stage time-to-clot and the chromogenic TriniCHROM FVIII:C® assays. FVIII, Factor VIII

The 10 plasma sample from patients on emicizumab therapy demonstrated the effect of emicizumab on the time-toclot assay delivering detectable FVIII levels. However, TriniCHROM FVIII:C® chromogenic assay reflected the correct endogenous FVIII level which was similar to that prior to commencement of therapy (Table 2).

Table 2: FVIII levels of patients on emicizumab therapy with one-stage time-to-clot and TriniCHROM FVIII:C® assays.

Patient no	Time-to-clot FVIII assay (IU/dL)	Chromogenic FVIII assay (IU/dL)	Pretreatment FVIII level (IU/dL)
1	291	1	1
2	457	1	1
3	7	1	1
4	9	1	1
5	8	1	1
6	247	1	1
7	412	1	1
8	600	1	1
9	565	1	1

10	7	1	1

DISCUSSION

The HAVEN trials demonstrated excellent responses to emicizumab therapy in patients with hemophilia A with and without FVIII inhibitors and the use of this drug is therefore expected to increase [5, 14, 15]. This is especially true for patients with high titer FVIII inhibitor levels and associated with frequent bleeds with high annualized bleeding rates. Measurement of endogenous FVIII and associated inhibitor levels is however challenging in patients on emicizumab therapy as the commonly used one-stage time-to-clot assay overestimates the FVIII concentration due to the homeostatic activity of the drug [8, 11].

Alternative laboratory assays are therefore required to accurately assess endogenous FVIII and FVIII inhibitor levels in patients on emicizumab therapy. A chromogenic FVIII assay based on bovine coagulation factors such as the TriniCHROM FVIII:C® assay is required since emicizumab does not recognize or interact with the factors in this reagent [11].

The current study evaluated the performance of the TriniCHROM FVIII:C® (STAGO®) chromogenic FVIII reagent. Acceptable precision (repeatability) was demonstrated on N and P commercial controls with clinically acceptable accuracy of both FVIII and FVIII inhibitor results in comparison with a one-stage time-to-clot aPTT based assay in samples (n 50) from patients with and without hemophilia not exposed to emicizumab. The R^2 value of 0.92 on the linear regression analysis of the FVIII levels obtained with the 2 assays is within the recommendation of the ICSH for method comparison of 0.9-1.1 [16]. The overestimation of FVIII levels with the one-stage time-to-clot aPTT based assay was confirmed in patients on emicizumab therapy. The chromogenic assay however accurately reflected these patients' endogenous FVIII level despite the presence of the drug.

Patients with mild or moderate hemophilia A could have discrepant FVIII levels with the one-stage time-to-clot versus the chromogenic FVIII assay with resultant misclassification of the severity of the bleeding disorder which has been detailed in a recent publication by Bowyer et al. [12]. This discrepancy was also demonstrated in the current study in which 3 patients with hemophilia had higher FVIII results with the chromogenic TriniCHROM FVIII:C® reagent versus the one-stage assay with potential misclassification of the hemophilia severity category. Multiple genetic pathogenic variants in patients with hemophilia A have been shown to cause disagreement between different FVIII testing assays [17]. Further details of the identified pathogenic variants are outside the scope of the current research but it is recommended that laboratories attached to hemophilia treatment centers have access to both one-stage and chromogenic FVIII assays [18], and genotype analysis be offered to all people with hemophilia A [17,18]. In circumstances were discrepancy does occur, it is suggested that the lowest factor level by any assay correlates best with the phenotype but other studies suggest that chromogenic assays better reflect bleeding tendency [17].

Limitations of this study are inclusion of only 10 samples from patients on emicizumab therapy. The possible interference of hemolysis, icterus and lipemia on chromogenic assays was assessed during the installation of the STAGO® analysers but not re-evaluated in the current study. Low curve analysis of samples with FVIII levels between

1-10 IU/dL and linearity of the TriniCHROM FVIII:C® assay were also not performed. These omissions relate to limited availability of samples from patients on emicizumab therapy as well as limited TriniCHROM FVIII:C® reagent and should be evaluated in the future.

Despite these limitations the results of this study indicate that the TriniCHROM FVIII:C® reagent delivers accurate and repeatable FVIII and FVIII inhibitor results in patients with hemophilia A on emicizumab therapy. The assay is further compatible with standard automated coagulation analyzers and can be introduced in the routine coagulation laboratory.

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Appendix 1: Ethics approval

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R14/49 Dr Tlangelani Vivian Masia

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M210830

NAME: (Principal Investigator)	Dr Tlangelani Vivian Masia
DEPARTMENT:	Molecular Medicine and Haematology NHLS - Charlotte Maxeke Johannesburg Academic Hospital
PROJECT TITLE:	Verification and implementation of a chromogenic Factor VIII assay to monitor Factor VIII levels in patients with haemophilia A on emicizumab therapy
DATE CONSIDERED:	27/08/2021
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr S. Louw
APPROVED BY:	CB Penny Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	21/09/2021

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions.Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. I **agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in **August** and will therefore be due in the month of **August** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

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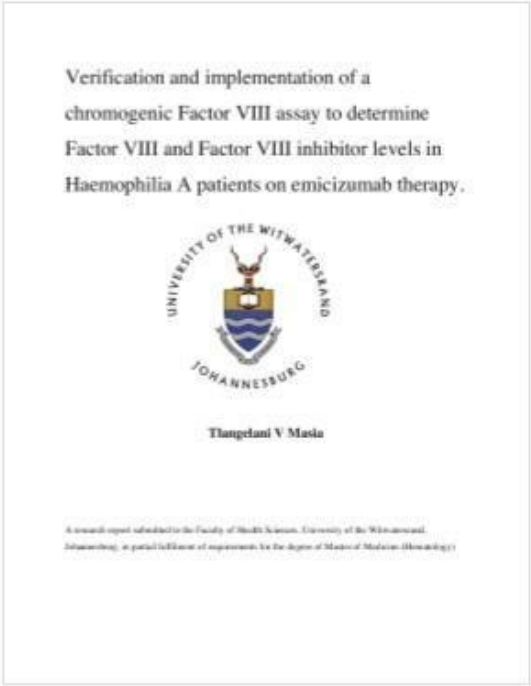
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Verification and implementation of a chromogenic Factor VIII assay to determine Factor VIII and Factor VIII inhibitor levels in Haemophilia A patients on emicizumab therapy.

Tlangelani V Masia

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Damien P. Higgins, Susan Hemsley, Paul J. Canfield. " Association of Uterine and Salpingeal Fibrosis with Chlamydial Hsp60 and Hsp10 Antigen-Specific Antibodies in -Infected Koalas ", Clinical and Vaccine Immunology, 2005

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Jenny Kwak, Michael Mazzeffi, Lisa N. Boggio, Mindy L. Simpson, Kenichi A. Tanaka. "Hemophilia: A Review of Perioperative Management for Cardiac Surgery", Journal of Cardiothoracic and Vascular Anesthesia, 2020

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Appendix 3: Precision study raw data

		Repeatability STA MAX 1:			Repeatability STA MAX 2:	
		Control P	Control N		Control P	Control N
1						
2						
3		Control P	Control N		Control P	Control N
4	1	43.73	95.95		41.3	124.14
5	2	42.17	90.8		42.25	135.09
6	3	42.33	80.23		40.75	123.55
7	4	42.23	82.24		41.41	123.78
8	5	42.69	83.61		43.65	130.98
9	6	43.45	85.65		40.26	123.35
10	7	45.68	85.69		41.11	130.26
11	8	42.15	84.34		42.73	123.19
12	9	44.24	84.5		43.89	125.13
13	10	43.54	83.37		40.56	123.82
14	11	42.73	88.28		41.57	125.27
15	12	43.62	85.42		42.73	123.37
16	13	44.92	83.55		40.57	126.82
17	14	43.54	83.72		41.37	125.89
18	15	42.32	83.73		41.81	127.85
19	16	43.19	82.99		42.16	125.86
20	17	44.05	83.92		42.16	131.64
21	18	46.03	90.69		42.89	132.51
22	19	46.18	89.45		40.57	128.21
23	20	42.72	91.49		44.31	126.18
24	**Mean**	**43.62052632**	**85.69105263**		**41.77578947**	**126.8445**
25	**Median**	**43.495**	**84.42**		**41.69**	**125.875**
26	**Standard**	**1.278985926**	**3.884061764**		**1.17906687**	**3.530420394**
27	**%CV**	2.94	4.53		2.83	2.78

Appendix 4: Research Protocol

Verification and implementation of a chromogenic Factor VIII assay to monitor Factor VIII levels in patients with haemophilia A on emicizumab therapy.

Student:

Dr Tlangelani Vivian Masia

Student number 573016

Molecular medicine and Hematology registrar

Supervisor:

Dr Susan Louw

Haematopathologist

Department of Molecular Medicine and Hematology

University of Witwatersrand (WITS)

National Health Laboratory Service (NHLS)

Background

Hemophilia A is an X-linked inherited bleeding disorder characterized by deficient coagulation Factor VIII (FVIII) with an incidence of 1:4000 live male births. This disorder is the result of a genetic defect in the long arm of the X chromosome at position 28 (Xq28). The most common genetic mutation is a large inversion with translocation of exons 1-22. Other defects include point mutations, gene deletions and insertions.

FVIII is an amino acid glycoprotein containing 26 exons and 25 non-coding introns with a molecular weight 293 kDa. This protein has 6 domains, namely A1-A2-B-A3-C1-C2, which is subdivided into 2 polypeptide chains, a heavy (A1A2-B) and light (A3-C1-C2) chain which is joined together by a covalent bond. Upon proteolytic activation by thrombin there is cleavage of the B domain forming a heterodimer consisting of A1/ A2/ A3-C1-C2 (1–4). After production by hepatocytes, endothelial cells and lymphatic tissue, FVIII circulates in plasma as a heterodimer bound to von-Willebrand factor (vWF) via a non-covalent bond. FVIII dissociates from its complex with vWF when it is activated and binds to the surface of activated platelets and acts as a co-factor for activated coagulation factor IX (FIXa). In the presence of

phospholipid and calcium the FVIIIa-FIXa complex activates coagulation factor X (FX) which, with activated coagulation factor V (FVa), have prothrombinase activity and converts prothrombin to thrombin (5).

The disease severity in patients with hemophilia A is classified according to the FVIII plasma level as per recommendation by the Subcommittee of the International Society of Thrombosis and Hemostasis (ISTH). Three severity groups are recognized: mild (FVIII of >5-40 IU/dL), moderate (FVIII of 1-5 IU/dL) and severe (FVIII <1 IU/dL) (6,7). Patients suffer recurrent, often unprovoked deep-seated bleeding episodes into their joints, muscles, soft tissue and internal organs. These episodes lead to chronic joint and muscle damage with significant morbidity and even mortality.

The management of patients with hemophilia A includes regular replacement therapy with FVIII concentrates to maintain adequate levels to achieve hemostasis and prevent bleeding. Apart from on demand treatment of bleeding episodes, regular prophylactic infusions 3 to 4 times per week or every other day has been successful in patients with severe hemophilia (6).

Concentrates of human FVIII however have short half-lives requiring multiple, frequent infusions which negatively impacts on the quality of life of both patients and their caregivers. Elucidation of the protein structure of the coagulation factors as well as the amino acid sequences have led to the commercial manufacturing of recombinant factor products with extended half-life properties. Half-life prolongation techniques of recombinant products include PEGylation, fusion with long half-life proteins such as albumin and the constant regions of immunoglobulins (iGg-Fc) (4,8) which prolong the half-life of recombinant factors by 2.5-5 fold and reduce infusion rate to weekly or fortnightly (8).

The major complication of factor replacement therapy is the development of neutralizing alloantibodies against the replacement FVIII rendering therapy ineffective with recurrent bleeds and reduced quality of life. Antibodies occur in 20-30% patients with severe and 5-10% with mild-moderate hemophilia A (5,9).

Additional therapeutic options have been developed to overcome the effect of inhibitors which include non-factor agents with increased half-lives and less immunogenicity including procoagulants such as emicizumab.
Emicizumab, also known ACE910, is a bispecific antibody i.e. recombinant, monoclonal antibody that recognizes and simultaneously binds to 2 distinct target antigens (2,7,10) i.e. the active sites of FIXa and FX (2). The drug therefore simulates the action of FVIIIa and has the ability to simultaneously bind to both FIXa and FX at single specific antigenic sites, thereby bringing these coagulation factors in close proximity resulting in proteolytic activation of FX. This bispecific antibody binds with similar affinity to both inactive and active forms of FIX and FX without requiring preactivation by thrombin and therefore results in more rapid activation of FX, as compared to endogenous FVIII which requires activation prior to exerting a proteolytic action on FX (2). The FIX produced by activated FVII-Tissue Factor (TF) complex in the extrinsic pathway is also readily available as a co-factor for emicizumab which is constitutively active. The natural anticoagulants, Proteins C and S, do not have any proteolytic action on emicizumab and it therefore has prolonged hemostatic activity (7).

Emicizumab was well tolerated with no serious adverse effects, no detectable changes in endogenous FVIII, IX or X levels, no detectable changes on D-dimers, platelet count nor the concentration of thrombin-antithrombin complexes in human trials. Anti-drug antibodies develop in 4.2% of trial patients which is similar to that documented after exposure to most other humanized antibodies (2,7).

The greatest challenge with emicizumab therapy is however the monitoring of the hemostatic activity of therapy which is often required prior to invasive surgery and in patients with spontaneous bleeding episodes while on therapy. The commonly used intrinsic pathway monitoring assays based on the activated Partial Thromboplastin Time (aPTT), which measures time to clot based on thrombin dependent FVIIIa generation, results in falsely shortened results due to the fact that emicizumab does not depend on thrombin for its activation and overestimate the FVIII concentration (2,7). In addition, due to its prolonged half-life of approximately 28 days, the results of routine coagulation assays based on the aPTT will be affected for up to 5-6 months following the last administered dose. Chromogenic FVIII assays, based on bovine coagulation factors such as TRINICHROM: CHROMOGENIC FACTOR VIII reagent accurately assess endogenous FVIII as well as FVIII inhibitor levels and the hemostatic balance in patients on emicizumab therapy. Chromogenic FVIII assays containing human coagulation factors are however sensitive to emicizumab and will result in an overestimation of endogenous FVIII activity and hemostatic balance in patients receiving emicizumab (7).

Study goal:

Verify the accuracy of the TRINICHROM: CHROMOGENIC FACTOR VIII reagent to monitor FVIII levels as well as FVIII inhibitor levels in local hemophilia patients on emicizumab therapy.

Study objectives:

- Determine the accuracy of the TRINICHROM: CHROMOGENIC FACTOR VIII reagent by comparing the results obtained with this reagent on samples from patients with and without hemophilia but not on emicizumab therapy with the results obtained with the already validated STAGO® time-to-clot FVIII assay.
- Demonstrate reproducibility of the chromogenic FVIII assay reagent with repeat analysis of TRINICHROM: CHROMOGENIC FACTOR VIII reagent internal control material.
- Determine the endogenous FVIII levels with both the TRINICHROM®: CHROMOGENIC FACTOR VIII and the time-to clot FVIII assay in samples from patients on emicizumab therapy and compare the results with the already determined factor levels prior to commencing emicizumab therapy.

Methodology:

1. This verification study will be performed as far as possible following the guidelines by the International Committee on Standardisation in Haematology (ICSH 1993), the Clinical and Laboratory Standards Institute recommendations (CLSI, USA) and NHLS validation guidelines (GPQ 0007). In order to minimize interoperator bias, the analyses will be performed by the same dedicated, technically competent staff following the instrument standard operating procedure (SOP). As the principal investigator, I will be involved in majority of the activities needed in analysis, including preparation and processing of samples and control material, extracting data and analyzing for accuracy and reproducibility.
- This analysis will be carried out over a period of four weeks.

Setting: Charlotte Maxeke Johannesburg Academic Hospital (CMJAH), National Health Laboratory Service (NHLS) haematology coagulation laboratory.

Analyzer:

STAGO® MAX automated coagulation analysers, that has already been set up with verification that all reagents can be utilized successfully.

Reagents:

- FVIII chromogenic assay: TRINICHROM® CHROMOGENIC FACTOR VIII reagent.
- FVIII time-to-clot assay: STA® FVIII deficient plasma and Automate aPTT reagent.

Calibrator and Control material:

- TRINICHROM® FACTOR VIII reagent calibrator and Control I and II.
- STA® System calibrator and Control N&P.

Samples:

- Residual plasma, after the analysis of requested routine tests, from 50 venous blood samples received in the main NHLS laboratory at CMJAH from patients with and without hemophilia will be utilized to investigate agreement between the time-to-clot and the chromogenic FVIII reagents.
- 10 residual samples of patients known with factor VIII inhibitors not on emicizumab will be used to investigate agreement between time-to-clot and chromogenic FVIII reagents utilizing the Bethesda (inhibitor) method.
- 10-20 blood samples from hemophilia patients on emicizumab from the hemophilia trial clinic and collected during the approved emicizumab clinical trial from consenting trial participants, as arranged with Prof JM Mahlangu.
    - No additional blood will be collected from patients for the purpose of this verification study.
    - All patient samples will be centrifuged and stored at -80˚C prior to analysis for a maximum period of 60 days (2 months).
    - Controls and reagents will be stored and processed as per manufacturer instruction.

Reproducibility study on control material:

The within run reproducibility study will be performed on normal (i.e. within reference range) and abnormal (i.e. below reference range) commercial controls supplied by the manufacturers. Each control will be analyzed 20 times on the STA® MAX analyzers. The results will be collated on an Excel® spread sheet, tabulated and graphically summarized using standard statistical methods.

Accuracy study:

1. 50 Citrate plasma samples will be processed on the already validated STAGO® MAX automated coagulation analyzers in routine use in the NHLS laboratory with both the FVIII time-to-clot (STAGO STA FVIII Deficient plasma and STA® aPTT Automate) and the TRINICHROM : FACTOR VIII chromogenic assay.
    - Each sample will be split in to 2 aliquots which will be analyzed with each of the 2 reagents on one of the STAGO® Max analyzers.
    - Calibration curves and positive and negative controls will be processed and samples will only be processed after satisfactory calibration and performance of the control material.
    - The analytical accuracy will be determined via statistical analysis including Bland Altman plots and linear regression analysis. Acceptability of correlation will be based on biological databases (11,12).
    - The test principals of the 2 reagents are as follows:
        - Time to clot formation assay: measured in seconds after agonist introduced to plasma.
        - Chromogenic assay: change in colour detected upon enzymatic action on a chromogenic substrate.

2. 10 Citrate plasma samples of patients with known FVIII inhibitors will be processed on the STAGO® MAX coagulation analyzers with the FVIII time-to-clot and the chromogenic factor VIII assays to determine the inhibitor levels using the Bethesda method.

3. 10-20 samples from hemophilia patients on emicizumab will then be processed with the chromogenic and timeto-clot FVIII reagents and the results compared with the pre-treatment endogenous FVIII levels that was previously determined prior to the commencement of emicizumab therapy

Data analysis:

Precision analysis: The data from the precision analysis will be presented in tabular form considering mean, standard deviation (SD) and coefficient of variation (CV) for the test. The results will be compared to known acceptable precision standards from the manufacturer, the International Committee on Standardisation in Haematology (ICSH) and European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) variation database.

Accuracy analysis: The results of the FVIII levels obtained on patient samples with the 2 reagents will be captured in Excel® and analyzed on Excel® data analysis software with Linear regression and Bland-Altman analyses to investigate the accuracy of the chromogenic FVIII reagent. The y-intercept and the coefficient of determination, the R^2, will be determined with the linear regression and the standard deviation (SD) of the bias and 95% limits of agreement will be determined with the Bland-Altman analysis.

Study Limitations

To mitigate against emicizumab contamination due to sample carry-over during analysis an additional needle rinse step will be performed although no clinical significant sample carry-over was detected during the validation of the STAGO analyzers.

Ethics:

An ethic clearance application for this verification study has been made to the University of the Witwatersrand (WITS) Ethics Committee (HREC) and approval is awaited.

Timing:

	May-Aug 2020	Feb-Mar 2021	April 2021	May-July 2021	Aug- Oct 2021	Nov 2021-Jan 2022	Feb-April 2022
Literature review							
Preparing protocol							
Ethics application							
Protocol assessment							
Collecting data							
Data analysis							
Writing up thesis and paper							

Funding:

Reagents for the verification study will be funded by Roche diagnostic company as an educational grant.

References:

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Appendix 5: Submission Guidelines: Clinical Laboratory Journal

Original Articles

The majority of articles published in the Journal report original research into laboratory medicine, transfusion medicine and cell therapy. All articles are subject to review and authors are urged to be brief; long papers with many tables and figures may require shortening if they are to be accepted for publication. The text is limited to 7,000 words (not counting summary, tables, figure legends, and references), with a summary, a maximum of 7 figures and 7 tables and up to 40 references.

PREPARATION OF MANUSCRIPTS

Only manuscripts in American English are accepted. Databases used as the basis for analyses must also be available in English. Manuscripts, including figures, tables and figure legends, should be submitted as an e-mail attachment attended by a cover letter. The text should be typed 1½-spaced, on one side of the paper, with a margin of 3 cm. The pages should be numbered consecutively as well as the lines beginning with the title page. Use a separate sheet of paper for each of the following sections: title page, summary and key words, text, acknowledgements, references, individual tables and figure legends, in that order. Preferably use the type Times New Roman or Courier, and Symbol, as these can be converted into pdf files without problems.

Title Page

The title page should contain: 1) the title of the article, which should be concise but informative and should not include abbreviations or brand names. **The title is limited to a total of 120 characters and may not exceed 2 lines when written in Times New Roman with font size 16**; 2) a short "running title" of no more than 50 characters (count letters and spaces) typed at the bottom of the title page and identified as such; 3) **first name (additional first names must be given as initials with dots)** and last name **(double last names must be hyphenated)** of each author but not his/her titles, degrees, positions, or academic ranks; 4) the name(s) of department(s) and institution(s) to which the work should be attributed; 5) disclaimers, if any; 6) name, address, telephone and FAX numbers, and e-mail address of the author responsible for correspondence about the manuscript; 7) the source(s) of support in the form of grants, equipment, or drugs; 8) Declaration of Interest. 1) Please note on the title page of your word document any Declaration of Interest of any of the authors. If there are no conflicts, please state that fact.

Summary and Keywords

The second page must carry a summary of not more than 350 words. The summary should consist of four sections labeled BACKGROUND, METHODS, RESULTS, and CONCLUSIONS. These sections should briefly describe, respectively, the objectives of the study or investigation, the basic procedures (study subjects and analytic methods), the main findings (giving specific data and the statistical significance, if possible), and the principal conclusions. Emphasize new and important aspects of the study. Do not use first-person pronouns in the summary. **Keywords** should follow the summary on the same page.

Text

The text of observational and experimental articles is usually divided into sections using the headings INTRODUCTION, MATERIALS AND METHODS, RESULTS, and DISCUSSION. Long articles may need subheadings within some sections to clarify content, especially in the Materials and Methods, Results, and Discussion sections. Other types of articles, such as reviews and editorials, may require other formats; authors should consult the Editor for guidance. Use as much active voice as possible in writing.

Introduction. Clearly state the purpose of the article. Summarize the rationale used for the study or observations. Give only pertinent references: do not review the subject extensively. Do not include data or conclusions from work being reported.

Case Report. Include only for manuscripts that focus on individual histories.

Materials and Methods. Describe your selection of observational or experimental subjects (patients or animals, including controls, and the number in each study group) clearly. Identify the methods, apparatus, equipment, reagents, and procedures used in sufficient detail to allow other colleagues to reproduce the results. Publication of an original research article implies that the authors are prepared to distribute freely to academic researchers

for their own use any materials (e.g., cell lines, DNA clones, antibodies) used in the experiments. Provide model name or number, and the manufacturer's name (in parentheses) the first time a reagent, an apparatus, or any equipment is mentioned. Give references to established methods; provide references and brief descriptions of methods that are not well-known; describe new or substantially modified methods, give reasons for using them, and describe their limitations.

Statistics. The basis for these guidelines is described in Bailar JC III, Mosteller F. Guidelines for statistical reporting in articles for medical journals: amplifications and explanations. Ann Intern Med 1988;108:266-73. Describe statistical methods in detail to enable a knowledgeable reader with access to the original data to verify the reported results. When possible, quantify findings and present them with appropriate indicators of measurement error or uncertainty (such as confidence intervals). Avoid sole reliance on statistical hypothesis testing, such as the use of p values, which fails to convey important quantitative information. References for study design and statistical methods should be to standard works (with pages stated) when possible, rather than to papers in which designs or methods were originally reported. Specify any general-use computer programs used. Put general descriptions of methods in the Methods section. Define statistical terms, abbreviations, and most symbols.

Results. Present your results in logical sequence in the text, tables, and/or illustrations. Do not repeat in the text any data presented in tables and/or illustrations; emphasize or summarize only important observations, and avoid tables displaying data showing insignificant differences among groups.

Discussion. Emphasize the new and important aspects of the study and the conclusions that follow from them. Do not repeat in detail the data given in the Results section or in tables or illustrations. Include in the Discussion the implications of the findings and their limitations, and relate the observations to other relevant studies. Link the conclusions with the stated purpose of the study, but avoid unqualified statements and conclusions not completely supported by the data presented. Avoid claiming priority and alluding to work that has not been completed. Recommendations, when appropriate, may be included.

JOURNAL STYLE

Tables

Type each table on a separate sheet. **Each table must not be larger than ONE page (vertical or horizontal) written in Times New Roman and no smaller than font size 10**. Larger tables have to be submitted as "Supplemental Table S1 — Sx" in a seperate file. Tables should be numbered consecutively with Arabic numerals. A short descriptive heading should be given above each table and footnotes and/or explanations below. Please prepare your tables in "Word" by the "Table" function or use xls.-files (Excel). Do not use the tab key for tables.

Illustrations

Please send figures as an e-mail attachment. If possible, use Word or jpg files. Illustrations in color will be published only if the author pays for the extra cost involved. **For more detailed information on manuscripts see:** International Committee of Medical Journal Editors. Uniform requirements for manuscripts submitted to biomedical journals. Brit Med J 1991;302:338-41.

References

EACH REFERENCE GIVEN MUST HAVE AT A MINIMUM AN ENGLISH ABSTRACT. REFERENCES TO ARTICLES AVAILABLE ONLY IN NON-ENGLISH LANGUAGE WILL NOT BE ACCEPTED.

References must be 1½-spaced and numbered consecutively, with Arabic numerals in brackets, as they are cited. References first cited in a table or figure legend should be numbered so that they will be in sequence with references cited in the text at the point where the table or figure is first mentioned. List all authors when there are

six or fewer; when there are seven or more, list the first three, followed by “et al.” Please include the PMID (PMID: 8xxxxx4) for each reference. Should a reference NOT have PMID, please include the link or a link/address where the article can be obtained in the internet (please make sure that the article is displayed not just the journal reference - at least an English abstract must be available for reading) **e.g.**

1. **Jan-Inge Henter, Annacarin Horne, Maurizio Aricó, et al. Diagnostic and therapeutic guidelines for hemophago¬cytic lymphohistiocytosis. Pediatr Blood Cancer 2007 Feb;48(2):124-31 (PMID: 16937360).**
2. **Bjerregaard B, Talts JF, Larsson LI. The endogenous envelope protein syncytin is involved in myoblast fusion. Cell Fusions: Springer; 2011:267-75 https://link.springer.com/chapter/10.1007/978-90-481-97729_13?no-access=true**

Key references only should be given, and exhaustive lists will not be accepted. Few original communications justify more than 40 references. Reviews may contain more references. Try to avoid using summaries as references; ‘unpublished observations’ and ‘personal communications’ may not be used as references, although references to written, not oral, communications may be inserted (in parentheses) in the text. Give the name of the person from whom the communication was received and the date (month and year) of the communication. References to manuscripts accepted but not yet published may be included; designate the journal name, followed by ‘in press’ (in parentheses). Information from manuscripts submitted but not yet accepted should be cited in the text as ‘unpublished observations’ or ‘submitted for publication’ (in parentheses).

Abbreviations, Units

Except for units of measurement, abbreviations are strongly discouraged. The full term for which an abbreviation stands must precede its first use in the text for ALL abbreviations with the exception of units of measurement. Use only standard abbreviations. No abbreviations should be used in the title of the manuscript.
Solutes should be expressed in g/L, mg/L etc. Concentrations of solutes of known molecular weight may be stated in mol/L or recognized submultiples thereof (mmol/L, µmol/L etc. according to the international system of units (SI)). Enzyme activities should be reported in U/L. (http://www.bipm.org/en/si/si_brochure/).

Acknowledgments

Acknowledgments include one or more statements that specify: 1) contributions that should be acknowledged but do not justify authorship, such as general support by a departmental chairman; 2) technical help received; 3) financial and material support, specifying the nature of the support; and/or 4) financial relationships that may pose a conflict of interest. Persons who have contributed intellectually to the paper, but whose contributions do not justify authorship, may be named and their function or contribution described as, for example, ‘scientific adviser’, ‘critical review of study proposal’, ‘data collection’, or ‘participation in clinical trial’. Such persons should have given written permission to be named. Authors are responsible for obtaining this written permission from persons acknowledged by name, because readers may infer their endorsement of the data and conclusions. Technical help should be acknowledged in a paragraph separate from paragraphs acknowledging other contributions.