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Effects of Emicizumab on APTT, FVIII assays and FVIII Inhibitor assays using different reagents: Results of a UK NEQAS proficiency testing exercise.

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Dear Editors,

My co-authors and I would like to submit the manuscript with a word count of 3,233 entitled "*Effects of Emicizumab on APTT, FVIII assays and FVIII Inhibitor assays using different reagents: Results of a UK NEQAS proficiency testing exercise*" by Anna Lowe, Steve Kitchen, Ian Jennings, Dianne P Kitchen, Tim AL Woods, Isobel D Walker to be considered for publication in *Haemophilia.*

We believe these findings will be of interest to readers of your journal.

We declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

We know of no conflicts of interest associated with this publication and there has been no financial support for this work that could influence the outcome. As corresponding author I can confirm that the manuscript has been read and approved for submission by all the named authors.

We look forward to receiving your decision.

Best regards,

Anna Lowe (on behalf of the co-authors)

Abstract

Introduction: Emicizumab (Hemlibra: Roche Switzerland) is a, humanised, bi-specific monoclonal modified immunoglobulin G4 (IgG4) which binds human FX, FIX and activated FIX (FIXa) to mimic activated FVIII activity.

Aim: Evaluate the effect of APTT, surrogate FVIII activity and FVIII inhibitor results on samples containing emicizumab

Methods: Two samples were provided, one obtained from an emicizumab treated severe haemophilia A patient with FVIII inhibitors and one constructed by in-vitro addition of emicizumab using plasma from a severe haemophilia A patient without FVIII inhibitors. An APTT screen, surrogate FVIII and FVIII inhibitor tests were performed on both samples by participating centres.

Results: APTT results were below the lower limit of normal range. Chromogenic FVIII assay results with the Hyphen/Biophen assay gave higher than expected coefficient of variation (CV) results, 38-40%. The modified one-stage FVIII assay with emicizumab calibrators, showed similar results regardless of the APTT reagent. Inhibitor assay median results for sample S18:23= 1.43BU (range 0.9-3.0 BU/ml, CV 38%). S18:24 was classified as below the lower limit of detection.

Conclusion: APTT screens showed a consistent shortening. Unmodified one stage Factor VIII assay results were remarkably high. APTT based assays are unsuitable for measurement of coagulation factors or inhibitors in patients treated with emicizumab. Bovine origin chromogenic assays are insensitive to emicizumab and should be used to monitor FVIII levels/FVIII inhibitors in emicizumab treated patients. Product specific calibrators should be implemented to reduce result variability.

Effects of Emicizumab on APTT, FVIII assays and FVIII Inhibitor assays using different reagents: Results of a UK NEQAS proficiency testing exercise.

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Running title: The effects of emicizumab on APTT, FVIII & FVIII inhibitor assays.

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Introduction

Emicizumab (Hemlibra: F Hoffman-La Roche Basel Switzerland) is a, humanised, bi-specific monoclonal modified immunoglobulin G4 (IgG4) which binds human FX, FIX and activated FIX (FIXa). Unlike FVIII, it does not require activation by thrombin to act as a bridging mechanism between activated FIX (FIXa) and FX. Factor X activation occurs in the tenase complex, where emicizumab mimics activated FVII. Emicizumab has a half-life of around 4-5 weeks in the circulation

In 2017 the US Food and Drug Administration (FDA) and in 2018 the European Medicines Agency (EMA) approved the use of emicizumab in patients with haemophilia A with inhibitors as it had been shown to substantially reduce the bleeding rates in this group(1). Following review of data submitted from the HAVEN 3 trial demonstrating superior efficacy when emicizumab was compared to FVIII prophylaxis in haemophilia A subjects without inhibitors (2) the FDA and EMA approved emicizumab for prophylaxis in haemophilia A subjects without inhibitors. Currently it has been approved for use in a number of countries including USA, Australia, European Union member states and UK.

Patients receiving emicizumab therapy may have haemostasis laboratory tests and assays performed for a number of possible reasons and therefore data are needed for the different reagents and methods used routinely by haemophilia centres.

UK National External Quality Assessment Scheme for Blood Coagulation (UK NEQAS BC) performs regular external quality assessment (EQA) exercises to assess the proficiency of laboratories that are monitoring haemophilia treatments. The data collected from such exercises can also be used to assess not just local laboratory performance but also to identify reagent and method specific effects.

We report here on the first exercise in which samples containing emicizumab were analysed in multiple laboratories using a range of different reagents and methods for determination of APTT, surrogate FVIII activity and FVIII inhibitors.

Materials and Methods

This survey incorporated 2 samples. One sample (18:23) was from a severe haemophilia A patient who had a FVIII inhibitor and who had been on emicizumab therapy for 7 weeks prior to sample collection and was on maintenance doses of 1.5 mg/kg per week of emicizumab at the time of donation. The other sample (18:24) was prepared by in vitro addition of emicizumab into plasma from a patient with severe Haemophilia A, who did not have any inhibitors, at a calculated concentration of 50 µg/ml (based on the labelled potency of the drug).

Samples were collected after obtaining informed consent as approved by local ethics/clinical governance authorities. Samples were buffered with 0.8g% HEPES and 1.0% glycine and then lyophilised before being distributed through the post at room temperature. The stability of samples prepared in this way has been demonstrated . (6)

Samples were sent out to 31 centres with participants asked to use their local methods/reagents to perform the following tests:

1) An APTT,

2) An assay to detect the presence of emicizimab,

3) Any other established Factor VIII assay that the participating centre might perform in patients receiving emicizumab therapy.

Results

APTT Results

Responses came from 25 centres that performed APTTs. Results were reported as either a ratio or, seconds (which were then converted to a ratio by dividing the test result by the mid-point of the local normal range). In total six different APTT reagents were used. The majority of centres that

provided results reported APTT results which were below or around the lower limit of the normal range. Table 1 show results grouped with respect to which APTT reagents were used. four APTT groups had a sufficient amount of users to provide statistically relevant results.

FVIII assay result

Participants used a variety of different chromogenic FVIII assay kits (containing Bovine and/or Human FIXa/FX) for FVIII surrogate activity measurement for sample S18:23 and S18:24.12. A number of the participating centres performed assays for emicizumab using a modified one-stage APTT-based assay and product specific calibrators. Table 2 shows the results from both NEQAS samples S18:23 & S18:24.

Interestingly for both samples the chromogenic FVIII assay results with the Hyphen/Biophen assay gave higher than expected coefficient of variation (CV) results at 38-40% which was investigated. Centres that used the Hyphen/Biophen assay were asked to provide further information to assess the high CV. A variety of different dilutions of plasma in the assay was reported. To assess the effect of the use of the different dilutions both test plasmas were analysed at four different dilutions of sample in a single centre alongside two control samples that did not contain emicizumab, one with a normal FVIII level and one with a moderately reduced level. Results are shown in table 3. The results obtained for both controls were similar over a range of different dilutions whereas the two samples containing emicizumab showed a non-parallelism relationship so that results were higher at higher dilutions.

Emicizumab Assay results.

12 centres performed specific one-stage APTT-based assays for emicizumab, known as the modified one-stage FVIII assay. 9 of these centres reported the use of r2 Diagnostics emicizumab calibrator

material. Table 4 shows the results obtained in this exercise.

Irrespective of the type of APTT reagent used in the modified one-stage FVIII assay with emicizumab calibrators, the results that were reported from testing sample 18:24 by participants were to be similar to one another , as shown in figure 1.

FVIII inhibitor results

Ten centres performed bovine based FVIII Bethesda inhibitor assays using Siemens or Coamatic chromogenic kits. The median result for sample S18:23 was 1.43BU (range 0.9-3.0 BU/ml, CV 38%). S18:24 was classified by all participants as below the lower limit of detection, or as a negative result.

Two centres also performed Porcine FVIII Bethesda assays, reporting <0.4BU and 1.3BU for sample S18:23, and <0.4BU and 'no inhibitor detected' for sample S18:24.

Discussion and conclusion.

The introduction of emicizumab into routine clinical use raised a number of issues for haemostasis laboratories.

We report here on the first inter laboratory comparison of laboratory testing in samples containing emicizumab in a UK NEQAS Blood Coagulation EQA exercise. One sample (18:23) was obtained from a patient with severe haemophilia A with inhibitor who had been on emicizumab for 7 weeks and the other sample was prepared by in vitro addition of emicizumab into plasma from a patient with severe haemophilia A, who did not have any inhibitors, at a calculated concentration of 50 µg/ml.

Due to the mode of action of emicizumab, haemostasis tests which involve FVIII and FIX are most affected (4). There are preliminary data on the impact of emicizumab on some methods for

determination of the activated partial thromboplastin time (APTT), or APTT-based assays including single factor assays, Bethesda FVIII inhibitor titre assay and APTT-based activated protein C resistance (APCV)(5). Even at sub-therapeutic concentrations of drug, an effect can be noted when patients are commencing treatment. Data has been published to show that the APTT screen results tend to be shortened, usually below the normal reference range (4). One-stage assays, when used to monitor surrogate FVIII activity in the presence of emicizumab, can also give a falsely elevated result which has been confirmed in ouratudyfor multiple reagents. The emicizumab present in S18:23 and S18:24 produced a consistent shortening of APTT by all methods and the reported APTTs were around the lower limit of the reference range for all 6 reagents used by participants, including reagents incorporating ellagic acid, kaolin and silica activators, which was found to be consistent with the published impact of emicizumab on several APTT reagents (4, 5 & 7).

Factor VIII assays performed using unmodified one stage assays calibrated with conventional plasma standards gave surrogate FVIII activities of between 300 and 1200 IU/dl. This marked yet variable emicizumab interference indicates that these unmodified one stage assays would be unsuitable for use during emicizumab therapy There is also evidence to suggest that the degree of FVIII activity overestimation varies with different APTT reagents. These findings are consistent with single centre study data (4& 5) and support the comment by UK HCDO that APTT based assays are unsuitable for measurement of coagulation factors or inhibitors in patients treated with emicizumab (8).

A number of chromogenic substrate assays are used for FVIII activity monitoring in patients receiving conventional FVIII therapies. These vary in source of clotting factors -human or bovine, mechanism of activation of FVIII, incubation time, phospholipid/calcium concentrations and buffer type. Emicizumab binds human FIXa and human FX so the chromogenic assays that use both human FIXa and FX components show significant interference in FVIII surrogate activity levels in a concentration dependant manner; however these kits can be used to provide an assessment of FVIII surrogate activity (4,5 & 8).

The results of chromogenic FVIII assays were similar in both samples for five kits utilising bovine FIXa and FX with minimal or no interference by emicizumab detected. This was also the case for one kit with human FIXa and bovine FX as there is no interference and such assays are insensitive to emicizumab (5) as it does not bind to bovine FIXa or bovine FX). This confirms that the UK HCDO recommendation that these methods are suitable for determination of FVIII in the presence of emicizumab (8) can be applied for a number of different commercial kits.

All centres which used a chromogenic assay that incorporated human FIXa/FX in the reagents reported similar surrogate FVIII activity in both samples, at mean levels of 34 and 37 IU/ml against conventional human plasma calibrators. The inter- laboratory variability as indicated by CV results of 38-41% was higher than what has been reported for chromogenic assays in samples containing FVIII. Upon investigation we demonstrated that there was non-parallelism/dilutional non linearity which showed that the surrogate FVIII activity increased as the test samples were increasingly diluted. This should therefore be taken into account if the human FVIII chromogenic assay kit is used and the use of product specific calibrators should be assessed

To quantify the emicizumab drug concentration a modified version of the one-stage FVIII activity assay can be used. This involves a higher dilution of plasma than a standard one-stage FVIII assay and the use of CE marked emicizumab-specific calibrators (R2 diagnostics, Enzyme Research Laboratories), to calibrate the assay. (9-10). 12 centres used a modified one stage FVIII assay in where an extra 1 in 8 pre dilution of test sample prior to assay in a one stage test system calibrated with calibrators containing emicizumab (and no FVIII) was used. Despite the use of different APTT reagents, deficient plasmas and analysers, there was good agreement between reported emicizumab concentrations by this assay as indicated by the inter laboratory CVs of 11-13% This helps to confirm that this modified One stage assay is suitable for use with different APTT reagents for determination of active emicizumab present in patient samples as recommended elsewhere (9).

The proportion of patients developing clinically relevant anti-drug antibodies was low in clinical trials however centres may still use laboratory assays to monitor the presence and level of emicizumab if there is clinical suspicion of neutralising antibody development. In this case interference of emicizumab can cause false negative or false low inhibitor titres. Even the incorporation of a heat inactivation step into the method, which is normally used to inactivate residual FVIII before testing, does not completely remove the emicizumab IgG4 antibodies from the patient plasma. Within this survey the detection of FVIII inhibitors in a sample from a severe haemophilia A patient known to have inhibitors was successful regardless of the type of bovine kit used. The variability between centres was similar to that reported in laboratory proficiency testing exercises utilising samples that did not contain emicizumab (10). This was also the same in samples without inhibitors, where participants reported results that were lower than the lower limit of detection/absence of inhibitors again independent of the type of bovine based chromogenic Bethesda assay.

For proficiency testing it is important that test materials behave in a similar way to patient samples ie are commutable (6). In the present exercise the results obtained on a constructed/spiked sample were similar to those obtained on a sample from a patient treated with emicizumab in all the tests studied confirming that spiked samples are commutable and are therefore suitable for use in EQA between- laboratory studies

Regular participation in proficiency testing exercises like the one reported here is essential so that individual laboratories can obtain the evidence that their current techniques are fit for purpose. It also helps to assess the suitability of future methods in use by haemophilia centres seeking to make use of emicizumab in their patient population.

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Tables legends

Table 1: The APTT screen results for Sample S18:23 (Patient on emicizumab therapy) and S18:24 (FVIII deficient plasma spiked with emicizumab)

Table 2: The chromogenic FVIII assay results, including bovine, human and human-bovine kits for sample S18:23(Patient on emicizumab therapy) and sample S18:24 (FVIII deficient plasma spiked with emicizumab).

Table 3: The effects of dilution on both a sample containing emicizumab and a quality control sample.

Table 4: Emicizumab assay results from participating centres that used r2 Diagnostics emicizumab calibrators within a modified one-stage FVIII assay.

Table 1

		Sample S18:23			Sample S18:24		
Reagent	**N**	**Median (APTT ratio)**	**CV (%)**	**Range (APTT ratios)**	**Median (APTT ratio)**	**CV (%)**	**Range (APTT ratios)**
Siemens Actin FS	8	0.80	6.2	0.72-0.86	0.81	7.8	0.71-0.88
Siemens Pathromtin SL	2	0.91	-	0.87-0.94	0.92	-	0.88-0.95
Stago STA CK Prest	1	0.81	-	-	0.80	-	-
Triniclot APTT-HS	1	0.73	-	-	0.80	-	-
Not Stated	2	0.81	-	0.76-0.85	0.77	-	0.76-0.78
Werfen HemosIL APTT-SP	1	0.80	-	-	0.87	-	-
Werfen HemosIL Synthasil	10	0.79	3.7	0.74-0.83	0.80	4.0	0.74-0.83
Overall	25	0.80	6.3	0.72-0.94	0.81	6.6	0.71-0.95

Table 2.

			Sample S18:23			Sample S18:24		
Assay	**Kit**	**n**	**Median Surrogate FVIII IU/dl**	**CV (%)**	**Range Surrogate FVIII IU/dl**	**Median Surrogate FVIII IU/dl**	**CV (%)**	**Range Surrogate FVIII IU/dl**
Chromogenic: human FIXa /FX in reagents	Hyphen Biophen	9	33.6	38.3	15.1-57.0	37.0	40.7	16.5-63.0
Chromogenic: bovine FIXa/FX in	Chromogenix	1	<5	-	-	<5	-	-

reagents*	Coamatic FVIII	4	<1 – 4.4	-	<1 – 4.4	<1 – 4.4	-	<1 – 4.4
	HemosIL Electochrome (bovine)	1	<5.8	-	-	<5.8	-	-
	Siemens bovine	9	<1	-	0.1-<3.0	<1	-	0.1-<3.0
	Trinichrom	1	1			1	-	-
Chromogenic: mixed human/bovine FIXa/FX	Technochrom	1	1.7	-	-	2.4	-	-
1-stage assay (unmodified assay with plasma calibrants)	All reagents	10	470.5	45.9	302-1168	499.1	30.6	355-942

** these data exclude 1 centre stating use of a chromogenic assay but reporting results of 499u/dl, in line with 1-stage APTT results.*

Table 3

Sample dilution	**Patient sample S18:23**	**Spiked sample S18:24**	**Human Plasma control #1**	**Human Plasma control #2**
	Surrogate FVIII IU/dl	**Surrogate** FVIII IU/dl	FVIII IU/dl	FVIII IU/dl
1 in 40	35	39	80	31
1 in 80	43	48	81	30
1 in 160	51	58	77	30
1 in 240	59	64	73	34

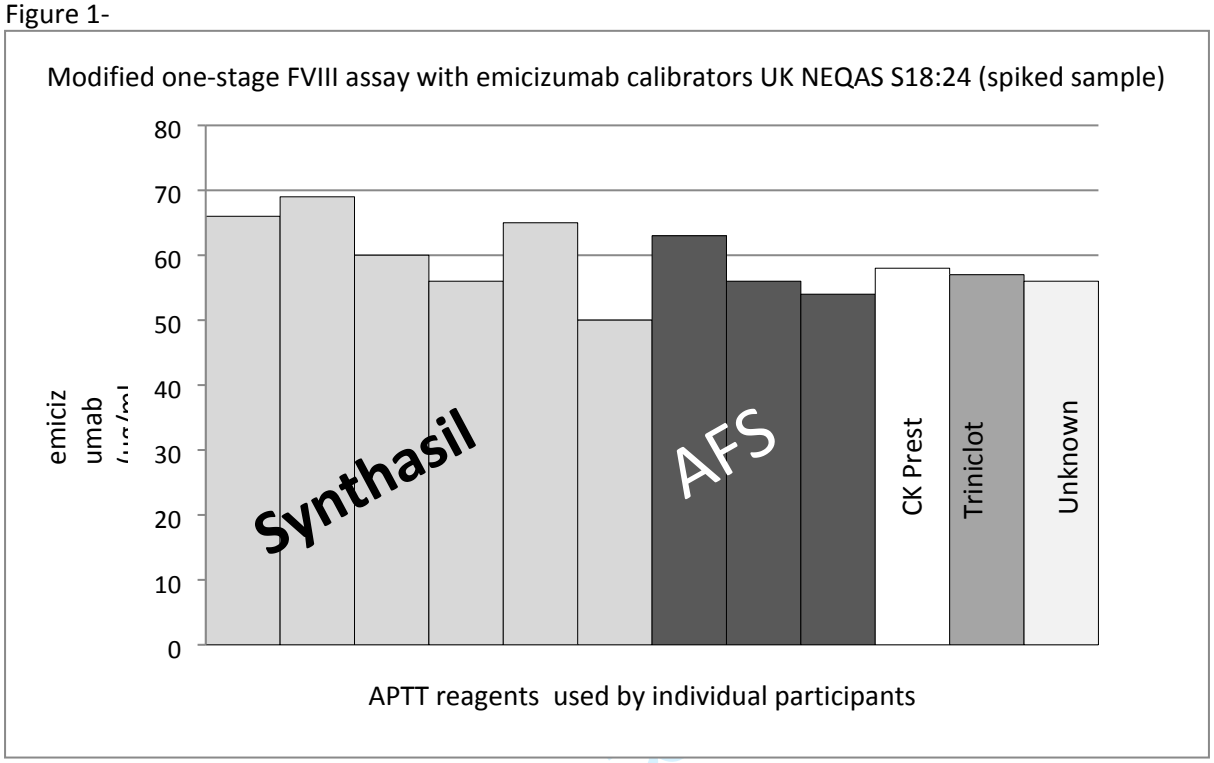
Table 4.

Sample	**S18:23**	**S18:24**
n	12	12
Median emicizumab (µg/ml)	51.2	58.1
CV (%)	13.2	11.0
Range emicizumab (µg/ml)	41.3-66.6	47.1-69.2

Figure legend

Figure 1:The modified one-stage FVIII assay results with emicizumab calibrators using different APTT reagents.

Figure 1-



Abstract

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Aim: Evaluate the effect of APTT, surrogate FVIII activity and FVIII inhibitor results on samples containing emicizumab

Methods: Two samples were provided, one obtained from an emicizumab treated severe haemophilia A patient with FVIII inhibitors and one constructed by in-vitro addition of emicizumab using plasma from a severe haemophilia A patient without FVIII inhibitors. An APTT screen, surrogate FVIII and FVIII inhibitor tests were performed on both samples by participating centres.

Results: APTT results were below the lower limit of normal range. Chromogenic FVIII assay results with the Hyphen/Biophen assay gave higher than expected coefficient of variation (CV) results, 38-40%. The modified one-stage FVIII assay with emicizumab calibrators, showed similar results between different methods regardless of the APTT reagent. Inhibitor assay median results for sample S18:23= 1.43BU (range 0.9-3.0 BU/ml, CV 38%). S18:24 was classified as below the lower limit of detection.

Conclusion: APTT screens showed a consistent shortening. Unmodified one stage Factor VIII assay results were remarkably high. Conventional APTT based assays are unsuitable for measurement of coagulation factors or inhibitors in patients treated with emicizumab. Bovine origin chromogenic assays are insensitive to emicizumab and should be used to monitor FVIII levels/FVIII inhibitors in emicizumab treated patients. Product specific calibrators should be implemented in one stage APTT based assays to reduce result variability.

Keywords: FVIII, APTT, Emicizumab, inhibitors, chromogenic, Bovine, One-stage

Acknowledgements.

Author contribution

AL collated and analysed the data. All authors participated in the writing of the manuscript.