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BRIEF REPORT

Effect of emicizumab-neutralizing antibodies on activated partial thromboplastin time–based clotting time test results in patients treated with emicizumab

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Abstract

Background: Emicizumab is a bispecific humanized monoclonal antibody that shortens the activated partial thromboplastin time (aPTT), making aPTT-based tests unreliable.

Objectives: To evaluate the efficacy of a mixture of 2 anti-idiotype monoclonal antibodies (anti-emi) in neutralizing emicizumab in samples from persons with hemophilia A treated with emicizumab.

Methods: Fifty samples from persons with hemophilia A treated with emicizumab were analyzed for aPTT and factor VIII procoagulant activity; FVIII inhibitor titer was measured using Nijmegen-Bethesda assay in 50 plasma samples of additional patients (positive for FVIII inhibitor) treated with emicizumab. FVIII procoagulant activity and inhibitor titer were measured using 1-stage (Actin FS, Siemens) and chromogenic assays with bovine regents (Factor VIII Chromogenic Assay, Siemens). Emicizumab concentration was measured by modified a 1-stage assay calibrated with a drug-specific calibrator (r^2 Diagnostics Inc). All the tests were performed on Sysmex CS-2400 (Sysmex) before and after the addition of anti-emi (Chugai Pharmaceutical).

Results: Emicizumab concentrations measured after neutralization were <1.6 μg/mL in all samples. FVIII levels were >480 IU/dL with an aPTT of <30.8 seconds in all samples before neutralization and were <1 IU/dL with an aPTT of >70 seconds after adding anti-emi. FVIII inhibitor resulted in a false negative result in 44 of 50 samples measured with the 1-stage assay before neutralization. A good correlation (r = 0.98) was found between inhibitor titer measured using the chromogenic (insensitive to emicizumab) and 1-stage assays after neutralization.

Conclusion: The anti-emi antibodies were shown to completely neutralize emicizumab, making samples pretreated with anti-emi analyzable with the 1-stage assay.

KEYWORDS
activated partial thromboplastin time, antibodies, emicizumab, factor VIII, hemophilia A

Essentials

- Emicizumab is a monoclonal antibody used as nonreplacement therapy in persons with hemophilia A.
- Activated partial thromboplastin time–based tests are unreliable in the presence of emicizumab.
- A mixture of 2 monoclonal antibodies can neutralize emicizumab.
- Samples pretreated with a mixture of 2 monoclonal antibodies can be accurately analyzed with a 1-stage assay.

1 | INTRODUCTION

Emicizumab (Hemlibra, Roche) is a genetically engineered, humanized, bispecific monoclonal antibody that binds to a single site of factor IX/FIXa with one arm and to the FX/FXa with the other, with no distinction between zymogen and enzyme [1]; it represents a novel therapeutic approach [2,3] already used in persons with severe hemophilia A (HA) with or without inhibitor. It has no structural homology to FVIII, and thus, it would not be expected to induce inhibitors to FVIII or be inhibited by existing FVIII inhibitors. Moreover, emicizumab is infused subcutaneously once a week or every 2 to 4 weeks [4,5].

In contrast to FVIII, emicizumab does not require thrombin-mediated activation, and this characteristic leads to significant shortening of activated partial thromboplastin time (aPTT)–based clotting times even at subtherapeutic plasma concentrations of emicizumab, making the results of aPTT-based tests not representative of the actual hemostatic condition.

During a breakthrough bleeding episode or in case of surgery, the patients on prophylaxis with emicizumab (with or without inhibitor) need to be treated with additional drugs (replacement therapy or bypassing agents); laboratory monitoring, even during emicizumab treatment, could therefore include the measurement of factor VIII procoagulant activity (FVIII:C) and FVIII inhibitor titer with the Bethesda assay, for which the aPTT-based tests usually used need to be replaced by a chromogenic assay using bovine reagents insensitive to emicizumab [6,7].

Recently, an additional method has been proposed as an alternative to the aforementioned chromogenic assay whose availability may vary depending on countries; it is based on the standard 1-stage assay and the use of anti-idiotype antibodies for neutralizing emicizumab present in plasma samples [8,9].

The aim of the present study was to evaluate the effect of a mixture of 2 anti-idiotype monoclonal antibodies (anti-emi mAbs) against emicizumab on some aPTT-based laboratory tests performed on samples from persons with HA treated with emicizumab.

2 | METHODS

The study was performed using the plasma samples already collected during the emicizumab clinical trials sponsored by Hoffmann-La Roche and Chugai Pharmaceutical (BH29884, BH29992, and BH30071) and biobanked in our Institute according to the informed consent previously signed by patients. Blood samples were collected in 109-mM sodium citrate and centrifuged (1800*g*, 15 minutes, room temperature) after collection to prepare platelet-poor plasma. Plasma samples were stored at −80 °C and thawed at 37 °C for 3 to 4 minutes before being tested. The study was conducted according to the Declaration of Helsinki guidelines for research on human subjects, and written informed consent to use the stored samples was obtained from adult patients (or parental consent for minors). The study was approved by the Ethics Committee of the Fondazione Istituto di Ricovero e Cura a Carattere Scientifico [IRCCS] Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy, and was registered on November 19, 2019. The patients gave permission for using plasma samples for this study by signing a specific informed consent and privacy information statement.

Fifty plasma samples from 10 patients affected with severe HA with FVIII:C less than 1 IU/dL without inhibitor and additional 50 plasma samples from 23 persons with HA with an inhibitor against FVIII at a titer ranging from 0.6 to 178 Bethesda units (BU) were analyzed.

2.1 | Sample preparation

All the samples were analyzed before and after the addition of a mixture of 2 recombinant anti-emi mAbs provided by Chugai Pharmaceutical at a dilution of 1:20 (1 volume of anti-emi into 19 volumes of emicizumab-containing plasma) [8,9].

The mixture contains the recombinant chimeric rcAQ8 recognizing the FIX/FIXa-binding Fab and the recombinant chimeric rcAJ540 recognizing the FX/FXa-binding Fab of emicizumab [8]. The samples mixed with anti-emi were analyzed without a preincubation time.

2.2 | Determination of emicizumab concentration

The blood concentration of emicizumab was measured using the modified 1-stage FVIII:C assay calibrated by a specific emicizumab calibrator (r^2 Diagnostics Inc).

This modified 1-stage assay was performed on plasma samples before (in order to measure emicizumab concentration) and after anti-emi addition (in order to verify the neutralization of emicizumab).

2.3 | Reagents

Determinations of the aPTT and the FVIII:C concentrations were performed on plasma samples of persons with HA without inhibitor

TABLE 1 Results obtained on samples from persons with hemophilia A without inhibitor.

Test	Before neutralization	After neutralization using anti-emi
Emicizumab (μg/mL)	48.2 (32.6 to 99.2)	<1.6
aPTT (s)	20.9 (18.4 to 30.8)	71.1 to >120
FVIII:C 1-stage assay (IU/dL)	>480	0.25 (0.2 to 0.3)
FVIII:C chromogenic assay (IU/dL)	<3.3	Not done

The results are reported as median and range (minimum to maximum); otherwise, the detection limit or the upper linearity cutoff is reported.
anti-emi, a mixture of 2 anti-idiotype monoclonal antibodies; aPTT, activated partial thromboplastin time; FVIII:C, factor VIII procoagulant activity.

using Actin FS and FVIII-deficient plasma (Siemens) before and after the addition of anti-emi. FVIII:C was also measured on the same samples by using a bovine chromogenic assay (Siemens).

The FVIII inhibitor titer was measured by performing the Nijmegen-Bethesda assay (clot-based) on the additional 50 plasma samples from persons with HA with inhibitor before and after the addition of anti-emi. Moreover, FVIII inhibitor titer was evaluated on plasma samples (without anti-emi) using the Nijmegen-Bethesda assay and measuring residual FVIII by the chromogenic assay. The latter (with bovine origin reagents), being insensitive to emicizumab and giving reliable quantification of FVIII levels, was used as the reference method in the study [6,7]. All the tests were performed on a Sysmex CS-2400 analyzer (Sysmex).

2.4 | Statistical analysis

Descriptive statistics were used to report the results of each group. Pearson correlation coefficient was calculated, and Passing and Bablok regression was performed to assess the relationship between assays by means of a linear regression procedure. Bland–Altman plots were also used to graphically assess the agreement between the 2 methods by plotting the differences between the 2 techniques against the averages of the 2 techniques. Statistical analysis was performed by MedCalc(R) Statistical Software (MedCalc Software Ltd).

3 | RESULTS AND DISCUSSION

Table 1 contains the results obtained on 50 plasma samples from persons with HA without inhibitor.

The samples showed a wide range of emicizumab concentrations that were completely neutralized by the addition of an anti-emi reagent. The aPTTs were markedly shortened, and FVIII:C was higher than the linearity cutoff when measured before neutralization; after adding anti-emi, the aPTT and FVIII:C values were in line with those usually observed in persons with severe HA. Factor VIII:C measured with the chromogenic assay was lower than the detection limit in all the samples.

TABLE 2 Results obtained on samples from persons with hemophilia A with inhibitor.

Test	Before neutralization	After neutralization using anti-emi
Emicizumab (μg/mL)	41.65 (18.0 to 100.4)	<1.6
Inhibitor titer 1-stage assay (BU)	<0.5 (<0.5 to 203)	3.5 (0.5 to 194)
Inhibitor titer chromogenic assay (BU)	3.9 (0.5 to 178)	Not done

The results are reported as median and range (minimum to maximum); otherwise, the detection limit is reported.
anti-emi, a mixture of 2 anti-idiotype monoclonal antibodies; BU, Bethesda units.

Table 2 shows the results of the 50 samples from persons with HA with anti-FVIII inhibitor. Emicizumab concentrations ranged from 18 to 100 μg/mL before the addition of anti-emi and resulted in less than 1.6 μg/mL after neutralization.

All 44 samples from patients with inhibitor titer up to 17 BU showed false negative results (with inhibitor titer less than 0.5 BU) when analyzed with the 1-stage assay before the addition of anti-emi. The same samples measured after neutralization gave results ranging from 0.5 to 17 BU (median, 3 BU), in line with those obtained with the chromogenic assay, as shown in Figure 1, representing the Passing and Bablok regression analysis. The Spearman's rank correlation coefficient (r) was 0.98.

Emicizumab interference seemed to be irrelevant with a high titer of inhibitor (>50 BU), giving similar results with the 1-stage assay before and after neutralization and with the chromogenic assay in the 6 samples with inhibitor titer >50 BU. This is probably due to the

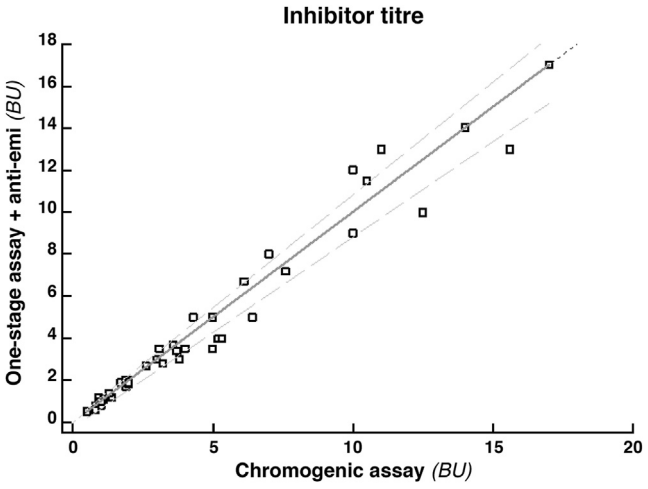

FIGURE 1 Passing and Bablok regression analysis comparing factor VIII inhibitor titer (<20 Bethesda units [BU]) obtained with the chromogenic assay and with the 1-stage assay after neutralization. anti-emi, a mixture of 2 anti-idiotype monoclonal antibodies.

samples' preanalytical treatment consisting of high dilutions of the plasmas and, consequently, the emicizumab present in the samples.

Also considering the 50 samples altogether, a good correlation was found between inhibitor titer measured with the chromogenic assay (the reference method) and with the 1-stage assay after neutralization, with a Spearman's rank correlation coefficient (r) of 0.98.

The agreement of FVIII inhibitor measured with the chromogenic assay and 1-stage assay after neutralization was also analyzed using Bland–Altman plots on the samples with low and medium titer (Figure 2). The mean difference between the values obtained with the 2 methods was 0.2 BU, and for the majority of the analyzed samples, the absolute difference was within ±1.96 SD.

The increasing use of emicizumab as an alternative therapeutic option for prophylaxis in HA is having a great impact on clinical management of the patients, as confirmed by clinical trials [4,10–14] and real-world data [15–17].

Due to its mode of action, emicizumab shortens significantly, and not linearly, the aPTT of plasmas of treated patients with HA; as a consequence the aPTT is oversensitive and not useful to evaluate emicizumab activity, and all the aPTT-based assays are not accurate for laboratory monitoring of these patients in different clinical settings, such as in the case of FVIII inhibitor titer over time assessment [7,18]. Moreover, although greatly efficacious, the prophylaxis with emicizumab results is not sufficient in the case of surgery or major bleeding, and the use of additional FVIII replacement therapy must be considered in persons with HA who are inhibitor-negative, with the need for FVIII:C measurement. Chromogenic kits with bovine origin reagents are commercially available for FVIII:C measurement and, being insensitive to emicizumab, are the methods of choice for analyzing plasmas containing emicizumab [6,7]. Nevertheless, this assay is not universally available.

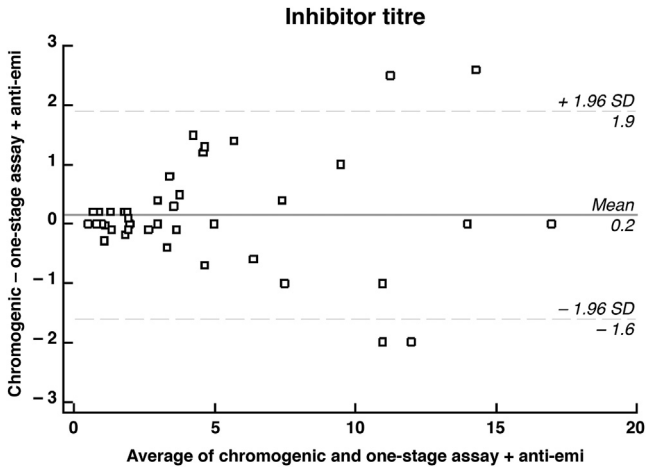

FIGURE 2 Bland–Altman plot showing the absolute differences of factor VIII inhibitor titer (<20 Bethesda units [BU]) measured with the chromogenic assay and with the 1-stage assay after neutralization. anti-emi, a mixture of 2 anti-idiotype monoclonal antibodies.

The present study aimed to evaluate the neutralizing efficacy of a mixture of 2 anti-emi mAbs and if this pretreatment could be considered an alternative approach for bypassing emicizumab interference when standard aPTT-based coagulation assays are performed. Our results confirm the neutralizing efficacy of anti-emi mAbs as shown by emicizumab concentrations measured after anti-emi addition that resulted to be less than 1.6 μg/mL in all the samples. Moreover, the use of this mix of anti-emi mAbs has proved to be reliable for measuring FVIII:C and FVIII inhibitor titers using the Nijmegen-Bethesda assay, as shown by the agreement between the results obtained using the 1-stage assay after neutralization and the chromogenic assay, considered the reference method.

Our results confirm the data previously reported by other authors [8,9] on *in vitro* emicizumab-spiked samples and demonstrate the utility of anti-idiotype mAb use in patients with and without inhibitor in treatment with emicizumab.

Our results need to be confirmed by further evaluation, possibly on other aPTT-based tests; however, the importance of our data stems from being obtained from patients treated with emicizumab, confirming those observed on plasmas prepared artificially.

It must be said that the laboratory approach described in the present study includes an additional, although not overly time-consuming, step of neutralization, and it is not feasible at the moment because the anti-emi mAbs are not yet commercially available. However, availability of the bovine chromogenic assay varies depending on countries. Even if the chromogenic assay is marketed in a country, not all laboratories necessarily have the capability to perform the chromogenic assay while 1-stage assay is performed possibly because of financial or other restrictions. For those laboratories, the approach of pretreatment of samples could be an alternative option to measure FVIII:C or inhibitor titer without emicizumab interference.

In conclusion, considering the widespread use of emicizumab worldwide, another choice of laboratory assay for emicizumab-containing samples may be beneficial in terms of patient care.

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ETHICS STATEMENT

The study was conducted according to the Declaration of Helsinki guidelines for research on human subjects, and written informed consent to use the stored samples was obtained from adult patients (or parental consent for minors). The study was approved by the Ethics Committee of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy, and was registered on November 19, 2019. The

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AUTHOR CONTRIBUTIONS

C.N. designed all experiments, performed experiments, analyzed the data, interpreted the data, and wrote and edited the manuscript. M.-B.A. and E.G. performed experiments. S.S. contributed to design of the experiments. F.P. supervised the manuscript.

RELATIONSHIP DISCLOSURE

S.S. is an employee of Sysmex Corporation. F.P. serves on the advisory boards of Sanofi, Sobi, Takeda, Roche, and BioMarin. All other authors have no competing interests to disclose.

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REFERENCES

[1] Lenting PJ, Denis CV, Christophe OD. Emicizumab, a bispecific antibody recognizing coagulation factors IX and X: how does it actually compare to factor VIII? *Blood*. 2017;130:2463–8.

[2] Shima M, Hanabusa H, Taki M, Matsushita T, Sato T, Fukutake K, et al. Factor VIII-mimetic function of humanized bispecific antibody in hemophilia A. *N Engl J Med*. 2016;374:2044–53.

[3] Uchida N, Sambe T, Yoneyama K, Fukazawa N, Kawanishi T, Kobayashi S, et al. A first-in-human phase 1 study of ACE910, a novel factor VIII-mimetic bispecific antibody, in healthy subjects. *Blood*. 2016;127:1633–41.

[4] Pipe SW, Shima M, Lehle M, Shapiro A, Chebon S, Fukutake K, et al. Efficacy, safety, and pharmacokinetics of emicizumab prophylaxis given every 4 weeks in people with haemophilia A (HAVEN 4): a multicentre, open-label, non-randomised phase 3 study. *Lancet Haematol*. 2019;6:e295–305. https://doi.org/10.1016/S2352-3026(19)30054-7

[5] Oldenburg J, Mahlangu JN, Kim B, Schmitt C, Callaghan MU, Young G, et al. Emicizumab prophylaxis in hemophilia A with inhibitors. *N Engl J Med*. 2017;377:809–18.

[6] Tripodi A, Chantarangkul V, Novembrino C, Peyvandi F. Advances in the treatment of hemophilia: implications for laboratory testing. *Clin Chem*. 2019;65:254–62.

[7] Bowyer AE, Lowe AE, Tiefenbacher S. Laboratory issues in gene therapy and emicizumab. *Haemophilia*. 2021;27:142–7.

[8] Nogami K, Soeda T, Matsumoto T, Kawabe Y, Kitazawa T, Shima M. Routine measurements of factor VIII activity and inhibitor titer in the presence of emicizumab utilizing anti-idiotype monoclonal antibodies. *J Thromb Haemost*. 2018;16:1383–90.

[9] Ogiwara K, Furukawa S, Shinohara S, Tabuchi Y, Arai N, Noguchi-Sasaki M, et al. Anti-idiotype monoclonal antibodies against emicizumab enable accurate procoagulant and anticoagulant assays, irrespective of the test base, in the presence of emicizumab. *Haemophilia*. 2023;29:329–35.

[10] Shima M, Hanabusa H, Taki M, Matsushita T, Sato T, Fukutake K, et al. Long-term safety and efficacy of emicizumab in a phase 1/2 study in patients with hemophilia A with or without inhibitors. *Blood Adv*. 2017;1:1891–9.

[11] Callaghan MU, Negrier C, Paz-Priel I, Chang T, Chebon S, Lehle M, et al. Long-term outcomes with emicizumab prophylaxis for hemophilia A with or without FVIII inhibitors from the HAVEN 1-4 studies. *Blood*. 2021;137:2231–42.

[12] Parnes A, Mahlangu JN, Pipe SW, Paz-Priel I, Lehle M, Trask PC, et al. Patient preference for emicizumab versus prior factor therapy in people with haemophilia A: results from the HAVEN 3 and HAVEN 4 studies. *Haemophilia*. 2021;27:e772–5. https://doi.org/10.1111/hae.14421

[13] Schmitt C, Emrich T, Chebon S, Fernandez E, Petry C, Yoneyama K, et al. Low immunogenicity of emicizumab in persons with haemophilia A. *Haemophilia*. 2021;27:984–92.

[14] Yang R, Wang S, Wang X, Sun J, Chuansumrit A, Zhou J, et al. Prophylactic emicizumab for hemophilia A in the Asia-Pacific region: a randomized study (HAVEN 5). *Res Pract Thromb Haemost*. 2022;6:e12670. https://doi.org/10.1002/rth2.12670

[15] Barg AA, Livnat T, Budnik I, Avishai E, Brutman-Barazani T, Tamarin I, et al. Emicizumab treatment and monitoring in a paediatric cohort: real-world data. *Br J Haematol*. 2020;191:282–90.

[16] Mason JA, Young G. Emicizumab prophylaxis in infants with severe haemophilia A without inhibitors: illustrative real-world cases to support shared decision-making. *Haemophilia*. 2021;27:724–9.

[17] Arcudi S, Gualtierotti R, Marino S, Nicolò G, Biguzzi E, Ciavarella A, et al. Real-world data on emicizumab prophylaxis in the Milan cohort. *Haemophilia*. 2022;28:e141–4.

[18] Recommendation on the Use and Management of Emicizumab-kxwh (Hemlibra®) for Hemophilia A with and without Inhibitors. National Hemophilia Foundation; 2022. https://www.hemophilia.org/healthcare-professionals/guidelines-on-care/masac-documents/masac-document-268. [accessed December 3, 2023].