Emicizumab augments thrombus formation in whole blood from patients with hemophilia A under high shear flow conditions

Journal:	*Thrombosis and Haemostasis*
Manuscript ID	TH-20-02-0067.R2
Manuscript Type:	Original Article: Coagulation and Fibrinolysis
Category:	Basic Science
Date Submitted by the Author:	05-Jul-2020
Complete List of Authors:	Yaoi, Hiroaki; Nara Medical University, Pediatrics Shida, Yasuaki; Nara Medical University, Pediatrics Kitazawa, Takehisa; Chugai Pharmaceutical Co., Ltd., Research Division Shima, Midori; Nara Medical University, Pediatrics NOGAMI, KEIJI; Nara Medical University, Pediatrics
Keywords:	emicizumab, Factor VIII, thrombus formation, bypassing therapy, flow chamber

Emicizumab augments thrombus formation in whole blood from patients with hemophilia A under high shear flow conditions

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Running heads; Effect of emicizumab under whole blood flow

Category; Original Article

Word counts;

Summary; 247, Text; 3,174

Figure/table counts: Figure 4 (color, black and white)

Reference counts; 34

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Abstract

Background*:** Emicizumab is a bispecific antibody to factor (F)IXa and FX that mimics FVIIIa cofactor function. Emicizumab prophylaxis markedly decreases bleeding episodes in patients with hemophilia A (PwHA), irrespective of the presence of FVIII-inhibitors. However, thrombotic microangiopathy (TMA) was reported when repeated high doses of activated prothrombin complex concentrates (aPCC) was concomitantly used with emicizumab. Although bypassing agents (BPAs) are vital in the hemostatic treatment for PwHA with inhibitors, the mechanism of emicizumab-related TMA remains unclear. ***Aim*:** To assess the risk of excessive thrombus formation associated with BPAs and emicizumab under high shear condition. ***Methods: Perfusion flow-chamber experiments under high shear conditions were performed using whole blood from PwHA in the presence of emicizumab without or together with FVIII or BPAs *ex vivo*. ***Results*:** Emicizumab (100 μg/ml) added *ex-vivo* to whole blood from PwHA improved defective thrombus formation in a similar manner to that observed with the addition of recombinant FVIII at the early phase, while FVIII continued to be important at the later stages. aPCC (1.2 U/ml equal to 100 U/kg) or recombinant FVIIa (rFVIIa: 1.1 μg/ml; equal to 90 μg/kg) together with emicizumab further promoted platelet interactions and fibrin formation *ex vivo* but did not induce excessive thrombus formation. ***Conclusion*:** Emicizumab enhanced thrombin generation at local sites and improved defective hemostasis in whole blood from PwHA under high shear conditions. Simple concomitant use of BPAs with emicizumab did not mediate excessive thrombus formation and remains an option for hemostatic management of emicizumab-treated PwHA with inhibitors.

Key words; emicizumab, factor VIII, thrombus formation, bypassing therapy, flow chamber

What is known about this topic?

- Emicizumab improves impaired coagulation function in severe patients with hemophilia A (PwHA).
- Emicizumab-related TMA has been reported when repeated high doses of aPCC was concomitantly used with emicizumab; however its mechanism(s) remains unclear.

What does this paper add?

- Emicizumab improved defective thrombus formation in PwHA under high shear conditions at the early phase, while FVIII continued to be important at the later stages.
- The simple co-presence of bypassing agents and emicizumab did not mediate untoward thrombus formation.

Introduction

Hemophilia A (HA) is a congenital bleeding disorder caused by a defect or deficiency of factor (F)VIII. Intravenous infusion of FVIII concentrates is the first-line therapeutic option for patients with HA (PwHA) without inhibitor.[1] Regular prophylaxis with FVIII concentrates reduces the incidence of bleeding episodes, delays the onset of arthropathy and contributes greatly to improvements in quality of life in these individuals.[1-4] There are some barriers with current therapies, however. The need for multiple intravenous infusions is associated with a substantial mental and physical burden especially in children, and hemostatic treatment for PwHA with inhibitors is especially difficult.[2] Anti-FVIII alloantibodies neutralize FVIII coagulant activity, and severely limit the effectiveness of FVIII concentrates.[5] Bypassing agents (BPAs) including recombinant FVIIa (rFVIIa) and activated prothrombin complex concentrates (aPCC) are used as alternative hemostatic agents in these circumstances.[6,7] Clinical responses to treatment with BPAs vary among patients, however.[8,9]

In this context, emicizumab, a recombinant, humanized bispecific monoclonal antibody (mAb) that binds to FIX/FIXa and FX/FXa and mimics FVIIIa cofactor activity in the tenase complex, has been developed.[10,11] This antibody mediates an appropriate conformational structure for FIXa-catalyzed activation of FX. In the clinical trials, once-weekly, bi-weekly, and tetra-weekly subcutaneous administration of emicizumab substantially diminished bleeding episodes in PwHA, irrespective of the presence of inhibitor.[12-18] The antibody has received regulatory approval for routine prophylaxis in PwHA with or without inhibitors in US, EU, and Japan.

Along with the emergence of emicizumab, novel assays that can precisely assess the hemostatic effect of emicizumab are required. In this context, careful attention should be paid to introduce the conventional assays for this purpose. Unlike FVIII, emicizumab does not require activation by thrombin, and the shortening effect of the antibody on the activated partial thromboplastin time (aPTT) is more pronounced than that of FVIII.[10,19,20] The results of aPTT-based one-stage assays, therefore, do not directly reflect emicizumab activity. Global coagulation tests, including rotational thromboelastometry (ROTEM),[21] thrombin generation assays (TGA),[22] and clot waveform analysis (CWA),[23] have been reported as alternatives for assessing hemostasis in these circumstances. These

assays depend on a variety of trigger reagents, however, and are performed under static (TGA and CWA) or semi-static (ROTEM) conditions. The data obtained from these assays may not represent physiological hemostasis, because one of the obvious differences in this mimicry of emicizumab to FVIII is the lack of direct phospholipid binding ability[10] and may lead to a somewhat different way of action in the intravascular flow condition. However, techniques to confirm the potential role of emicizumab in thrombus and fibrin formation in native whole blood from PwHA under the flow conditions remain challenging and has not been reported as of yet.

While rFVIIa and aPCC are widely used to treat bleeding episodes in PwHA with inhibitors,[6,7] thrombotic adverse events are the potential risk related with BPAs. The reported incidence of thrombosis related with rFVIIa is limited to 1−2 %.[24,25] aPCC can be also prothrombotic and complications such as venous thromboembolism, myocardial infarction, and disseminated intravascular coagulation (DIC) have been reported.[26] Recent reviews of phase 3 clinical trials have noted that three PwHA with inhibitor had developed thrombotic microangiopathy (TMA).[14,27] These adverse events occurred only when the emicizumab-treated PwHA received repeated administration of aPCC for breakthrough bleeding. Notably, the TMA was not associated with more widespread DIC, although as expected, the concomitant therapy of aPCC with emicizumab consolidated overall coagulation potential. The mechanism(s) of emicizumab-related TMA in these circumstances remains unclear. The major reported causes of TMA include a disintegrin-like and metalloproteinase with thrombospondin type 1 motifs 13 (ADAMTS13) deficiency, abnormalities of complement regulation, and enteric infections with Shiga-toxin producing organisms.[28] None of these complications were evident in the emicizumab-treated patients, however.[14] Platelet micro-thrombi in small arterioles and capillaries at high shear stress may, therefore, contribute to TMA more than disordered blood coagulation. The pathogenesis of TMA is difficult to investigate using static assays, and we hypothesized that the modern, real-time, micro capillary perfusion methods using whole blood under high shear may reproduce the physiological conditions relevant to TMA. In fact, flow-based perfusion technique has been utilized to reproduce the condition of micro-vessel thrombosis for the ADAMTS13 study, since the ADAMTS13 function to cleave von Willebrand factor is regulated by the hydrodynamic shear force.[29]

Our studies focused, therefore, on novel perfusion experiments using whole blood from PwHA, to analyze emicizumab-mediated platelet thrombus at high shear stress and to assess the risk of excessive thrombus formation associated with BPA and emicizumab under the condition, attempting to reflect the pathogenic mechanisms of TMA and to evaluate a current risk management for emicizumab-related TMA in clinical.

Materials and Methods

This study was approved by the Medical Research Ethics Committee of Nara Medical University. Blood samples were collected after informed consent following the Universities ethical guidelines.

Reagents - Emicizumab and recombinant (r) anti-idiotype mAb to emicizumab (anti-emicizumab mAb; rcAQ8 to anti-FIXa-Fab)[30] were obtained from Chugai Pharmaceutical Co (Gotemba, Shizuoka, Japan). rFVIII (Advate®, Takeda Pharma Co. Ltd, Tokyo, Japan), aPCC (FEIBA®, Takeda), rFVIIa (NovoSeven®, Novo Nordisk, Bagsværd, Denmark), collagen I/III (MP Biomedicals, Santa Ana, CA), r-hirudin (Enzo Life Science, Farmingdale, NY), phalloidin-Alexa 488 (Molecular Probes, Eugene, OR), Cytoperm/Cytofix (BD, Franklin Lakes, NJ), anti-thrombin antibody (Abcam, Cambridge, United Kingdom), anti-fibrin antibody (DAKO, Santa Clara, CA), Alexa 568 and 647 labeling kit (Molecular Probes, Eugene, OR) were purchased from the indicated vendors. An anti-FVIII C2 polyclonal antibody (polyAb) was obtained from a severe PwHA with inhibitor, and was used for immunostaining as previously described.[31,32]

Perfusion chamber experiments - Perfusion experiments were performed as previously described.[33] Briefly, microscopy slides designed for cell culture and high resolution microscopy (μ-slide VI 0.1; Ibidi, Martinsried, Germany) were coated with collagen I/III (300 μg/ml) in sodium carbonate/bicarbonate buffer at room temperature (RT) overnight, washed three times with phosphate buffer saline (PBS), blocked with 5 % bovine serum albumin for 1 hr at RT, and washed with PBS prior to use. Whole blood samples were obtained from PwHA with or without inhibitor, using hirudin (25 μg/ml) as an anticoagulant. Hirudin was selected to keep the platelet function as much as possible to form physiological platelet thrombus to reproduce the condition of TMA where platelet thrombus formation plays a major role. Preliminary experiments were performed and 25

μg/ml of hirudin was the minimum dose for the completion of this experimental system. (data not shown). The study subjects had not taken any medication that may have affected platelet function or blood coagulation in the two-week period prior to blood sampling. Each FVIII, emicizumab (100 μg/ml), rFVIIa (1.1 μg/ml; corresponded to 90 μg/kg) and aPCC (1.2 U/ml; corresponded to 100 U/kg) were added to the whole blood *ex vivo*, followed by perfusion into the prepared chamber at the high shear rates (2,500 s^{-1}) controlled by the syringe pump NE-1600™ (New Era Pump Systems, Farmingdale, NY). The final concentration of aPCC or rFVIIa in whole blood was calculated using the following formula; therapeutic dose (U/kg or μg/kg)/80 = final concentration (U/ml or μg/ml, respectively). In this experiment, we utilized higher concentrations of emicizumab (100 μg/ml) than clinically therapeutic concentrations (30−80 μg/ml) [14-18] to ensure maximum activity for thrombus formation *ex vivo*. Reactions on the collagen that was coated surface under these conditions represented thrombus formation at high shear.

Immunostaining - After the perfusion experiments, the developing thrombi were fixed with Cytoperm/Cytofix® and immunofluorescent staining was performed as previously reported.[33] The fixed thrombi were permeabilized with PBS containing 1 % Triton X-100 for 10 min at RT and blocked with serum free protein block (DAKO) for 20 min, followed by incubation with phalloidin-Alexa 488 (6 μg/ml), anti-thrombin antibody (10 μg/ml) labeled with Alexa 568, and anti-FVIII polyAb (1 μg/ml) overnight at 4 °C. In some experiments, fluorescent patterns were visualized with anti-fibrin antibody or anti-emicizumab mAb conjugated with Alexa 647. After 3 washes with PBS, thrombi were mounted in DAKO-fluorescence mounting medium prior to photo-microscopy. Preliminary experiments, confirmed sufficient infiltration of fluorescent antibodies into thrombi. Platelet, thrombin and fibrinogen/fibrin reactions were visualized using confocal laser scanning microscopy (FV-1000™, Olympus, Tokyo, Japan). The digital images were obtained at 1-μm intervals from the collagen surface to a height of 60 μm. Surface coverage was measured by calculating the percentage of the area covered by adhering platelets based on sliced images at the 2 μm from the bottom of the thrombus using ImagePro 6.0™ (Media Cybernetics, Rockville, MD). Thrombus volume was calculated by summing all sliced images of identical portions. The average thrombus height was also calculated.

Data analyses - All data and statistical analyses were performed using Graphpad prism 7.0 (Graphpad Software, San Diego, CA). The results are illustrated as means and standard deviations (SD). Statistical significance between two groups was assessed using Student's *t*-tests and differences between more than two groups were evaluated using 2-way ANOVA. *P*-values <0.05 were considered as statistically significant.

Results

Ex vivo FVIII or emicizumab enhanced thrombus formation in whole blood from PwHA under high shear - Thrombus formation with whole blood from PwHA without inhibitor was first compared with healthy control blood using the perfusion chamber technique. Whole blood samples from controls, severe PwHA in the presence or absence of FVIII (100 IU/dl) were perfused in the flow chamber and coated slides were fixed at the indicated time points. Representative images are shown in **Figure 1A**. Thrombus formation after the *ex vivo* addition of FVIII to whole blood from PwHA was enhanced and accelerated compared to that in the absence of FVIII. After 8 min perfusion, surface coverage (SC) and thrombus height (TH) in untreated PwHA were 5.2 ± 3.4 % and 0.64 ± 0.43 μm, respectively, and were 35.3 ± 6.8 % and 5.3 ± 0.48 μm, respectively in the presence of FVIII. These measurements indicated restoration of thrombus formation to approximately normal levels (SC; 36.2 ± 1.9 %, TH; 4.7 ± 0.44 μm) (**Figure 1B**). Similarly, the addition of emicizumab (100 μg/ml) also augmented thrombus formation, especially at the early stages of thrombus formation (**Figure 1A**).

Emicizumab enhanced thrombus formation especially at the early stages - To further understand the effect of emicizumab on thrombus formation, the data in **Figure 1B** was reformatted to 1 min, 4 min, and 8 min and comparison of time variation was made between FVIII and emicizumab on thrombus formation (**Figure 2**). After 1 min perfusion, the effects of emicizumab on thrombus formation (SC; 14.5 ± 1.8 %, TH; 1.25 ± 0.18 μm) were comparable to that of FVIII (SC; 14.3 ± 5.8 %, TH; 1.39 ± 0.43 μm). The final thrombus sizes in the presence of FVIII after 4 min (SC; 28.7 ± 8.0 %, TH; 0.45 ± 0.23 μm) and 8 min (SC; 35.3 ± 6.8 %, TH; 5.3 ± 0.48 μm) perfusion were significantly greater than after the addition of emicizumab at the same time-points, however (4 min: SC; 20.6 ± 1.9 %, TH; 2.00 ± 0.14 μm, and 8 min: SC; 23.9 ± 2.54 %, TH; 2.66 ± 0.13 μm).

Distribution of FVIII, emicizumab, and thrombin in thrombi under high shear - To confirm the role of enhanced coagulation reactions mediated by FVIII and emicizumab in thrombus formation at high shear, thrombi were examined by immunostaining with anti-thrombin antibody labeled with Alexa 567 and anti-FVIII polyAb labeled with Alexa 647 [31] (**Figure 3A**). Increases in the intensity of thrombin staining were observed (1,805 ± 785 and 5,295 ± 320 AU, respectively) after the *ex vivo* addition of FVIII (0 and 100 IU/dl; **Figure 3B**). An anti-emicizumab mAb (rcAQ8 to anti-FIXa-Fab)-labeled with Alexa 647 demonstrated that emicizumab was stained throughout the formed thrombus and in addition was co-localized with thrombin (**Figure 3A**). Moreover, emicizumab enhanced the intensity of thrombin (4,940±306 AU), equivalent to ~80% of *ex vivo* addition of FVIII (100 IU/dl) (**Figure 3B**). These findings indicated that emicizumab contributed to thrombus formation in these circumstances.

Impact of the co-presence of BPAs and emicizumab on ex-vivo thrombus formation in PwHA with inhibitor - The effects of BPAs without or with emicizumab on thrombus formation in PwHA with inhibitor were assessed using aPCC (1.2 U/ml; equivalent to 100 U/kg), rFVIIa (1.1 μg/ml; equivalent to 90 μg/kg), emicizumab (100 μg/ml), emicizumab/aPCC, and emicizumab/rFVIIa added to whole blood from PwHA with inhibitor prior to perfusion as described in Methods. The formed thrombi were fixed, and immunostained with phalloidin-Alexa 488, Alexa 647-labeled anti-thrombin antibody, and Alexa 567-labeled anti-fibrinogen/fibrin antibody. Representative images and analysis of thrombus formation in the presence of aPCC, rFVIIa, emicizumab, emicizumab/aPCC, and emicizumab/rFVIIa after 4 min perfusion are shown in **Figure 4A** and **4B.** The *ex vivo* addition of either aPCC or rFVIIa alone improved the parameters of thrombus formation (aPCC: SC; 17.7 ± 1.2 %, TH; 1.55 ± 0.16 μm: rFVIIa: SC; 29.8 ± 1.9 %, TH; 2.32 ± 0.17 μm). rFVIIa appeared to have a greater impact than emicizumab alone, but conversely, emicizumab seemed to be more effective than aPCC. The combined mixtures of emicizumab and BPAs further enhanced thrombus formation (aPCC plus emicizumab: SC; 34.4 ± 3.7 %, TH; 2.59 ± 0.15 μm: rFVIIa plus emicizumab: SC; 31.3 ± 1.7 %, TH; 2.97 ± 0.18 μm) (**Figure 4B**). These measurements were not equivalent, however, to those determined after the addition of FVIII (100 IU/dl) to PwHA whole blood (*see* **Figures 1B** and **2**).

Discussion

Emicizumab, a humanized bispecific antibody to FIX/IXa and FX/FXa that mimics FVIIIa cofactor function, is predicted to be a paradigm-shifting therapeutic agent for PwHA, irrespective of presence of inhibitor.[12,17] The pre-clinical studies using TGA demonstrated that emicizumab at 30-50 μg/ml could be comparable to nearly FVIII:C 10 IU/dl, in which the potency of emicizumab seemed to be a little underestimated.[12,34] This global assay may be beneficial for monitoring emicizumab concentrations in blood, but it does not fully reflect emicizumab activity. In this context, TMA has been reported after the concomitant use of aPCC in emicizumab-treated PwHA with inhibitor,[14] and this unexpected complication is difficult to predict using static assays. We have utilized a novel flow-chamber perfusion technique, therefore, to assess emicizumab activity under near-physiological, high shear conditions,[33] and have examined the characteristics of thrombus formation in the presence of emicizumab. The trough levels of emicizumab in plasma at clinically therapeutic doses have been estimated to be approximately 30-80 μg/ml.[14-18] In the present study, to reflect pathogenic mechanism(s) of TMA and to evaluate current risk management for emicizumab-related TMA in clinical settings, a concentration of 100 μg/ml as the maximum condition, comparable to plasma emicizumab concentration at high dose regimen (C3-cohort) in the phase 1/2 study,[12,13] was chosen.

Our results confirmed that platelet interactions and fibrin formation leading to thrombus formation in the whole blood from PwHA under the high shear conditions were severely impaired (Figure 1). The *ex-vivo* addition of FVIII significantly improved these interactions demonstrating that FVIII played a significant role in thrombus formation even under high shear. Our findings for the first time identified that FVIII continued to be important at the later stages (4 and 8 min), in contrast to emicizumab which significantly contributed to increased thrombus formation at early-reaction phases (within 1 min) but decreased its contribution at the later phase (4 and 8 min). It seemed likely that further thrombin would be generated at the later phases of thrombus formation, and FVIII could have a greater impact than emicizumab in the final thrombus size. These data suggested that FVIII and emicizumab play significant roles in thrombus formation even under high shear and this experimental system would be valuable to evaluate a risk of emicizumab-related TMA.

The immunofluorescent images indicated that emicizumab was co-localized with thrombin throughout the thrombi, and supported a role for emicizumab in coagulation under high shear. Emicizumab enhanced the thrombin intensity within the fibrin structures, which is in keeping with a potent generation of thrombin at sites of local thrombus formation. The findings also might support the clinical studies that have demonstrated a low incidence of bleeding episodes in PwHA receiving emicizumab prophylaxis. Since the dose of emicizumab (100 μg/ml) in this study was higher than that in patients in the HAVEN series of clinical trials,[14-18] emicizumab alone did not seem to be more thrombotic than FVIII.

Given that BPAs are the first choice for treatment of breakthrough bleeds in emicizumab-treated PwHA with high titer inhibitors and the reported TMA was associated with repeated concomitant use of high doses of aPCC with emicizumab,[14] we next investigated the influence of BPAs with and without emicizumab on thrombus formation using the same technique (Figure 4A). Both rFVIIa and aPCC enhanced thrombus formation in whole blood from PwHA with inhibitor. Interestingly, impact of rFVIIa on surface coverage and thrombus height was greater than that of aPCC (Figure 4B). This data were not consistent with the clinical data that the efficacy of rFVIIa and aPCC are comparable.[6] The reason may be explained by the differences in the pharmacokinetics between rFVIIa and aPCC. The plasma half-life of aPCC is 8−12 hours while that of rFVIIa is only 2−4 hours. By contrast, co-presence of emicizumab reduced the difference of thrombus size between rFVIIa and aPCC. The reason why emicizumab further enhanced thrombus size not in rFVIIa but in aPCC remains unclear. As one possibility, aPCC contains FIXa and FX, which may increase the FIXa-emicizumab-FX complex, while rFVIIa does not. The thrombus area and height increased with co-presence of emicizumab and a standard dosage of BPA did not exceed that formed in blood from control individuals, however.

TMA appears to be a clinical complication in emicizumab-treated PwHA only when frequent high doses of aPCC are used concomitantly for breakthrough bleeding.[14] The imbalanced coagulation interactions in these circumstances may not have been fully represented in our experimental system, and further analyses using whole blood from emicizumab-treated PwHA with various doses of

BPAs are required. The availability of patients in this category is limited, however. Nevertheless, our data indicated that a single standard dose of rFVIIa or aPCC seemed unlikely to severely modify the hemostatic balance in the presence of emicizumab (100 μg/ml).

Emicizumab-related TMA was not clearly reproduced in this experimental system. Therefore, further study will be required. Collagen was used in our model as a surrogate of damaged endothelial vessel wall. However, microvascular occlusion occurs at a distance from any endothelial damage and non-collagen bound component of active hemostatic components might help our understandings. Another possibility would be *in vivo* thrombosis model for the experiment although emicizumab does not cross-react with mouse proteins and its hemostatic effect cannot be reproduced in mice.

In conclusion, we have described the use of an *ex vivo* flow chamber technique to explore the possible mechanism of emicizumab-mediated TMA. Emicizumab appeared to augment platelet interactions and fibrin formation in whole blood from PwHA under high shear conditions, although the bispecific antibody was not as effective as conventional FVIII. Simple concomitant use of rFVIIa or aPCC with emicizumab did not mediate excessive thrombus formation and remains an option for the hemostatic management of emicizumab-treated PwHA with inhibitor.

Acknowledgements

The authors would like to thank Drs. Masahiro Takeyama, Kenichi Ogiwara, Koji Yada, Shoko Furukawa for the clinical assistance and helpful discussions. The authors also thank Dr. Hiroaki Minami for the technical assistance.

Authorship

Contribution; HY; performed experiments, analyzed and interpreted the data, and made the figures, YS; designed and performed experiments, interpreted the data and wrote the manuscript, TK; prepared emicizumab and interpreted the data, MS; supervised this study, KN; designed

experiments, interpreted the data and wrote the manuscript and approved the submission of this manuscript.

Grant support; This work was partly supported by a Grant-in-Aid for Scientific Research (KAKENHI) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) to YS (No. 26461593) and to KN (No. 18K07885).

Conflict-of-interests; HY, YS, MS, and KN have received research grants from Chugai Pharmaceutical Co., Ltd. (Chugai), and are engaged in clinical studies sponsored by Chugai and F. Hoffmann-La Roche. MS and KN have received (consulting) honoraria from Chugai. TK is employees of Chugai. TK possesses Chugai's stock. TK, MS and KN are inventors of the patents relating to emicizumab.

References

1. Srivastava A, Brewer AK, Mauser-Bunschoten EP, et al. Guidelines for the management of hemophilia. Haemophilia 2013;19:e1-47.
2. Berntorp E, Shapiro AD. Modern haemophilia care. Lancet (London, England) 2012;379:1447-1456.
3. Nilsson IM, Hedner U, Ahlberg A. Haemophilia prophylaxis in Sweden. Acta Paediatr Scand 1976;65:129-135.
4. Manco-Johnson MJ, Abshire TC, Shapiro AD, et al. Prophylaxis versus episodic treatment to prevent joint disease in boys with severe hemophilia. N Engl J Med 2007;357:535-544.
5. Witmer C, Young G. Factor VIII inhibitors in hemophilia A: rationale and latest evidence. Ther Adv Hematol 2013;4:59-72.
6. Astermark J, Donfield SM, DiMichele DM, et al. A randomized comparison of bypassing agents in hemophilia complicated by an inhibitor: the FEIBA NovoSeven Comparative (FENOC) Study. Blood 2007;109:546-551.
7. Leissinger C, Gringeri A, Antmen B, et al. Anti-inhibitor coagulant complex prophylaxis in hemophilia with inhibitors. N Engl J Med 2011;365:1684-1692.
8. Furukawa S, Nogami K, Ogiwara K, Yada K, Minami H, Shima M. Systematic monitoring of

hemostatic management in hemophilia A patients with inhibitor in the perioperative period using rotational thromboelastometry. Journal of thrombosis and haemostasis : J Thromb Haemost 2015;13:1279-1284.

9. Ogiwara K, Nogami K, Matsumoto T, Shima M. Tissue factor pathway inhibitor in activated prothrombin complex concentrates (aPCC) moderates the effectiveness of therapy in some severe hemophilia A patients with inhibitor. Int J Hematol 2014;99:577-587.
10. Kitazawa T, Igawa T, Sampei Z, et al. A bispecific antibody to factors IXa and X restores factor VIII hemostatic activity in a hemophilia A model. Nat Med 2012;18:1570-1574.
11. Sampei Z, Igawa T, Soeda T, et al. Identification and multidimensional optimization of an asymmetric bispecific IgG antibody mimicking the function of factor VIII cofactor activity. PLoS One 2013;8:e57479.
12. Shima M, Hanabusa H, Taki M, et al. Factor VIII-Mimetic Function of Humanized Bispecific Antibody in Hemophilia A. N Engl J Med 2016; 374:2044-2053.
13. Shima M, Hanabusa H, Taki M, 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;1:1891-1899.
14. Oldenburg J, Mahlangu JN, Kim B, et al. Emicizumab Prophylaxis in Hemophilia A with Inhibitors. N Engl J Med 2017;377:809-818.
15. Young G, Liesner R, Chang T, et al. A multicenter, open-label phase 3 study of emicizumab prophylaxis in children with hemophilia A with inhibitors. Blood 2019;134:2127-2138.
16. Pipe SW, Shima M, Lehle M, 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-e305.
17. Mahlangu J, Oldenburg J, Paz-Priel I, et al. Emicizumab prophylaxis in patients who have hemophilia A without inhibitors. N Engl J Med 2018;379:811-822.
18. Shima M, Nogami K, Nagami S, et al. A multicentre, open-label study of emicizumab given every 2 or 4 weeks in children with severe haemophilia A without inhibitors. Haemophilia 2019;25:979-987.

19. Kitazawa T, Esaki K, Tachibana T, et al. Factor VIIIa-mimetic cofactor activity of a bispecific antibody to factors IX/IXa and X/Xa, emicizumab, depends on its ability to bridge the antigens. Thromb Haemost 2017;117:1348-1357.
20. Uchida N, Sambe T, Yoneyama K, et al. A first-in-human phase 1 study of ACE910, a novel factor VIII-mimetic bispecific antibody, in healthy subjects. Blood 2016;127:1633-1641.
21. Yada K, Nogami K, Kitazawa T, et al. Mode of enhancement in the global hemostatic potentials with concomitant use of bypassing agents and emicizumab in hemophilia A patients with inhibitor evaluated by ROTEM. Res Pract Thromb Haemost 2017;1(Suppl 1):163-164.
22. Dargaud Y, Lienhart A, Janbain M, Le Quellec S, Enjolras N, Negrier C. Use of thrombin generation assay to personalize treatment of breakthrough bleeds in a patient with hemophilia and inhibitors receiving prophylaxis with emicizumab. Haematologica 2018;103:e181-e183.
23. Nogami K, Matsumoto T, Tabuchi Y, et al. Modified clot waveform analysis to measure plasma coagulation potential in the presence of the anti-factor IXa/factor X bispecific antibody emicizumab. J Thromb Haemost 2018;16:1078-1088.
24. Goodnough LT, Lublin DM, Zhang L, Despotis G, Eby C. Transfusion medicine service policies for recombinant factor VIIa administration. Transfusion 2004;44:1325-1331.
25. Bosch J, Thabut D, Bendtsen F, et al. Recombinant factor VIIa for upper gastrointestinal bleeding in patients with cirrhosis: a randomized, double-blind trial. Gastroenterology 2004;127:1123-1130.
26. Lusher JM. Use of prothrombin complex concentrates in management of bleeding in hemophiliacs with inhibitors--benefits and limitations. Semin Hematol 1994;31(2 Suppl 4):49-52.
27. Nogami K, Shima M. New therapies using nonfactor products for patients with hemophilia and inhibitors. Blood 2019;133:399-406.
28. Moake JL. Thrombotic microangiopathies. N Engl J Med 2002;347:589-600.
29. Shida Y, Swystun LL, Brown C, et al. Shear stress and platelet-induced tensile forces regulate ADAMTS13-localization within the platelet thrombus. Res Pract Thromb Haemost 2019;3:254-260.
30. 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;16:1383-1390.

31. Shima M, Scandella D, Yoshioka A, et al. A factor VIII neutralizing monoclonal antibody and a human inhibitor alloantibody recognizing epitopes in the C2 domain inhibit factor VIII binding to von Willebrand factor and to phosphatidylserine. Thromb Haemost 1993;69:240-246.
32. Nogami K, Shima M, Nakai H, et al. Identification of a factor VIII peptide, residues 2315-2330, which neutralizes human factor VIII C2 inhibitor alloantibodies: requirement of Cys2326 and Glu2327 for maximum effect. Br J Haematol 1999;107:196-203.
33. Shida Y, Rydz N, Stegner D, et al. Analysis of the role of von Willebrand factor, platelet glycoprotein VI-, and α2β1-mediated collagen binding in thrombus formation. Blood 2014;124:1799-1807.
34. Muto A, Yoshihashi K, Takeda M, et al. Anti-factor IXa/X bispecific antibody (ACE910): hemostatic potency against ongoing bleeds in a hemophilia A model and the possibility of routine supplementation. J Thromb Haemost 2014;12:206-213.

Figure Legends

Figure 1. Time course changes of thrombus formation in severe PwHA without inhibitor under high shear conditions - (*Panel* **A**) Representative images of thrombi in whole blood from healthy individuals (normal) and PwHA without inhibitor at the indicated reaction-times (1, 4, and 8 min) after perfusion. FVIII (100 IU/dl) or emicizumab (100 μg/ml) was added to the whole blood from PwHA, followed by staining the platelets with phalloidin-Alexa 488 as described in Methods. The scale bar is set at 30 μm. (*Panel* **B**) Statistical analyses corresponding to the images shown in (A); Values and error bars represent the mean (± SD) of surface coverage and thrombus height in 15 defined areas (each 133×100 μm) randomly selected in 5 separate flow experiments. Surface coverage (SC; *left panel*) and thrombus height (TH; *right panel*) are shown. *p<0.05, **p<0.01, ***p<0.001

Figure 2. Comparisons between emicizumab and FVIII on thrombus formation under high shear at each time point - The data in Figure 1 was reformatted and summarized at 1, 4, and 8 min.

Statistical analyses corresponding to the images shown in Figure 1A; Surface coverage (SC; *left panel*) and average thrombus height (TH; *right panel*) are shown. *p<0.05, **p<0.01, ***p<0.001

Figure 3. Thrombin localized in formed thrombi under high shear after the addition of emicizumab and FVIII - (*Panel* **A**) Thrombi in whole blood from PwHA with *ex vivo* FVIII (final concentration of 0 and 100 IU/dl) after 4 min perfusion were stained with phalloidin-Alexa 488 (green), anti-thrombin antibody-Alexa 567 (red pseudo-color), and anti-FVIII antibody Alexa 647 (blue pseudo-color). Emicizumab (100 µg/ml) was added instead of FVIII. Anti-emicizumab mAb conjugated with Alexa 647 was used to visualize the localization of emicizumab on thrombi (blue pseudo-color). Representative images of multi-color immunostaining of thrombi are shown. The scale bar is set at 30 µm. (*Panel* **B**) Intensity measurements of thrombin were derived and expressed as arbitrary units (AU). Statistical analyses corresponding to the images in (A) are shown. Values and error bars represent the mean (± SD) of the intensity measurement in 15 defined areas (each 133×100 µm) randomly selected in 5 separate flow experiments. *p<0.05, **p<0.01, ***p<0.001

Figure 4. *Ex vivo* effects of BPAs in the absence or presence of emicizumab on thrombus formation in PwHA with inhibitor - (*Panel* **A**) aPCC (1.2 U/ml; equivalent to 100 U/kg) (**a**), rFVIIa (1.1 µg/ml; equivalent to 90 µg/kg) (**a**), emicizumab (100 µg/ml) (**b**), aPCC (1.2 U/ml)/emicizumab (100 µg/ml) (**b**), and rFVIIa (1.1 µg/ml)/emicizumab (100 µg/ml) (**b**) were added to whole blood from PwHA with inhibitor prior to perfusion. Thrombi were stained with phalloidin-Alexa 488 (*green*), anti-thrombin antibody-Alexa 567 (*red pseudo-color*), and anti-fibrin antibody-Alexa 647 (*blue pseudo-color*). Representative images of immunostaining are shown. Normal comparator was also shown. The scale bar is set at 30 µm. (*Panel* **B**) Statistical analyses corresponding to the images shown in (A). Values and error bars represent the mean (± SD) of surface coverage (SC) and thrombus height (TH) in 15 defined areas (each 133×100 µm) randomly selected in 5 separate flow experiments. Surface coverage (SC; *left panel*) and average thrombus height (TH; *right panel*) are shown. *p<0.05, **p<0.01, ***p<0.001

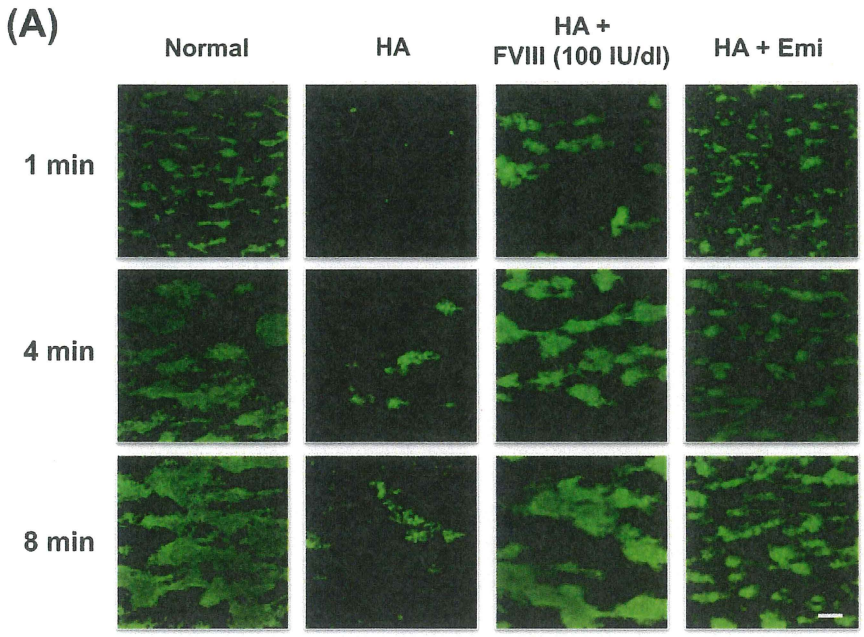

Figure 1A

Figure 1A

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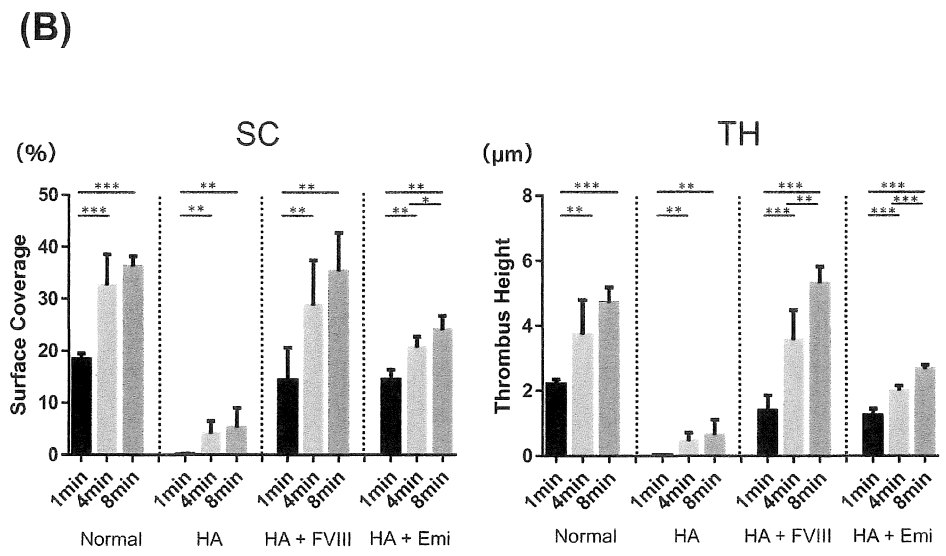

Figure 1B

Figure 1B

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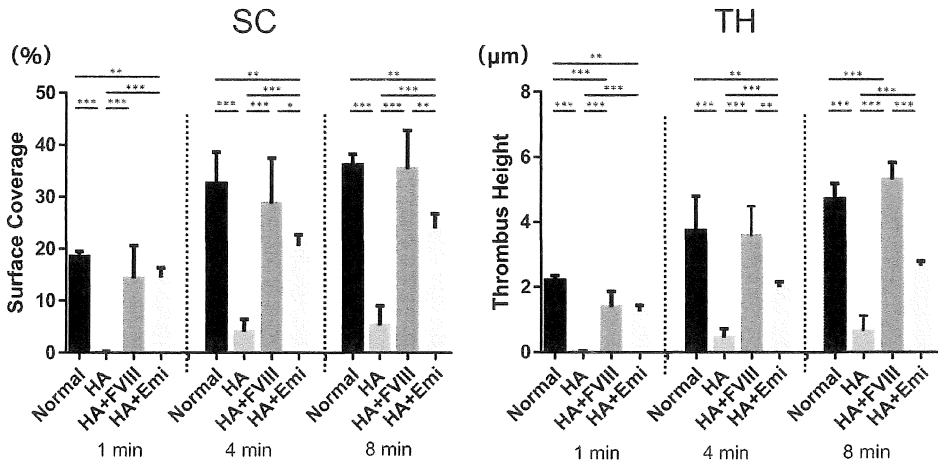

Figure 2

Figure 2

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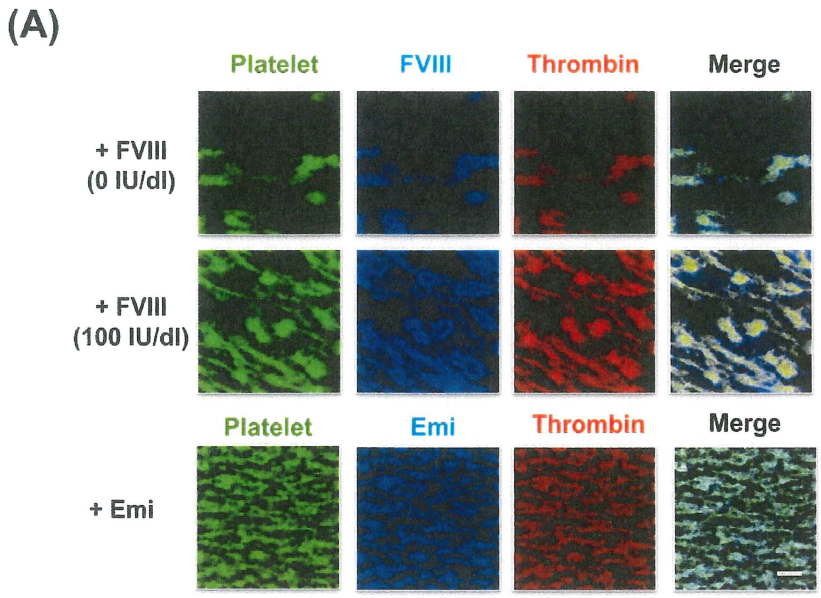

Figure 3A

Figure 3A

254x190mm (600 x 600 DPI)

(B)

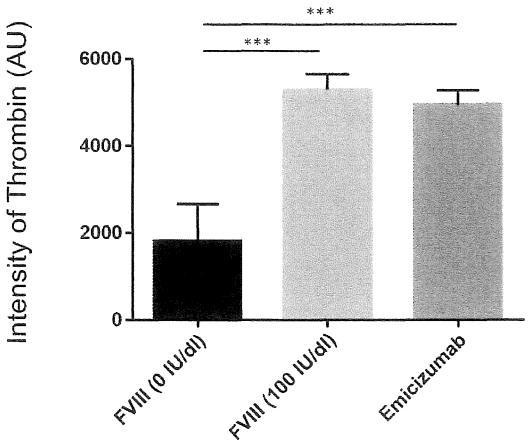

Figure 3B

Figure 3B

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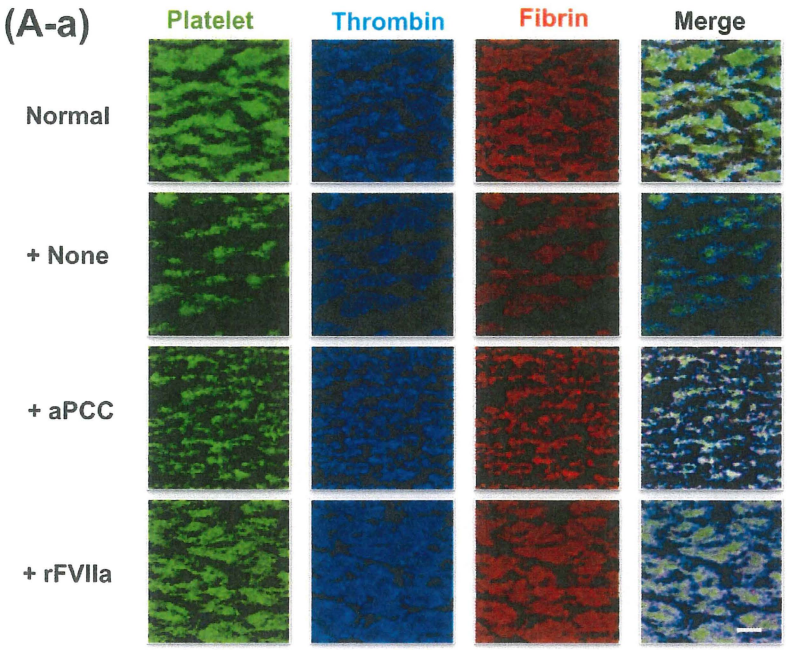

Figure 4A-a

Figure 4A-a

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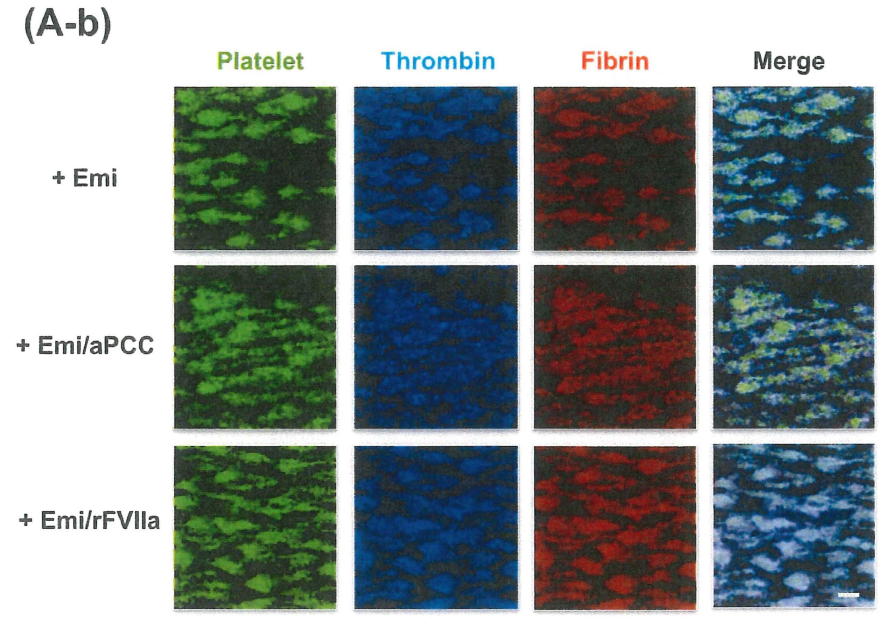

Figure 4A-b

Figure 4A-b

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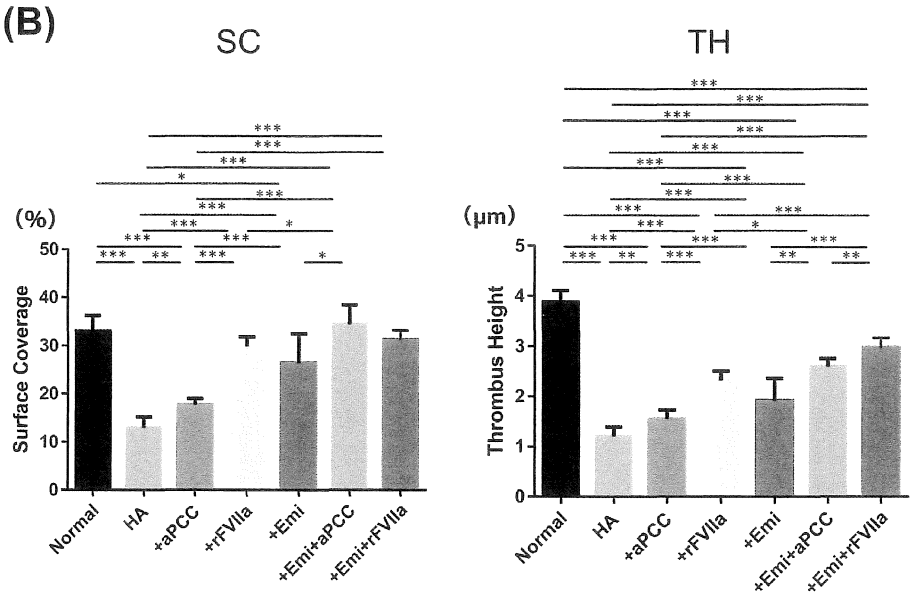

Figure 4B

Figure 4B

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