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REPLICATION STUDIES

The effect of emicizumab and bypassing agents in patients with hemophilia – An in vitro study

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Abstract

Background: Emicizumab is a nonfactor replacement therapy for hemophilia A (HA) and is a bispecific monoclonal antibody mimicking factor VIII by binding both factors IXa and X. Although it reduces the frequency of bleeding episodes, there is still need for bypassing agents in case of breakthrough bleeds or need for surgery. The HAVEN-1 study showed an increased risk of thrombotic events and episodes of thrombotic microangiopathic hemolytic anemia with simultaneous treatment with emicizumab and activated prothrombin complex concentrate (aPCC) in high doses (>100 U/kg daily) for more than 1 day, and it is suspected that these drugs have a synergistic hemostatic effect.

Objectives: To evaluate and compare the hemostatic effect of bypassing agents in vitro in people with HA before and after starting treatment with emicizumab to investigate if dosing should be adjusted to optimize treatment.

Patients/Methods: Blood collected before and after start of treatment with emicizumab was spiked with aPCC and recombinant factor VIIa (rFVIIa) at different concentrations. The effect of aPCC and rFVIIa was assessed by thrombin generation assay and thromboelastometry.

Results: Six people with HA were included. The response to aPCC in thrombin generation after starting emicizumab was significantly stronger than before. This synergistic effect was less pronounced for emicizumab and rFVIIa. Furthermore, aPCC shortened thromboelastometry clotting time more effectively after starting emicizumab than before starting this treatment.

Conclusions: We demonstrated a strong synergistic effect of emicizumab and aPCC and a similar but less pronounced effect of rFVIIa in people treated with emicizumab.

KEYWORDS
activated prothrombin complex concentrate, blood coagulation tests, emicizumab, hemophilia A, rFVIIa

Essentials

- A synergistic effect of emicizumab and activated prothrombin complex concentrate (aPCC) has been hypothesized.
- Reducing the dose of aPCC after starting emicizumab is warranted.
- The combination of emicizumab and aPCC caused hypercoagulability.
- We investigated the in vitro effect of aPCC before and after the start of emicizumab.

1 | INTRODUCTION

Treatment of hemophilia A (HA) has traditionally been replacement therapy with factor VIII (FVIII). This treatment may represent a burden to the patients because of frequent intravenous administrations and challenges in maintaining venous access. More importantly, some patients develop antibodies (inhibitors) that rapidly reduce the level of FVIII, rendering replacement therapy ineffective.[1]

In people with HA and inhibitors, bypassing agents (BPAs) are used prophylactically or on demand in case of bleeding episodes or need of surgery.[2] BPAs provide hemostasis by bypassing FVIII and FIX in coagulation and generating thrombin despite their absence. The effect of BPAs is unpredictable, which warrants individualization of the dosage based on bleeding history under treatment and coagulation assays.[3,4] Two BPAs are currently available. Activated prothrombin complex concentrate (aPCC) containing activated factor VII, factor X (FX), and thrombin in addition to factor II, factor IX (FIX), and FX in their inactive forms targets processes in both the extrinsic and intrinsic pathways of coagulation.[5] Recombinant factor VIIa (rFVIIa) affects hemostasis via the extrinsic pathway of coagulation.[6]

Emicizumab is a nonfactor replacement therapy approved for prophylactic treatment in people with HA, which can be administered subcutaneously once weekly or even less frequently. It consists of recombinant monoclonal antibodies that simultaneously bind to FIXa and FX, leading to activation of FX without the involvement of FVIIIa.[7] Thus, coagulation is not impaired by FVIII inhibitors. Even though the efficacy of emicizumab appears to be sufficient for bleeding prophylaxis in people with HA, BPA administration is still required in some situations such as episodes of breakthrough bleeding or need for major surgery. In the HAVEN-1 study, which included people with HA and inhibitors,[8] prophylaxis with emicizumab was associated with a significantly lower rate of bleeding than no prophylaxis. However, eight people on emicizumab prophylaxis required aPCC administration in high doses, five of which experienced a thrombotic episode.[9] The mechanism for this adverse effect is not clear, but it has been observed that emicizumab and aPCC exert a synergistic effect on hemostasis. No complications were reported for the concomitant use of emicizumab and rFVIIa.

A synergistic effect of emicizumab and aPCC on thrombin generation (TG) and viscoelastic coagulation assays has been demonstrated in in vitro studies in which blood samples were spiked with both emicizumab and aPCC.[10,11] In a recent study, Kizilocak et al[12] performed thromboelastography and TG assay in aPCC and rFVIIa spiked samples of emicizumab-treated people with HA. They demonstrated that aPCC in concentrations higher than 0.05 U/mL resulted in excess thrombin generation, compared with normal pooled plasma.

The objective of the present study was to confirm the results of Kizilocak et al[12] in patients starting with emicizumab treatment. The present study differs from the former studies in that blood samples from the same people with HA before starting emicizumab were used as controls instead of normal pooled plasma. Furthermore, we aimed to investigate to which extent the concentrations of aPCC should be reduced in people treated with emicizumab to obtain a hemostatic effect similar to before starting this treatment.

2 | MATERIALS AND METHODS

2.1 | Participants

People with severe HA and inhibitors were enrolled consecutively when treatment with emicizumab was planned. Patients started subcutaneous emicizumab with 3 mg/kg once weekly for 4 weeks as loading dose, thereafter 1.5 mg/kg once weekly. Blood samples were drawn before and 4 weeks after starting treatment with emicizumab. The patients or their guardian signed an informed consent form up front.

2.2 | Blood collection and preanalytical issues

Blood samples were collected using minimal stasis and a 21G × 19 mm butterfly needle (Vacuette, Greiner Bio-One GmbH, Kremsmünster, Austria). Blood was collected in tubes containing 0.109 M buffered citrate (Monovette, Sarstedt, Nümbrecht, Germany) manually prefilled with additional corn trypsin inhibitor (Haematologic Technologies Inc., Essex Junction, VT, USA) at a final concentration of 20 µg/mL.[13]

The BPAs aPCC (FEIBA, Baxter AG, Vienna, Austria) and rFVIIa (Novoseven, NovoNordisk, Copenhagen, Denmark) were prepared according to the instructions for use. Whole blood was spiked with the BPAs at different concentrations corresponding to subtherapeutic, therapeutic, and supratherapeutic doses: aPCC 0.2–3.2 U/mL (15-200 U/kg) and rFVIIa 0.7-5.6 µg/mL (45–360 µg/kg). The concentrations were calculated assuming that the human body consists of 65 mL of blood/kg.

2.3 | Thrombin generation

TG was measured in platelet-poor plasma (PPP) using the Calibrated Automated Thrombogram (CAT; (Diagnostica Stago, Asnière, France)

with Thrombinoscope software (Thrombinoscope BV, Maastricht, The Netherlands).[14,15] PPP was prepared by double centrifugation, first for 15 minutes at 2500 *g*, thereafter for 10 minutes at 10 000 *g*. For the youngest patients (patients 1 and 4), a smaller amount of blood was collected than for the adult patients, and only a single centrifugation was performed. TG in PPP containing the different BPAs in different concentrations, including one unspiked sample, were run in triplicates. The PPP reagent "low," containing 1 pM of tissue factor (TF) and 4 µM phospholipids (Diagnostica Stago, Asnière, France), was used to initiate TG.

2.4 | Thromboelastometry

In this study, we used rotational thromboelastometry initiated by minimal TF activation as described by Sørensen et al.[16] Clotting time (CT; seconds), clot formation time (CFT; seconds), and maximum clot firmness (MCF; mm) were measured by rotational thromboelastometry (TEM Innovations, Munich, Germany) in duplicate. The plastic test cups were prepared with 40 µL of buffer containing 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 150 mM NaCl, and 100 mM CaCl, pH 7.4, and recombinant relipidated TF (Innovin, Dade Behring, Liederbach, Germany) diluted in 20 µL of buffer containing 20 mM HEPES and 150 mM of NaCl. Thereafter, whole blood (280 µL) was added to initiate the coagulation process. The final TF dilution was 1:70 000, corresponding to a theoretical TF concentration of 0.35 pM.

2.5 | Statistical analysis

The data were expressed as medians with interquartile ranges (IQRs). The one-way analysis of variance with the post hoc test by Tukey correcting for multiple testing, was used to calculate the differences in effect between the BPAs before and after starting emicizumab treatment. Statistical calculations were performed by using SPSS version 26 (SPSS Inc., Chicago, IL, USA). Statistical significance was set to $P < .05$.

3 | RESULTS

3.1 | Participants

Six people with HA with inhibitors planning to start emicizumab treatment were included between 2019 and 2020 at Oslo University Hospital. Treatment with emicizumab was commenced after inclusion in the study, and the characteristics are listed in Table 1.

3.2 | Thrombin generation

The baseline values of the TG parameters did not change significantly after starting treatment with emicizumab (Figure 1A-C). Before initiating emicizumab treatment, median lag time was 8.3 (IQR, 3.3) minutes, median peak thrombin concentration was 8.0 (IQR, 4.0) nM, and median endogenous thrombin potential (ETP) was 104 (IQR, 87) nM/min. After starting emicizumab, median lag time was 10.4 (IQR, 6.6), median peak thrombin was 9.3 (IQR, 18) and median ETP was 356 (IQR, 472). The response to the added bypassing agents was altered significantly after starting this treatment. Except for lag time, which was relatively constant for all the experiments, peak thrombin and ETP were elevated significantly when adding aPCC compared to before starting emicizumab. This effect was less pronounced in the samples spiked with rFVIIa (Figures 1 and 2).

In plasma from before starting emicizumab, aPCC and rFVIIa at any dose had similar effects on TG. After starting emicizumab, the increase in peak thrombin concentration was significantly larger at all tested concentrations of aPCC, also compared to the response to rFVIIa. aPCC at a concentration of 0.2 U/mL (corresponding to the dose 15 U/kg) increased the peak to a significantly higher level than the highest dose of rFVIIa ($P < .0001$) and to the same level as aPCC at a concentration corresponding to 200 U/kg before starting emicizumab ($P = .12$) (Figure 1B).

Also, for ETP the response to aPCC after starting emicizumab was significantly stronger than before (Figure 1C). For aPCC at the highest concentrations (1.6 and 3.2 U/mL, corresponding to 100 and 200 U/kg, respectively), thrombin generation was too

TABLE 1 Patient characteristics

-	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Age	8 mo	59 y	28 y	2 y	68 y	43 y
Type hemophilia A	Severe	Moderate	Severe	Severe	Severe	Severe
FVIII, %	<1	3-4	<1	<1	<1	<1
Peak inhibitor level, BU/mL	36.5	90	64	3000	18	17
Emicizumab dose, mg/kg	3	3	3	3	3	3
Previous treatment	ITI	SHL FVIII, ITI	ITI	ITI	aPCC P	ITI rFVIIa P
Previous need for BPA	rFVIIa OD	rFVIIa OD aPCC OD	aPCC OD	rFVIIa (P+ OD)	aPCC OD	rFVIIa OD

Abbreviations: aPCC, activated prothrombin complex concentrate; BPA, bypassing agents; ITI, immune tolerance therapy; OD, on demand; P, prophylactically; rFVIIa, recombinant factor VIIa; SHL FVIII, standard half-life factor VIII.

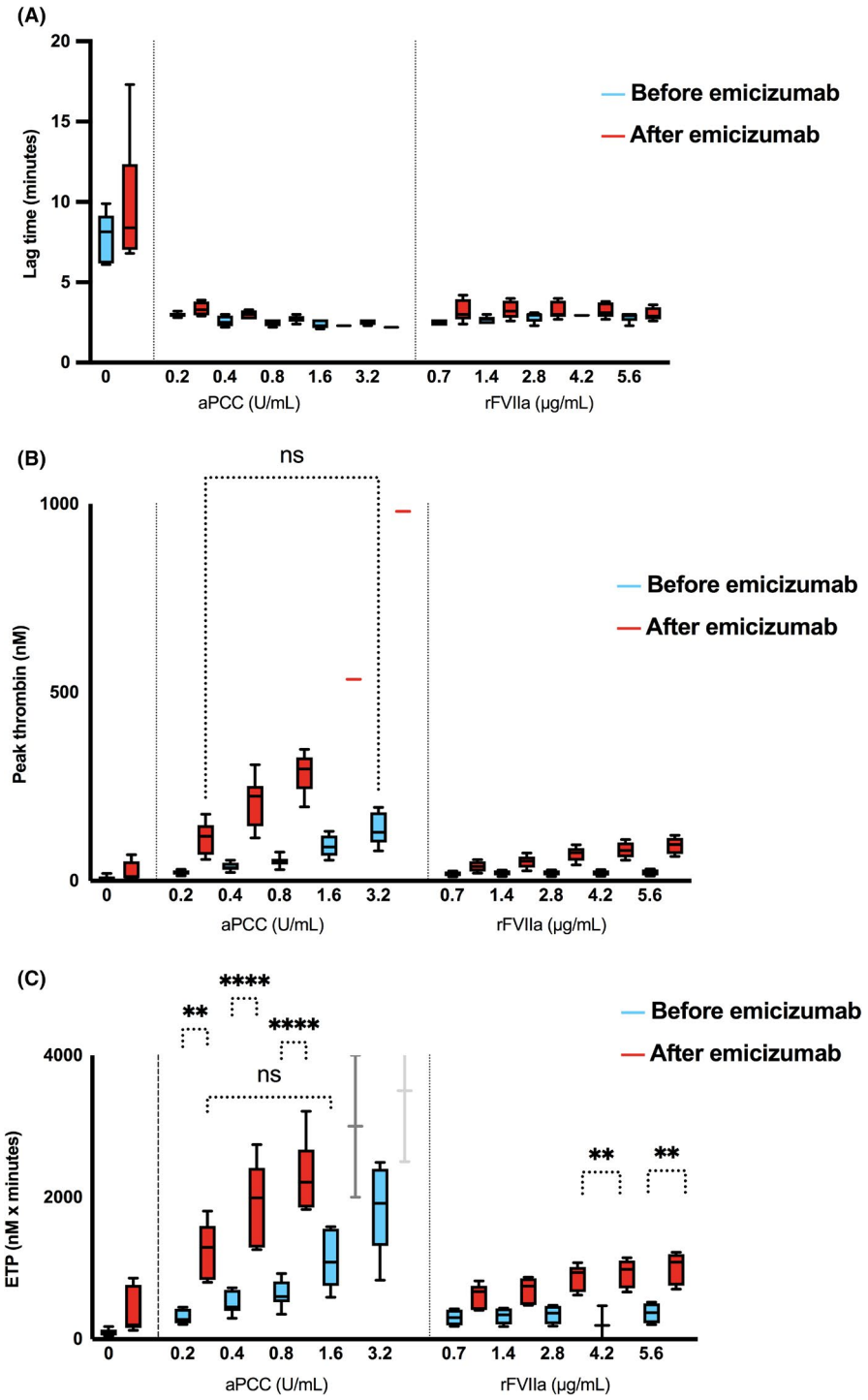


FIGURE 1 Thrombin generation parameters before (red box plots) and after (blue box plots) starting emicizumab. Samples were spiked with activated prothrombin complex concentrate (aPCC) (left) and with recombinant factor VIIa (rFVIIa) (right). The parameters lagtime (A), peak thrombin generation (B), and endogenous thrombin potential (ETP) (C) were assessed by thrombin generation assay. aPCC caused a stronger increase in peak and ETP after starting emicizumab that before. The same effect was demonstrated by rFVIIa, but to a lesser degree

high to be measured with the CAT instrument, hence TG curves and ETP could not be obtained. After initiating emicizumab treatment, an increased response was also seen for rFVIIa. ETP was increased to a significantly higher level after emicizumab than before at concentrations of 4.2 µg/mL (corresponding to 270 µg/kg) and above.

Lag time was shortened by both BPAs, but no significant differences were seen between aPCC and rFVIIa, or between the different concentrations before and after starting emicizumab (Figure 1A).

3.3 | Thromboelastometry

Median (IQR) CT before starting treatment with emicizumab was 806 (382) seconds, and it did not change significantly after starting emicizumab. CT was shortened after adding aPCC and rFVIIa both before and after starting emicizumab (Figure 3A,B). Before starting emicizumab, adding aPCC at a concentration of 0.4 U/mL caused a reduction of CT to 369 (166) seconds and rFVIIa 1.4 µg/mL to 448 (322) seconds (Figure 3A,B, blue boxplots). Higher doses did not significantly reduce CT further. After starting emicizumab, CT

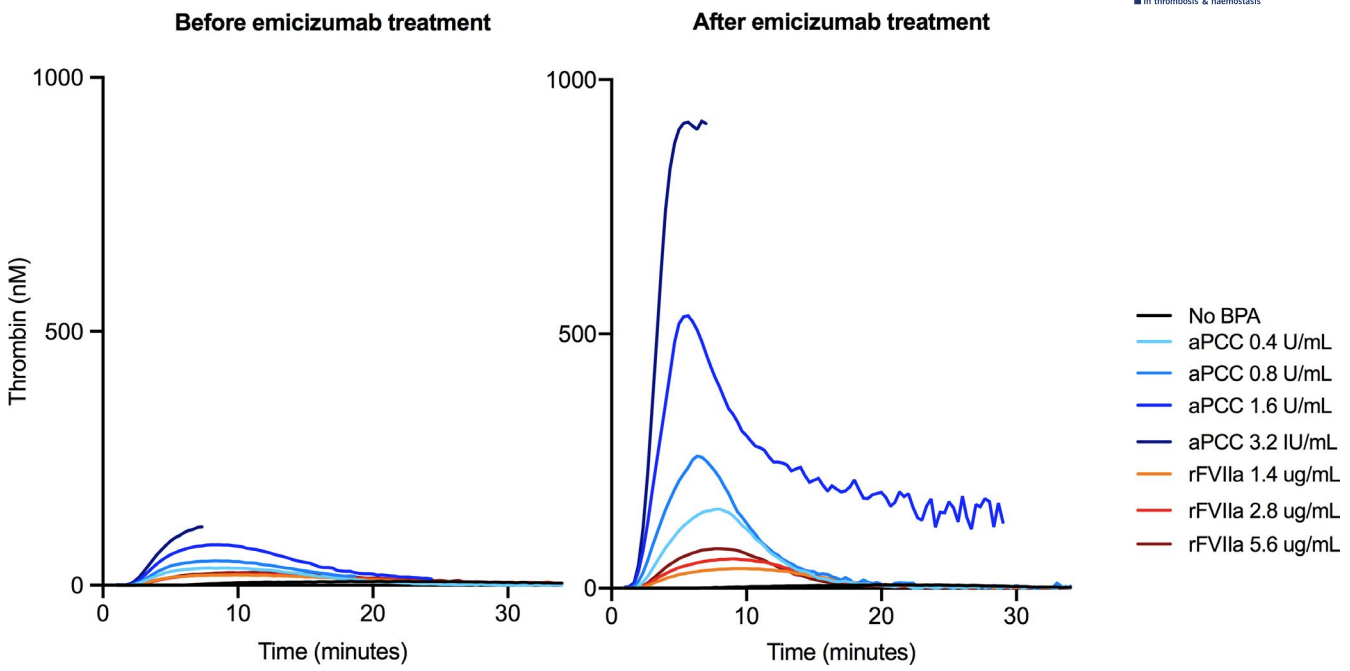
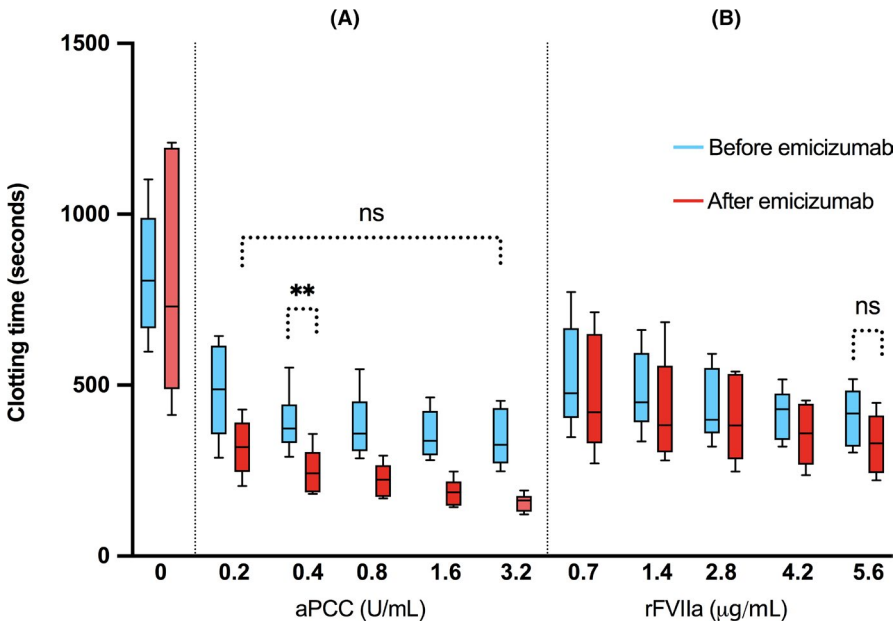


FIGURE 2 Representative thrombin generation curves before and after starting emicizumab. Samples were spiked with bypassing agents (BPAs) at different concentrations. The curves illustrate the increased response to activated prothrombin complex concentrate (aPCC) after starting treatment with emicizumab compared to before. A similar but much weaker increase in response was seen to recombinant factor VIIa (rFVIIa) after starting emicizumab treatment. For the highest concentrations of aPCC, thrombin generation was too high to obtain a complete curve

FIGURE 3 Thromboelastometry clotting time (CT) before (red box plots) and after (blue box plots) starting emicizumab. Samples were spiked with activated prothrombin complex concentrate (aPCC) (A) and recombinant factor VIIa (rFVIIa) (B). aPCC caused a stronger reduction in CT after starting emicizumab that before



was dramatically reduced in a dose-response relationship by adding aPCC, and at a concentration of 0.4 U/mL CT was 239 (IQR 87) seconds and at 0.8 U/mL, 195 (65) seconds (Figure 3A, red boxplots). A significant difference in response before and after emicizumab was not seen for rFVIIa (Figure 3B), as CT was reduced to 406 (204) seconds (P = .1) for rFVIIa 1.4 μg/mL. MCF was not affected by BPAs.

4 | DISCUSSION

Emicizumab is a convenient and effective prophylactic treatment option for patients with HA and inhibitors.[8,17] However, an increased risk of thrombotic events in patients receiving emicizumab in combination with aPCC has been described.[8] In the present in vitro study, we assessed the hemostatic response to aPCC and rFVIIa in people

with HA and inhibitors before and after starting emicizumab treatment by thromboelastometry and TG. The study demonstrated that even at low concentrations, aPCC had a significantly stronger hemostatic effect after starting emicizumab compared to before. This synergistic effect with emicizumab was less pronounced for rFVIIa.

By assessing the effect of BPAs before and after the start of emicizumab, we could observe the individual changes in coagulability provided by emicizumab in combination with BPAs. Such interindividual differences in response to BPAs among patients with HA and inhibitors have been shown previously,[3,18] but not yet in people treated with emicizumab. Based on recent publications concerning emicizumab and aPCC, experts recommend rFVIIa as the first-line treatment in case of breakthrough bleeds or need for invasive procedures in people receiving emicizumab treatment.[19-21] However, some people do not respond to rFVIIa, and in that case, aPCC remains the only option. It may therefore be beneficial to investigate the individual response in thrombin generation to BPAs before and after starting emicizumab. This may help to establish personalized treatment plans to avoid hypercoagulability when using BPAs in case of breakthrough bleeds or surgery. In the present study, it is shown that a low dose (30 U/kg) of aPCC in combination with emicizumab provides a hemostatic effect comparable to aPCC doses of 100–200 U/kg before the start of emicizumab treatment. As aPCC and rFVIIa in different doses demonstrated a dose-response relationship, finding an optimal individual dose of BPA should be possible.

The major limitation of the study is the small sample size. Therefore, the results must be interpreted with caution. Furthermore, the effects of aPCC and rFVIIa were assessed in vitro, and the results may not fully reflect the in vivo effect. However, we have found that the concomitant use of emicizumab and aPCC causes an excess TG, which has been demonstrated to be associated with increased risk of thrombosis.[22] Furthermore, the design of the present study in which people with HA are included, in contrast to in vitro studies with healthy volunteers, allowed for individual parameters affecting coagulation to be accounted for.

The global coagulation assays thromboelastometry with minimal tissue factor activation and TG assay are sensitive analyses to measure the coagulation potential of people with hemophilia.[23] A limitation of the present study is the assessment of TG in PPP and not in platelet-rich plasma (PRP) due to practical reasons and limited amount of patient plasma. It has been suspected that rFVIIa works also by a TF-independent mechanism over platelets. Therefore, the effect of rFVIIa may be different in assays with platelets present.[24] In fact, it has previously been shown that rFVIIa causes a stronger increase in TG in PRP than in PPP,[25] so the effect of this BPA may be underestimated in this study; however, a weaker coagulant response of rFVIIa than of aPCC was seen also in thromboelastometry, which is an assay in which platelet reactivity has an impact on the results, particularly on CT, the parameter reported in our study.

Previous studies of Hartmann et al[10] and Zong et al[11] had slightly different designs, but both found that aPCC caused excessive TG when combined with emicizumab or a sequence-identical analog. The present study is a replication study confirming the results from another study. In this recent study, Kizilocak et al,[12] performed thromboelastography and TG assay in people with HA treated with emicizumab and demonstrated that aPCC in concentrations above 0.05 U/mL resulted in excess TG compared with normal pooled plasma. In neither of the mentioned studies was the effect of bypassing agents compared with the hemostatic effect in people with HA before starting emicizumab treatment.

In addition to confirming the results of the above-mentioned study, a weaker synergistic effect of rFVIIa and emicizumab was demonstrated. Furthermore, unlike in previous studies, we compared the effect of BPAs before and after starting emicizumab treatment in the same individuals, which may help us establish appropriate doses of BPAs in individuals.

The increased TG in our experiments may reflect an interaction between emicizumab and aPCC. This interaction may cause the increased risk of thrombotic events after treatment of aPCC in people treated with emicizumab shown in the HAVEN-1 study.[17] The mechanism for this thrombogenic effect is not well understood, but it has been speculated that aPCC provides an increased availability of FIX and FX, the targets for emicizumab. This assumption is supported by the study of Hartmann et al[10] mentioned above. Another explanation may be that, unlike FVIIIa, emicizumab is not inactivated by the natural anticoagulant, protein C system, or another regulatory system,[26] and thus coagulation is enhanced.

aPCC is an established treatment for breakthrough bleeds in people with HA with inhibitors, in doses ranging from 100 to 200 IU/kg. In the present paper, we have demonstrated that the hemostatic effect of aPCC was obtained at much lower doses after starting emicizumab treatment; thus, a dose reduction is appropriate.

5 | CONCLUSIONS

This replication study confirms the strong synergistic effect of emicizumab and aPCC and demonstrates a similar but less pronounced effect of rFVIIa in vitro. Precautions should be made before administering BPAs to people treated with emicizumab. Clinical trials, assessing the effect and safety of the combination of emicizumab and BPAs, are needed.

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AUTHOR CONTRIBUTIONS

PAH, HG, and NHS designed the study, performed the research, analyzed and interpreted the data, and wrote the manuscript. SB contributed to the acquisition of data and performed the laboratory analyses.

RELATIONSHIP DISCLOSURE

NHS has received personal fees from Bayer, Pfizer and BMS. PAH has received personal fees from Takeda, SOBI, Bayer, Pfizer, Roche,

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