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ORIGINAL ARTICLE

In vitro reversal of direct factor Xa inhibitors: Direct comparison of andexanet alfa and prothrombin complex concentrates Cofact and Beriplex/Kcentra

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Abstract

Background: Both andexanet alfa and four-factor prothrombin complex concentrate (4F-PCC) are clinically applied reversal agents for direct factor Xa inhibitors (FXaIs) in emergency situations. Controversy exists whether 4F-PCC is as effective as andexanet alfa in correcting FXaI anticoagulation.

Objective: This in vitro study was designed to directly compare andexanet alfa with two different 4F-PCCs (Cofact and Beriplex/Kcentra) in their ability to correct FXaI anticoagulation.

Method: Normal plasma was spiked with apixaban or rivaroxaban. Reversal of anticoagulation was assessed using a thrombin generation assay and a fibrin generation–clot lysis test.

Results: Andexanet alfa, applied at clinically recommended doses, was effective in restoring thrombin generation as evidenced by correction of thrombin generation lag time, peak thrombin, and endogenous thrombin potential (ETP). Clotting time and clot resistance to fibrinolytic breakdown was corrected over the full range of applied FXaI (0–800 ng/ml). 4F-PCC in increasing doses (0.625, 1.25 and 2 IU/ml; approximately 25, 50, and 80 IU/kg) only partially restored thrombin generation lag time and clotting time. Partial correction to overnormalization of peak thrombin and ETP was observed, depending on FXaI concentration and PCC dose. Clot resistance to fibrinolytic breakdown was dose-dependently improved to above normal. Beriplex/Kcentra was consistently less effective than Cofact.

Conclusion: Both andexanet alfa and 4F-PCC improved coagulation that is hampered by FXaIs. While andexanet alfa corrected all thrombin generation parameters, 4F-PCC predominantly increased peak thrombin and ETP. Especially heparin-free 4F-PCC also improved clot stability against fibrinolytic breakdown. Beriplex/Kcentra contains heparin, and this may have caused reduced effectivity compared to Cofact.

KEYWORDS
anticoagulation reversal, apixaban, prothrombin complex concentrates, PRT064445, rivaroxaban

Essentials

- Andexanet alfa is a recombinant drug approved to reverse apixaban and rivaroxaban.
- Blood plasma–derived prothrombin complex concentrate (PCC) may also be used.
- Andexanet alfa restores thrombin generation, fibrin clot formation, and fibrinolytic resistance.
- PCC increases thrombin generation and improves clot stability against fibrinolytic breakdown.

1 | INTRODUCTION

Direct oral anticoagulant (DOAC) factor Xa inhibitors (FXaIs) have become widely used for both venous thromboembolism treatment and prophylaxis, as well as stroke prevention in atrial fibrillation and prevention of atherothrombosis.[1] The primary complication of DOAC anticoagulation is serious or life-threatening hemorrhage, a condition that may require prompt anticoagulation reversal. Because DOACs have short half-lives, they do not usually require reversal in patients with bleeding that is not life threatening. Similarly, DOAC reversal is usually not necessary in nonbleeding patients requiring surgery that is not urgent. However, urgent invasive procedures in nonbleeding DOAC patients may necessitate quick anticoagulation reversal.[2]

Andexanet alfa with trade names Ondexxya (European Union) and Andexxa (United States) is a recombinant modified human factor Xa (FXa) that was developed as a specific antidote for reversal of anticoagulation by FXaI as well as antithrombin-dependent anticoagulation by low-molecular-weight heparins. Andexanet alfa is catalytically inactive due to mutation of the active-site serine to alanine (S419A) and deletion of the membrane-binding gamma-carboxyglutamic acid domain but, on the other hand, has retained the ability to be bound by FXa-inhibiting drugs.[3] Andexanet alfa in contrast to its native counterpart cannot assemble with activated factor V on a phospholipid membrane to form the so-called prothrombinase complex to generate thrombin. Andexanet alfa acts as a decoy protein: It competes with native FXa for binding to FXaI. Andexanet alfa is approved by both the US Food and Drug Administration and the European Medicines Agency for patients taking apixaban or rivaroxaban who require reversal of anticoagulation due to life-threatening or uncontrolled bleeding. Two dosing regimens are advised: a low-dose 400-mg bolus followed by a 2-h infusion at 4 mg/min, or a high-dose 800-mg bolus with a subsequent 8 mg/min infusion for 2 h. The recommended regimen to be followed depends on the specific FXaI that is prescribed to the patient, dose of the inhibitor, and time since last FXaI intake.[4] Phase II and phase III trials have revealed prompt reversal of the anticoagulant activity of apixaban and rivaroxaban after andexanet alfa administration with a hemostatic effectivity rated as good or excellent in 82% of patients presenting with a FXaI-associated acute major bleeding and treated with andexanet alfa.[5,6]

Four-factor prothrombin complex concentrate (4F-PCC) is prepared from human blood plasma. It consists of a mixture of partially purified vitamin K–dependent coagulation factors II, VII, IX, X, and the anticoagulant proteins C and S. Presently, 4F-PCC is indicated for the treatment and perioperative prophylaxis of bleeding under conditions of acquired deficiency in vitamin K–dependent coagulation factors, such as a deficiency caused by treatment with vitamin K antagonists or in congenital deficiency in one or more of the vitamin K–dependent coagulation factors, provided that purified coagulation products are not available.[7] 4F-PCC may also be used off-label to reverse DOAC anticoagulation.[7] The rationale for using 4F-PCC as a reversal agent for DOACs is that by increasing the concentration of vitamin K–dependent coagulation factors, it pushes thrombin generation over a certain inhibition threshold.[8] An additional mechanism for reversal of DOAC anticoagulation by 4F-PCC may include increased clot resistance to degradation by the fibrinolytic system.[9] Current clinical guidelines recommend the off-label 4F-PCC administration at doses of 25–50 IU/kg in cases of serious or life-threatening bleeding in DOAC-treated individuals when specific reversal agents are not available.[2] Results from animal bleeding models suggest that the high-dose 50 IU/kg is preferred over the low-dose 25 IU/kg (reviewed in Brinkman[7]). The preference for a high-dose 4F-PCC regimen can also be concluded from studies with healthy volunteers anticoagulated with apixaban, rivaroxaban, or edoxaban. The primary outcome in most of these studies was normalization of in vitro thrombin generation and especially the endogenous thrombin potential (ETP).[10–15] In real-life patients on FXaI medication and presenting with a major bleeding, 4F-PCC restores thrombin generation immediately after infusion.[16] Two recently published meta-analyses (one on 4F-PCC, the other also including activated PCC) demonstrated similar to slightly higher rates of hemostatic effectiveness for PCC compared to andexanet alfa. Thromboembolic complications were more common in patients treated with andexanet alfa compared to those treated with PCC.[17,18]

An alternative to animal bleeding models and studies in healthy volunteers are in vitro experiments in which normal plasma is spiked with coagulation inhibitor and the reversal agent of interest. In one such approach that was recently reported by Lu and coworkers,[19] the effectivity of 4F-PCC to reverse apixaban as well as rivaroxaban anticoagulation was compared with that of andexanet alfa using the thrombin generation assay. Andexanet alfa normalized ETP over a wide FXaI range (19–2000 ng/ml), whereas 4F-PCC was able to normalize ETP only at inhibitor concentrations less than 75 ng/ml.[19] With respect to 4F-PCC, this is a quite surprising outcome that conflicts with the aforementioned studies in healthy volunteers with apixaban and rivaroxaban plasma concentrations significantly exceeding 75 ng/ml (average plasma concentration ranging from 189 to 351 ng/ml).[11–14] The outcome of the in vitro plasma spiking experiments performed by Lu and coworkers may have been influenced by the heparin content of the used 4F-PCC (Beriplex/Kcentra), a too-low 4F-PCC dosage, used reagents, and plasma source.[8,9,20–22] Also, the thrombin generation assay with derived ETP may not be the most appropriate in vitro test for FXaI reversal assessment. The

disadvantage of that test is that it does not look beyond thrombin, ignoring the subsequent formation of fibrin and features related to clot structure, such as clot resistance to fibrinolytic enzymes.[9]

The aim of the present in vitro study was to directly compare andexanet alfa with two 4F-PCCs, one containing heparin (Beriplex/Kcentra), the other not supplemented with heparin (Cofact), in reversing apixaban as well as rivaroxaban anticoagulation. FXaI reversal was assessed by in vitro thrombin and fibrin generation, and by determining fibrin clot stability against tissue-type plasminogen activator (t-PA)-induced lysis.

2 | MATERIALS AND METHODS

2.1 | Anticoagulated plasma and reversal agents

Normal plasma consisted of a pool of 10 single donor plasmapheresis units containing 12–19 mM tri-sodium citrate (Sanquin [the national blood bank of the Netherlands], where informed consent was obtained) and was stored in aliquots at −80°C. Lyophilized reversal agents Cofact (Prothya Biosolutions B.V.), Beriplex (CSL Behring GmbH) and andexanet alfa (Portola Pharmaceuticals LLC,) were dissolved in water according to the manufacturer's instructions, aliquoted, and stored at −80°C. In the United States, Beriplex is licensed under the trade name Kcentra (CSL Behring LLC). Cofact does not contain heparin while the amount of added heparin in Beriplex/Kcentra as provided by the manufacturer on the package insert ranges between 8 and 40 units per 500 units of factor IX. The FXaIs apixaban and rivaroxaban (both from Selleck Chemicals GmbH) were prepared as respectively 5 and 6.7 mg/ml dimethyl sulfoxide stocks and were stored at −30°C. Immediately before analysis, aliquots of plasma and reversal agents were quickly thawed in a 37°C water bath and handled further at room temperature. Thawed plasma aliquots (250 µl) were spiked with apixaban or rivaroxaban (0, 50, 100, 200, 400, and 800 ng/ml) and one of the reversal agents: andexanet alfa (0, 0.125, 0.25, and 0.50 mg/ml; 0, 3, 6, and 12 µM) or the 4F-PCCs Cofact and Beriplex (0, 0.625, 1.25, and 2 IU/ml). Total volume of added inhibitor and reversal agent was set at 28 µl, and stated concentrations are those in 100% plasma. Assuming 40 ml plasma/kg bodyweight (bw) and a population average bw of 80 kg, these 4F-PCC doses recalculate to 0, 25, 50, and 80 IU/kg bw. For andexanet alfa, it calculates to a 0-, 400-, 800-, and 1600-mg bolus. Dilutions of FXaI were made in 50 mM Tris, 150 mM NaCl, 1% human serum albumun (HSA), pH 7.4 (thrombin generation assay) or 5 mM HEPES, 137 mM NaCl, 0.1% HSA, pH 7.4 (fibrin generation–clot lysis test). Dilutions of 4F-PCCs and andexanet alfa were made in their respective formulation buffers. For PCCs: 10 mM trisodium citrate, 135 mM NaCl, pH 7.0. For andexanet alfa: 10 mM Tris, 45 mM arginine., 2% sucrose, 5% mannitol, 0.01% Tween80, pH 7.0. To avoid differences in citrate concentration between 4F-PCC and andexanet alfa dilutions, dilutions of andexanet alfa were adjusted to 10 mM trisodium citrate. Pilot experiments with dilution buffers and formulation solutions but without active ingredients (FXaI, andexanet alfa, 4F-PCC) revealed no interfering effects of these solutions in the thrombin generation assay and the fibrin generation–clot lysis test.

2.2 | Thrombin generation assay

Thrombin generation was assessed by calibrated automated thrombography (CAT, Thrombinoscope) on a Fluoroskan Ascent microplate reader (Thermo Scientific) under control of specialized CAT software (Thrombinoscope version 5.0). In this assay, 80 µl of plasma mixture was combined with 20 µl of premixed phospholipid–tissue factor (TF) (PPP-reagent, Thrombinoscope) or 20 µl of calibrator reagent (Thrombinoscope). Thrombin generation was started by the addition of 20 µl of premixed thrombin substrate z-Gly-Gly-Arg-AMC (Bachem) and $CaCl_2$. Final concentrations of TF and phospholipid during measurement were 5 pM and 4 µM, respectively. Final concentrations of $CaCl_2$ and the thrombin substrate were 15 mM and 0.5 mM, respectively. Parameters obtained were lag time (min), thrombin peak (nM), and area under the curve (ETP, nM.min).[23] Velocity index (nM/min) is defined as peak thrombin divided by the difference between time to thrombin peak and lag time. Thrombin generation parameters are presented as percentage of control (no FXaI and/or reversal agent). Absolute data can be found in the Supporting Information.

2.3 | Fibrin generation–clot lysis test

We used optical density tracings of fibrin formation and clot lysis performed in 96-well microplates to determine plasma clotting time (CT), lysis time (LT) and clot lysis time (CLT) as described previously.[24] In this assay, 100 µl of plasma mixture was combined with 50 µl of start reagent (TF, phospholipids, t-PA and $CaCl_2$ at final concentrations of 0.5 pM, 4 µM, 50 ng/ml, and 15 mM, respectively) and optical density (405 nm) was recorded for 2 h at 37°C on a Spectramax multimode microplate reader (Molecular Devices). Start reagent was prepared from commercially obtained ingredients as described.[24] We defined CT as the time from reagent addition to half maximal optical density. The second part of the tracing with a decline from maximal to baseline optical density represents clot lysis. We defined LT as the time from reagent addition to half maximal lysis. We defined CLT as the time between CT and half maximal lysis. CT and CLT are presented as percentage of control (no FXaI and/or reversal agent). Absolute data can be found in the Supporting Information.

3 | RESULTS

3.1 | FXaI reversal evaluated in the thrombin generation assay

Representative thrombin generation curves obtained in the absence or presence of 200 ng/ml of apixaban or rivaroxaban, with or without reversal agent, being either andexanet alfa (0.25 mg/ml, 800 mg bolus) or one of the 4F-PCCs Cofact or Beriplex (1.25 IU/ml, 50 IU/kg bw), are shown in Figure 1. Apixaban delayed and attenuated thrombin generation to a considerable extent, and this inhibitory effect

of apixaban was completely abolished in the presence of andexanet alfa (Figure 1). In fact, in the presence of andexanet alfa, a higher-than-normal peak thrombin was obtained. When using Cofact or Beriplex/Kcentra as reversal agent, thrombin generation was only partly restored (Figure 1). Under the experimental conditions used and in the presence of 200 ng/ml of rivaroxaban, the thrombin generation curve was less flattened than observed with apixaban (Figure 1). Again, andexanet alfa returned the thrombin peak to above normal. This was also observed with 1.25 IU/ml Cofact but not with the same dose of Beriplex (Figure 1).

Thrombin generation parameters obtained at increasing doses of apixaban or rivaroxaban (0–800 ng/ml) and in the absence or presence of 1.25 IU/ml 4F-PCC (Cofact or Beriplex, approximately 50 IU/kg bw) or 0.25 mg/ml andexanet alfa (approximately 800-mg bolus) are depicted in Figure 2. A gradual lag time prolongation with concomitant reduction in peak thrombin, ETP, and velocity index was observed at increasing concentrations of both apixaban and rivaroxaban. Andexanet alfa at 0.25 mg/ml completely reversed rivaroxaban- and apixaban-induced inhibition of thrombin generation up to 800 ng/ml of the inhibitor. Both PCCs partially corrected thrombin generation lag time over the applied FXaI range. In the absence of FXaI, peak thrombin, ETP, and velocity index was were 2-fold or greater increased by both PCCs, and these thrombin generation parameters gradually decreased with increasing inhibitor concentrations. ETP was still above normal at the 10th–90th percentile of inhibitor levels reported for patients presenting with major bleeding.[6] It should be noted that while 4F-PCCs increased peak thrombin, ETP, and velocity index in the absence of inhibitor, andexanet alfa increased only peak thrombin and velocity index but not the ETP. The increase of peak thrombin and velocity index by 0.25 mg/ml of andexanet alfa was on average 23% and 86%, respectively, and this increase over normal remained in the presence of both apixaban and rivaroxaban up to 800 ng/mL of the inhibitor.

3.2 | FXaI reversal evaluated with the fibrin generation–clot lysis test

Representative fibrin generation–clot lysis profiles obtained in the absence or presence of 200 ng/ml of apixaban or rivaroxaban, with or without reversal agent, being either andexanet alfa (0.25 mg/ml, 800-mg bolus) or one of the 4F-PCCs Cofact or Beriplex (1.25 IU/ml, 50 IU/kg bw), are shown in Figure 3. Both apixaban and rivaroxaban caused a significant delay in fibrin clot formation, a condition that

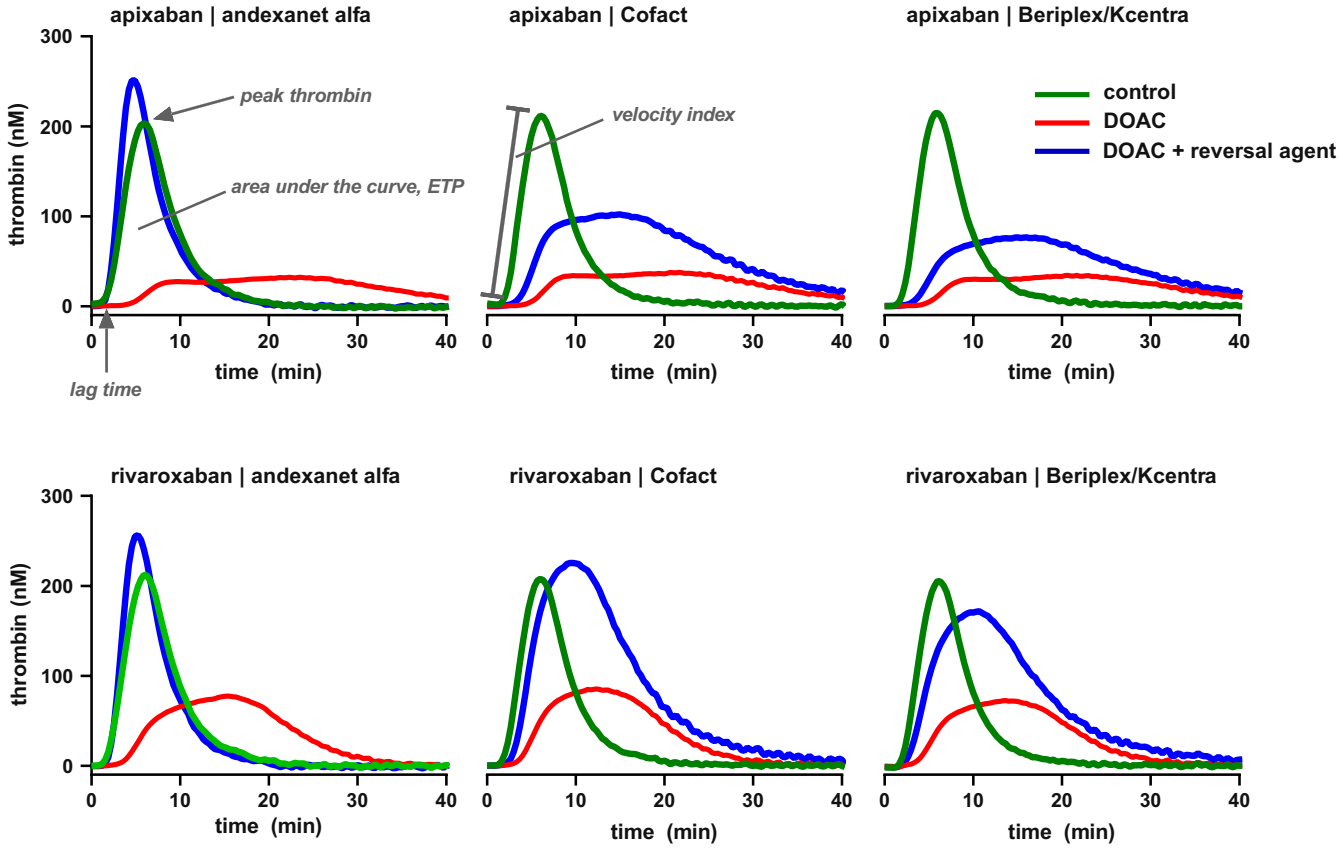

FIGURE 1 Representative thrombin generation curves obtained in the absence or presence of 200 ng/ml of DOAC with or without reversal agent, being either 0.25 mg/ml andexanet alfa or 1.25 IU/ml 4F-PCC. Derived parameters lag time, peak thrombin, and ETP are indicated in the upper left figure for the tracing with plasma without DOAC or reversal agent (control: no factor Xa inhibitor and no reversal agent added). For the derived velocity index, this is indicated in the upper middle panel. 4F-PCC, four-factor prothrombin complex concentrate; DOAC, direct oral anticoagulant; ETP, endogenous thrombin potential

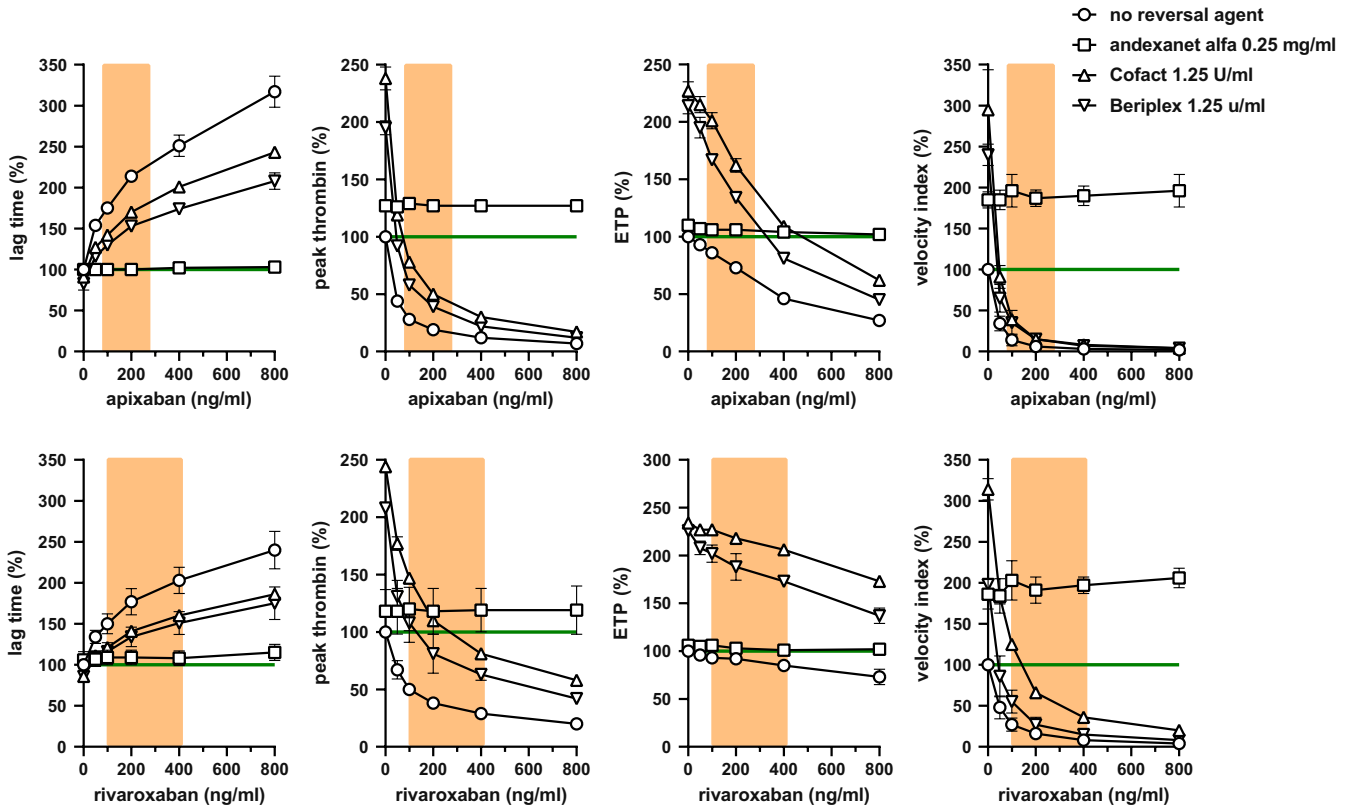

FIGURE 2 Thrombin generation parameters obtained at 0–800 ng/ml apixaban or rivaroxaban and in the absence or presence of 1.25 IU/ml of 4F-PCC (Cofact or Beriplex) or 0.25 mg/ml of andexanet alfa. Data are presented as percentage of control (no DOAC and no reversal agent) and represent the means ± SD of three separate DOAC titration experiments for each of the reversal agents. Horizontal green line: 100% control. Orange bar: 10th–90th percentile of rivaroxaban or apixaban levels in patients presenting with major bleeding.[6] 4F-PCC, four-factor prothrombin complex concentrate; DOAC, direct oral anticoagulant; ETP, endogenous thrombin potential; SD, standard deviation

was almost completely normalized when andexanet alfa was also present. Both PCCs only caused a partial correction of the by the FXaI prolonged CT. Important observation with 4F-PCCs, and especially with Cofact, is the increase in LT and consequently CLT.

Fibrin generation–clot lysis parameters CT and CLT obtained at increasing dose apixaban or rivaroxaban (0–800 ng/ml) and in the absence or presence of 1.25 IU/ml 4F-PCC (Cofact or Beriplex, 50 IU/kg bw) or 0.25 mg/ml andexanet alfa (800 mg bolus) are depicted in Figure 4. Both apixaban and rivaroxaban caused a dose-dependent increase in CT. Andexanet alfa almost completely normalized the CT in the presence of apixaban or rivaroxaban while CT correction was only partly with either PCC. Resistance of the fibrin clot to lysis by t-PA, as reflected by the parameter CLT, was somewhat reduced by both apixaban or rivaroxaban in a dose-dependent manner. In the absence of a factor Xa inhibitor, CLT was increased in the presence of 1.25 IU/ml (50 IU/kg bw) Cofact or Beriplex by 35% and 19%, respectively. CLT in the presence of Cofact remained high in the presence of apixaban or rivaroxaban levels in the 10th–90th percentile inhibitor-level range reported for patients presenting with major bleeding,[6] while overstabilization of the fibrin clot by Beriplex was rapidly lost at increasing inhibitor levels. Andexanet alfa did not cause an increase in clot resistance to t-PA in the absence of FXaI, but andexanet alfa was able to normalize decreased CLT, especially as seen at high apixaban levels.

3.3 | Dosing-dependent reversal of FXaIs

In addition to the aforementioned 0.250 mg/ml (6 μM, approximately 800-mg bolus) of andexanet alfa, we also examined two additional doses: 0.125 mg/ml (3 μM, approximately 400-mg bolus) and 0.500 mg/ml (12 μM, approximately 1600-mg bolus). At all three andexanet alfa doses, we observed a complete to near complete normalization of all thrombin generation parameters as well as CT and CLT up to 800 ng/ml FXaI (Figures S1–S4, Tables S1 and S2). In Figure 5, results obtained in the presence of 200 ng/ml of apixaban or rivaroxaban are shown. Three observations can be made: The slight increase in peak thrombin and the more prominent increase in velocity index independent of FXaI concentration that seems to disappear at very high andexanet alfa dose, and a slightly prolonged CT at very high andexanet alfa dose that appeared independent of the FXaI concentration (Figure 5, Figures S1–S4, Tables S1 and S2).

We also examined three doses of 4F-PCC for its reversal effectivity: 0.625, 1.25, and 2 IU/ml of plasma. These doses recalculate to 25, 50 (advised dose), and 80 IU/kg bw (see Materials and Methods). A summary of all data obtained is provided as Supporting Information (Figures S1–S4, Tables S3–S6). Peak thrombin, ETP, and CLT data are highlighted in Figure 6. Thrombin generation lag time and CT were only marginally corrected by Cofact or Beriplex/

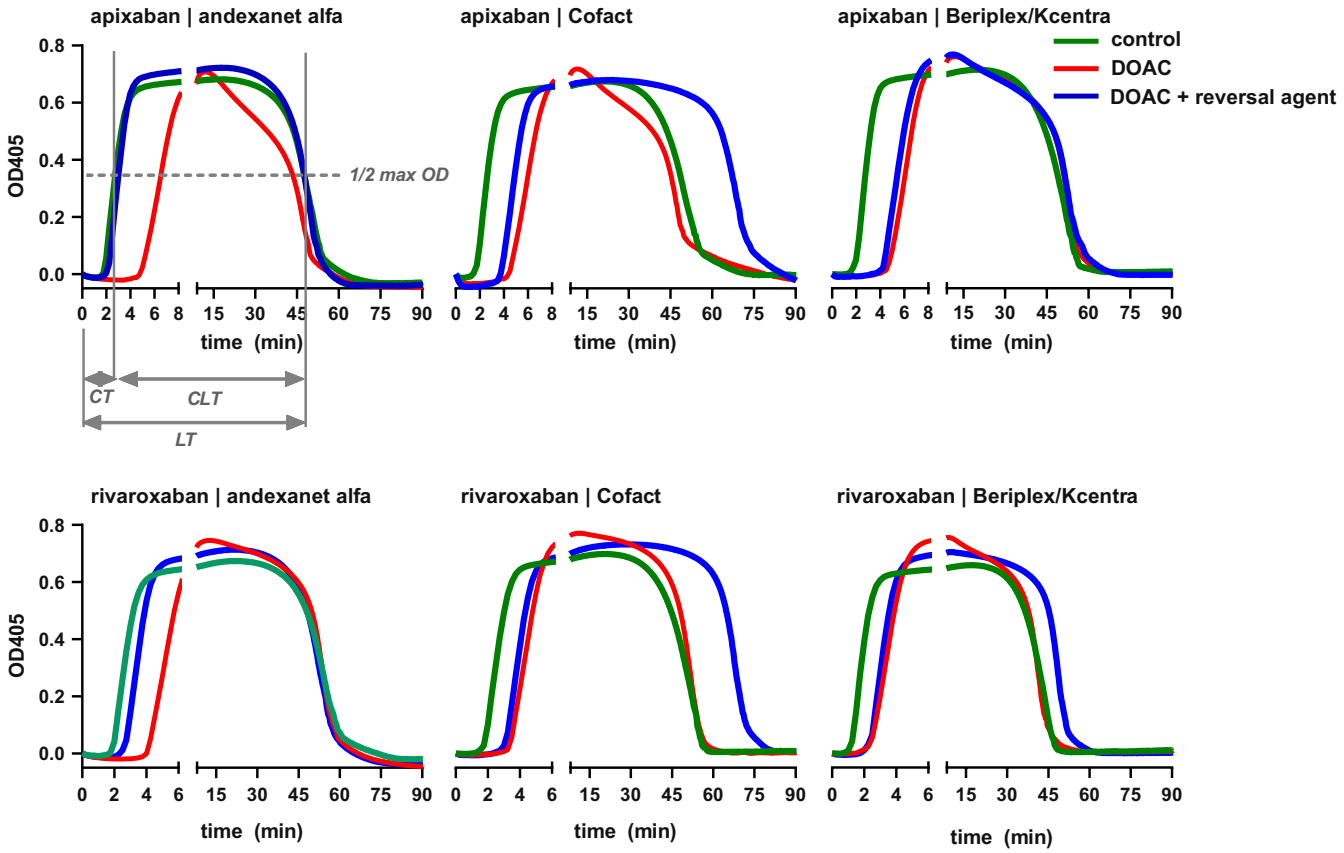

FIGURE 3 Representative fibrin generation-clot lysis curves obtained in the absence or presence of 200 ng/ml DOAC with or without reversal agent, being either 0.25 mg/ml andexanet alfa or 1.25 IU/ml 4F-PCC. Derived parameters CT, LT, and CLT are indicated in the upper left figure for the tracing with plain plasma (control: no factor Xa inhibitor and no reversal agent added). 4F-PCC, four-factor prothrombin complex concentrate; CLT, clot lysis time CT, clotting time; DOAC, direct oral anticoagulant; LT, lysis time

Kcentra, even at the lowest examined FXaI concentration (50 ng/ml) and highest PCC dose (2 IU/ml). Peak thrombin, ETP, and CLT, on the other hand, clearly responded to the addition of PCC (Figures S1–S4, Tables S3–S6). Cofact addition to apixaban anticoagulated plasma resulted in a dose-dependent increase in peak thrombin, ETP and CLT (Figure 6). Peak thrombin normalization was achieved only at a low apixaban concentration of 50 ng/mL and at least 1.25 IU/ml of PCC, or at 100 ng/ml of inhibitor and 2 IU/ml of PCC. ETP and CLT, on the other hand, were normalized or even increased to above normal with 1.25 IU/ml Cofact up to 400 ng/ml FXaI. Beriplex/Kcentra, compared to Cofact, was less effective in reversing apixaban anticoagulation. At 1.25 IU/ml or above, we observed leveling off of the increase in peak thrombin, ETP, and CLT, especially at high inhibitor concentrations. Beriplex/Kcentra even caused a decline in CLT above 100 ng/ml of apixaban. Differences between Cofact and Beriplex/Kcentra were also observed when reversing rivaroxaban anticoagulation, although, due to a less affected ETP and CLT compared to apixaban anticoagulation, differences were less clear. Most striking is the leveling off of the increase in peak thrombin at 1.25 IU/ml Beriplex/Kcentra or higher and the almost absent increase in CLT (Figure 6). The thrombin generation parameter velocity index essentially followed the same pattern as observed for peak thrombin, although the extent by which the velocity index in DOAC-anticoagulated plasma is corrected by 4F-PCC is less (Figures S1 and S3).

4 | DISCUSSION

Prothrombin complex concentrate exhibit similar, or slightly higher rates of hemostatic effectiveness compared to andexanet alfa in the treatment of major bleeding in patients using FXaI.[17,18] Nevertheless, there is still controversy over the usefulness of PCC as a reversal agent for FXaI that originates from in vitro thrombin generation studies. In a recent study by Lu and coworkers,[19] for example, 4F-PCC (Beriplex/Kcentra) was considerably less effective in restoring thrombin generation in apixaban or rivaroxaban-spiked plasma than andexanet alfa. Results from our present in vitro study, on the other hand, not only demonstrate effective reversal of thrombin generation by andexanet alfa but also show considerable improvement of thrombin generation by 4F-PCC (Cofact) up to approximately 400 ng/ml of FXaI compared to less than 75 ng/ml as reported by Lu and coworkers. In addition, we show that 4F-PCC (Cofact) in contrast to andexanet alfa, improves the stability of a clot against fibrinolytic degradation. Finally, we show that in in vitro spiking

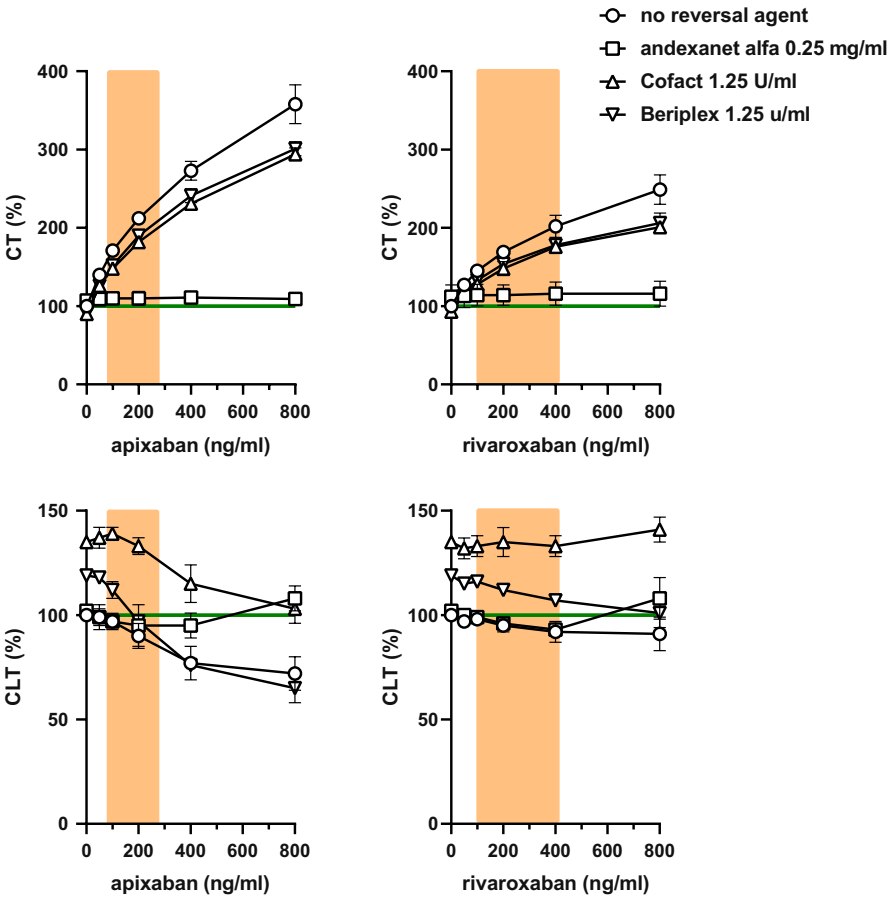

FIGURE 4 Clotting time and clot lysis time data obtained at 0–800 ng/ml apixaban or rivaroxaban and in the absence or presence of 1.25 IU/ml of 4F-PCC (Cofact or Beriplex/Kcentra) or 0.25 mg/ml of andexanet alfa. Data are presented as percentage of control (no DOAC and no reversal agent) and represent the means ± SD of three separate DOAC titration experiments for each of the reversal agents. Horizontal green line: 100% control. Orange bar: 10th-90th percentile of rivaroxaban or apixaban levels in patients presenting with a major bleeding.[6] 4F-PCC, four-factor prothrombin complex concentrate; CLT, clot lysis time CT, clotting time; DOAC, direct oral anticoagulant; SD, standard deviation

experiments Cofact is more effective in correcting FXaI anticoagulation than Beriplex/Kcentra.

A problem with applying the thrombin generation assay for assessing DOAC reversal is the complexity of this assay system and the lack of standardization.[21] The extent by which thrombin generation is inhibited by FXaI and the dose of PCC needed for subsequent normalization is highly dependent on the TF concentration (thrombin generation reagent).[8,20,21] Also, the presence of blood cells and platelets is of influence and whether the pooled normal plasma that is used for experimentation is solvent/detergent-treated or not.[9,20] For the present study, we used the same TF reagent as Lu and coworkers[19] did (PPP-reagent from Thrombinoscope, 5 pM TF final concentration). However, we prepared our pooled normal plasma ourselves from fresh apheresis plasma from the local blood bank, while Lu and co-workers used commercially available pooled platelet-poor plasma. This might have caused discrepancies between the studies in the response pattern of rivaroxaban and apixaban and subsequent reversal effectivity of the used PCC. Composition of PCC may also play a role, especially the heparin content.[22,25,26] Lu and coworkers performed a direct comparison between andexanet alfa and Beriplex. This 4F-PCC is known to contain added heparin. Lu and coworkers treated their PCC with heparinase and reported that similar results were obtained with untreated- and heparinase-treated plasma.[19] Lu and coworkers did not report whether heparin was indeed removed from the PCC by this treatment. In one of our previously published studies on the reversal of oral anticoagulation by 4F-PCC, we performed an in depth analysis on the role of heparin in different PCC brands. We effectively removed heparin from different PCCs using ecteola cellulose. We observed that heparin-containing PCCs severely hampered prohemostatic effects when therapeutic doses were added to anticoagulated plasmas. Upon heparin removal, comparable prohemostatic effects were observed.[22] Our present results showing that Beriplex/Kcentra (containing heparin) is less effective in reversing apixaban or rivaroxaban anticoagulation than Cofact (heparin free) are completely in line with these previously published results. Of importance is the notion that the anticoagulant effect of heparin supplement is additive to that of FXaI.[22] Also worth mentioning in this respect is the observation by Song and coworkers in apixaban-anticoagulated healthy volunteers, that Cofact administration resulted in a considerable higher ETP and peak thrombin than a similar dose of Beriplex/Kcentra.[14]

In a recent study in real-life patients on FXaI medication and presenting with a major bleeding, ETP was in the lower normal range and increased 2.5-fold to above normal 15–30 min after administration of Cofact with a concomitant increase in clot stability against fibrinolytic breakdown (CLT).[16] In our present in vitro study with normal plasma spiked with apixaban or rivaroxaban, we also observed a Cofact dose-dependent increase in CLT to above normal. This was

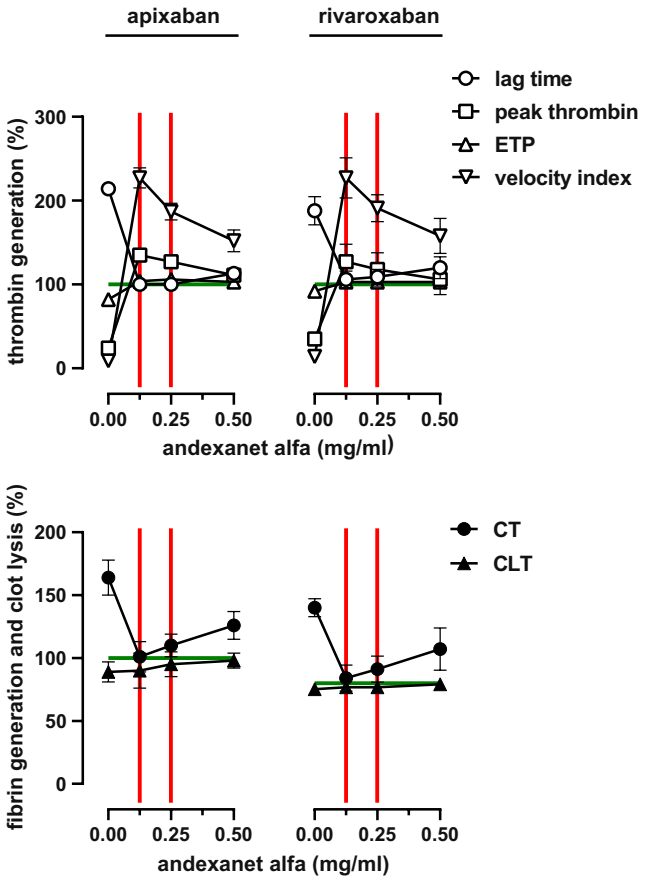

FIGURE 5 Thrombin generation parameters, CT and CLT at 200 ng/ml of apixaban or rivaroxaban and increasing andexanet alfa dose (0–0.5 mg/ml). Data are presented as percentage of control (no DOAC and no reversal agent) and represent the means ± SD of three separately performed experiments. Horizontal green line: 100% control. Vertical red lines: andexanet alfa doses corresponding to approximately 400-mg and approximately 800-mg bolus. CLT, clot lysis time CT, clotting time; DOAC, direct oral anticoagulant; ETP, endogenous thrombin potential; SD, standard deviation

not observed with Beriplex/Kcentra. With Beriplex/Kcentra we observed CLT inhibition, especially at high doses of both Beriplex/Kcentra and FXaI. It is very likely that this relates to heparin in Beriplex/Kcentra. The improved clot stability with Cofact was also not observed with andexanet alfa. The increase in CLT that we observed in our in vitro experiments coincides with an increase in ETP. This is not an unexpected observation when considering the importance of generated thrombin levels on clot structure.[27] Clot stability is a direct determinant of clinical outcome as evidenced by the successful use of antifibrinolytic agents in the treatment of bleeding disorders.[28,29]

Limitation of our present study is its in vitro nature as it remains to be established whether and how our in vitro results translate to correction of bleeding in patients on FXaI medication. Results from a recent study in real-life patients on FXaI medication and presenting with major bleeding do not show a relationship between outcome of coagulation tests (thrombin generation assay, fibrin generation–clot lysis test) and correction of bleeding after PCC treatment.[16] In healthy volunteers anticoagulated with rivaroxaban and treated with PCC, a correlation between thrombin generation and outcome of a punch biopsy bleeding test was also absent.[11] In contrast, a correlation between thrombin generation improvement and bleeding correction was evident in a similar study with edoxaban-anticoagulated individuals.[15] Also, in an animal bleeding model of rivaroxaban-treated pigs that underwent complex traumatic injury followed by PCC treatment, correction of hemostasis coincided with improvement of thrombin generation.[30] In the same polytrauma model, andexanet alfa rapidly restored thrombin generation and significantly reduced total blood loss in apixaban-treated animals.[31] We observed in our in vitro experiments that andexanet alfa increased peak thrombin and velocity index to above normal, irrespective of the presence of FXaI. A similar increase in peak thrombin by andexanet alfa was observed by Lu and coworkers.[19] This increase in peak thrombin and velocity index may be caused by residual thrombin-generating capacity of the FXa mutant or by neutralizing natural coagulation inhibitors including tissue factor pathway inhibitor (TFPI).[32] It has been shown that andexanet alfa can bind and sequester TFPI tightly as a result of its similarity to FXa.[33] At present, it is unclear whether this increase in peak thrombin relates to increased thrombotic risk associated with andexanet alfa compared to PCC as observed in some but not all clinical studies.[17,18]

The off-label use of PCC for FXaI reversal is endorsed by the Dutch medical community and may not be comparable to other countries. Considerable cost differences and the complicated dosing algorithm limit the use of andexanet alfa.[34] The present in vitro study clearly indicates that 4F-PCC like andexanet alfa is able to increase thrombin generation up to 800 ng/mL of FXaI. While andexanet alfa corrects all thrombin generation parameters, 4F-PCC predominantly increases peak thrombin and ETP. In addition, 4F-PCC, especially Cofact but not andexanet alfa, improves clot stability against fibrinolytic breakdown. FXaI anticoagulation reversal assessment by in vitro tests may be hampered by heparin in Beriplex/Kcentra. Whether heparin supplement to 4F-PCC counteracts its prohemostatic effectivity in bleeding patients on FXaI remains to be investigated.

AUTHOR CONTRIBUTIONS

HJMB conceived, designed, and supervised the project, analyzed and interpreted data, and wrote the first draft of the manuscript. MZ designed and performed experiments and acquired and analyzed data. JCMM conceived, designed, and supervised the project, analyzed and interpreted data, and edited the manuscript. All authors read and approved the final manuscript.

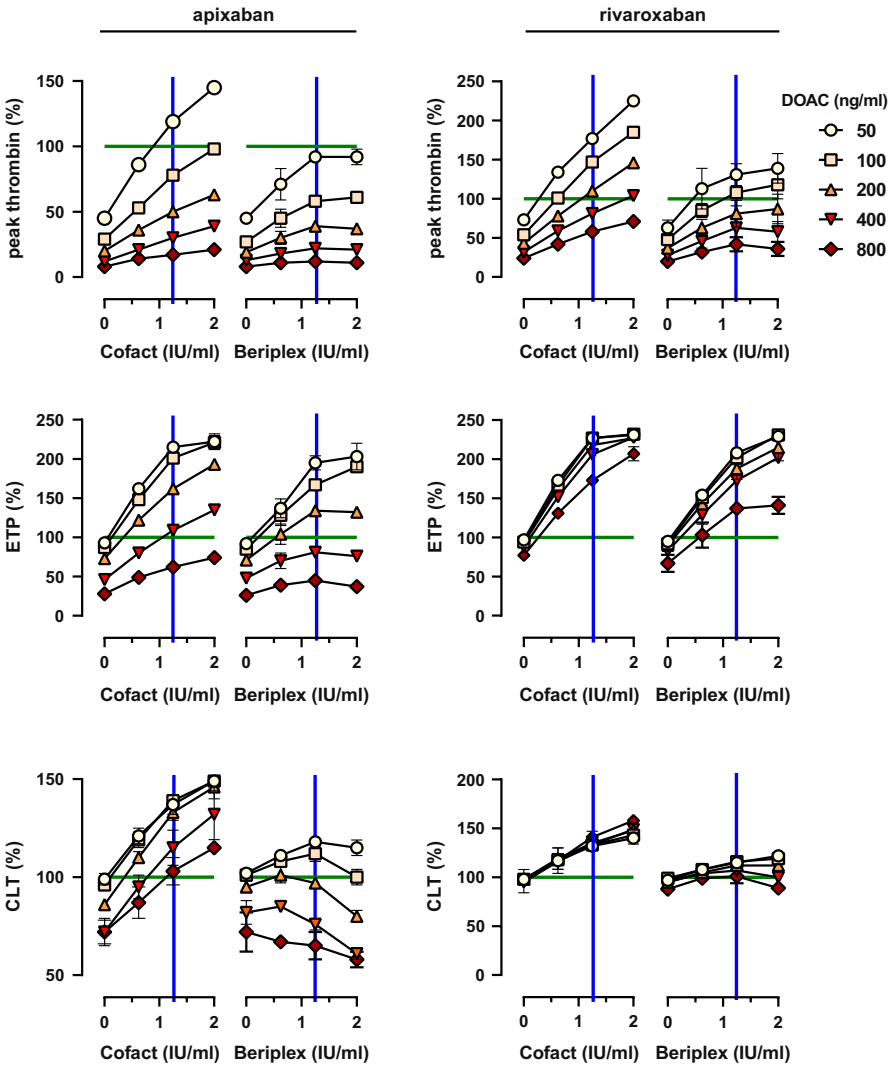

FIGURE 6 Peak thrombin, ETP, and CLT data obtained at 0–800 ng/ml of apixaban or rivaroxaban and increasing dose of 4F-PCC (Cofact or Beriplex/Kcentra). Data are presented as percentage of control (no DOAC and no reversal agent) and represent the means ± SD of three separately performed experiments. Horizontal green line: 100% control. Vertical blue line: advised 4F-PCC dose of 1.25 IU/ml (50 U/kg bw). 4F-PCC, four-factor prothrombin complex concentrate; bw, bodyweight; CLT, clot lysis time CT, clotting time; DOAC, direct oral anticoagulant; ETP, endogenous thrombin potential; SD, standard deviation

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RELATIONSHIP DISCLOSURE

HJMB, MZ, and JCMM are employed by Sanquin, until 2020 manufacturer of 4F-PCC (Cofact).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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