

ORIGINAL ARTICLE

Reversing direct factor Xa or thrombin inhibitors: Factor V addition to prothrombin complex concentrate is beneficial in vitro

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Abstract

Background: Prothrombin complex concentrate (PCC) is a human plasma-derived mixture of partially purified vitamin K-dependent coagulation factors (VKCF). Current therapeutic indication is treatment and perioperative prophylaxis of bleeding in acquired VKCF deficiency. Off-label uses include treatment of direct factor Xa- or thrombin inhibitor-associated bleeds, treatment of trauma-induced coagulopathy, and hemorrhagic complications in patients with liver disease.

Objective: Considering PCC as a general prohemostatic drug, we argued that its clinical efficacy can benefit from supplementation with coagulation factors that are absent in the current PCC formulation. In this study, we focused on factor V.

Methods: We mimicked a coagulopathy in vitro by spiking whole blood or derived plasma with the direct oral anticoagulants (DOAC) rivaroxaban or dabigatran. We studied DOAC reversal by PCC and factor V concentrate (FVC) using a thrombin generation assay, thromboelastography, fibrin generation clot lysis test, and microfluidic thrombus formation under flow.

Results: In DOAC-treated plasma, PCC increased the amount of thrombin generated. The addition of FVC alone or in combination with PCC caused a partial correction of the thrombin generation lag time and clotting time. In DOAC-treated whole blood, the combination of PCC and FVC synergistically improved clotting time under static conditions, whereas complete correction of fibrin formation was observed under flow. Clot strength and clot resistance toward tissue plasminogen activator-induced lysis were both increased with PCC and further enhanced by additional FVC.

Conclusion: Our in vitro study demonstrates a beneficial effect of the combined use of PCC and FVC in DOAC reversal.

KEYWORDS

anticoagulants, dabigatran, factor V, prothrombin complex concentrate, rivaroxaban

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Essentials

- Anticoagulants like rivaroxaban and dabigatran inhibit blood clotting but increase bleeding risk.
- Prothrombin complex concentrate (PCC) can be used as treatment when bleeding occurs.
- PCC is rich in vitamin K-dependent clotting factors but is devoid of clotting factor V (FV).
- This laboratory study shows that FV addition improves the effectivity of PCC to correct clotting.

1 | INTRODUCTION

Four-factor prothrombin complex concentrate (PCC) is a human plasma purified mixture of vitamin K-dependent coagulation factors (VKCFs) including the procoagulant factors II, VII, IX, and X and the anticoagulant proteins C and S. PCCs may contain copurified plasma constituents such as fibrinogen, vitronectin, inter-alpha-trypsin inhibitor, complement factors, albumin, ceruloplasmin, C4b-binding protein, and apolipoprotein A.¹ In addition, PCC preparations may contain added heparin and antithrombin.²⁻⁴ Presently, PCC is indicated for the treatment and perioperative prophylaxis of bleeding under conditions of acquired VKCF deficiency, such as a deficiency caused by treatment with vitamin K antagonists or in congenital VKCF deficiency, provided that purified coagulation products are not available.⁵ The off-label use of PCC includes reversal of anticoagulation resulting from direct factor (F) Xa- or thrombin-inhibiting oral anticoagulants (DOACs),^{6,7} treatment of trauma-induced coagulopathy and coagulopathy following surgery,^{8,9} and treatment of bleeds in patients with liver diseases¹⁰ and in infants after cardiopulmonary bypass.¹¹

Because of its enrichment in procoagulant vitamin K-dependent coagulation proteins, PCC appears to be more effective than fresh frozen plasma for emergency treatment of bleeds that are caused by acquired VKCF deficiency.^{12,13} It is therefore reasonable to assume also that clotting factor deficiencies after perioperative or traumatic bleeds can be treated with a PCC formula. A recently published systemic review and meta-analysis showed that the administration of PCC was associated with a reduced blood loss in cardiac surgery, and that PCC in addition to fresh frozen plasma reduced the mortality in trauma patients.¹⁴ The latter conclusion is in line with the observation made in an experimental porcine hemorrhagic shock model that plasma coadministration improved the animal resuscitation with PCC.¹⁵ In a recent *in vitro* study, we compared various resuscitation approaches upon rivaroxaban anticoagulation.¹⁶ It appeared that the dilution of plasma with saline or albumin induced a profibrinolytic state and deteriorated the impaired coagulation potential with rivaroxaban, even in the presence of PCC and fibrinogen. The combined use of plasma and PCC, on the other hand, improved coagulation and clot stability.¹⁶ This suggested that PCC preparations are lacking one or more plasma components other than fibrinogen to be fully effective.

The common concept behind using PCC as a reversal agent for DOACs is that by increasing the VKCF concentration, it pushes the coagulation potential over a certain inhibition threshold.¹⁷ That PCC administration is able to correct DOAC-associated bleeds has been convincingly demonstrated in animal models.¹⁸⁻²¹

Because of ethical reasons, placebo-controlled clinical trials on the efficacy of PCC as a reversal agent for DOAC-associated bleeds have not been performed in patients. On the other hand, two placebo-controlled punch biopsy bleeding studies in healthy volunteers on the reversal of the FXa inhibitors rivaroxaban or edoxaban by PCC gave conflicting results.^{22,23} The laboratory parameter endogenous thrombin potential (ETP), however, consistently improved after PCC supplementation to patients or healthy volunteers receiving direct FXa inhibitors.²²⁻²⁸ *In vitro* studies with DOAC-anticoagulated plasma samples revealed that not all thrombin generation parameters respond equally well to PCC addition. Although the ETP showed complete reversal of any DOAC by PCC, the thrombin peak did not normalize with PCC for all DOACs and the lag time in general was only partly corrected. Laboratory assays to monitor fibrin clot formation (prothrombin time, thromboelastography) also showed a partial correction of the delayed clotting.²⁹ The observation that not all coagulation parameters were normalized by PCC suggests a requirement for additional clotting factor support. In other words, PCC may be partly effective in treating complex coagulopathic conditions and may in particular be suboptimal for anticoagulation reversal in DOAC-treated patients.

Formulas of PCC do not contain a substantial amount of coagulation FV. In agreement with this, the plasma FV level in patients does not increase upon PCC infusion.³⁰ Activation of FV is essential in initiating the coagulation system.^{31,32} Activated FV is fundamental in coagulation as a cofactor for activated FX (FXa) in the conversion of prothrombin to thrombin, the key enzyme in the formation of a fibrin clot. On the other hand, FV can serve as a cofactor for tissue factor pathway inhibitor- α and activated protein C in the downregulation of thrombin formation.³³ A recent study pointed to the potential clinical applicability of plasma derived FV concentrate (FVC) for the correction of FV deficiency-related coagulopathies.³⁴ The aim of our *in vitro* study was to investigate whether the combined use of PCC and FVC is superior to PCC alone in the reversal of DOAC anticoagulation.

2 | MATERIALS AND METHODS

2.1 | Clotting factor concentrates and direct oral anticoagulants

Factor V concentrate was prepared from pooled normal human plasma by immunoaffinity and ion-exchange chromatography, as described.³⁵ A minor modification was that CaCl₂ in the final

dialysis step was omitted to rule out any coagulation activation when preparing FVC-spiked plasma or blood samples. Freeze-dried prothrombin complex concentrate (PCC, Cofact) came from Sanquin (Amsterdam, The Netherlands) and was prepared as a four-fold concentrated solution in citrate-buffered saline, as described.³⁶ PCC dosing was based on FIX international activity units (U).⁵ Relative content of the other vitamin K-dependent coagulation factors (U/U FIX) in the used PCC batch was: FII 0.99, FVII 0.51, FX 0.86, protein S 0.16, and protein C 0.88. For FV, this was <0.01. Validated assays were used to measure coagulation proteins in PCC. FII and FX chromogenic activity was determined with reference concentrate calibrated to the appropriate World Health Organization concentrate standard. Factor VII (chromogenic assay) and FIX (clotting assay) were determined against European Pharmacopoeia reference standards for FVII or FIX concentrates. FV (clotting assay), protein C (chromogenic assay), and total protein S antigen were determined with reference plasma calibrated to the appropriate World Health Organization plasma standard. Cofact contains antithrombin but lacks heparin.^{3,4} Rivaroxaban was a gift from Bayer HealthCare (Leverkusen, Germany).³⁶ Dabigatran was a gift from Dr. Harenberg (Clinical Pharmacology Mannheim, Ruprecht-Karls University, Heidelberg, Germany).³⁷

2.2 | Preparation of blood and plasma samples

Venous blood for thromboelastography and flow experiments was obtained from healthy volunteers after full informed consent, in accordance with the Declaration of Helsinki. Blood samples were collected in 1:10 (v/v) 3.2% trisodium citrate tubes. The first 3 ml of blood were discarded. Citrated whole blood was kept at room temperature and used for further experimentation within 5 h after collection. Normal plasma consisted of a pool of 32 single-donor plasmapheresis units (Sanquin) and was stored in aliquots at -80°C until further use. Immediately before analysis, citrated blood or plasma aliquots were spiked with DOAC (i.e., rivaroxaban [reversible FXa inhibitor], dabigatran [reversible thrombin inhibitor]), and/or with PCC, FVC, or solvent, as outlined in the text. For most experiments, we used a DOAC concentration of 300 ng/ml, which is in the upper range of peak concentrations measured in clinical samples.³⁸ We used this high DOAC dose to anticipate clinical situations in which bleeding patients are admitted to the hospital just after their last drug intake. In some experiments, we lowered the DOAC concentration to 50 ng/ml, which is in the lower clinical range. The advised PCC dose for DOAC reversal is 50 U per kg body weight (bw).³⁹ In the present in vitro experiments, we therefore used PCC at 0.25–2 U/ml, which recalculates to 10–80 IU per kg bw (assuming a plasma volume of 40 ml per kg bw). To provide a proof-of-concept, the dosing of FVC was in the same (reversal) range as was applied for PCC. The combination of PCC and FVC was consistently performed at a 1:1 ratio based on factor units.

2.3 | Thrombin generation assay

Thrombin generation was assessed by calibrated automated thrombography (CAT, Thrombinoscope, Maastricht, The Netherlands) on a Fluoroskan Ascent microplate reader (Thermo Scientific) using premixed phospholipid-tissue factor (TF) and calibrator reagents (Thrombinoscope). According to the manufacturer's instructions, the final plasma concentration was 67%. We lowered the final plasma concentration to 50% v/v, as described previously, enabling the addition of coagulation inhibitors and clotting factor concentrate.³⁶ Final concentrations of TF, phospholipids, and CaCl_2 were 5 pM, 4 μM , and 15 mM, respectively. The final concentration of the thrombin substrate z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland) was 0.5 mM. Parameters obtained were lag time, thrombin peak, and area under the curve (ETP).⁴⁰

2.4 | Fibrin generation and clot lysis by optical density measurement

A cell-free fibrin generation-clot lysis test was performed as described by Bakhtiari et al.⁴¹ In this assay, the final plasma concentration was set at 50% v/v. The remaining 50% was provided by Hepes buffered saline containing additives of interest (DOAC, clotting factor concentrate) and start reagents (TF, phospholipids, tissue plasminogen activator [tPA], and CaCl_2) at final concentrations of 0.5 pM, 4 μM , 50 ng/ml, and 15 mM, respectively. TF (Innovin) was from Dade Behring (Deerfield, IL, USA) and tPA (Actilyse) was from Boehringer Ingelheim GmbH (Ingelheim, Germany). Turbidity measurement was at 405 nm for 3 h. Clotting time (CT) was defined as the time to midpoint of the clear to maximum turbidity. Clot lysis time (CLT) was defined as the time from the midpoint of the clear to maximum turbid transition that characterizes clot formation, to the midpoint of the maximum turbid to clear transition, which represents clot lysis. To obtain reliable CT data, the used TF concentration was 10-fold lower compared with what was used in the CAT test. The tPA concentration used allowed completion of the test within 3 h.

2.5 | Thromboelastography

Thromboelastography (TEG) was performed using TEG 5000 equipment according to the manufacturer's description (Haemonetics Corporation, Braintree, MA, USA). For whole blood thromboelastography, citrated blood samples were spiked with FXa or thrombin inhibitor, clotting factor concentrate (PCC, FVC), or solvent. Other additives were TF and tPA, which were added just before the start of the measurement to a final concentration of 1 pM and 200 ng/ml, respectively. Additives accounted for 9% v/v of the final blood sample. A prepared sample (340 μl) was transferred into a TEG cup, and measurements were started directly after adding 20 μl of 0.2 mM CaCl_2 . Derived parameters were reaction time R (equivalent to CT), maximal amplitude (MA), and CLT (elapsed time between MA and

2-mm amplitude post-MA).⁴² Used concentrations of TF and tPA allowed completion of the test within 90 min under all experimental conditions.

2.6 | Determination of platelet-fibrin thrombi formation under flow

Blood perfusion was performed in a parallel-plate flow chamber at a wall shear rate of 1000 s^{-1} for 10 min as previously described.⁴³ Citrated blood samples spiked with DOAC, clotting factor concentrate, or solvent also contained Alexa Fluor (AF)568-conjugated annexin A5 (Fisher Scientific, Landsmeer, The Netherlands) to detect the phosphatidylserine (PS) exposure on activated platelets and AF647-conjugated fibrinogen (Molecular Probes-Life Technologies, New York, NY, USA) to detect fibrin(ogen). These additives accounted for 4% v/v of the final blood sample. Prepared samples were mixed with Hepes-buffered saline containing 63 mM CaCl_2 and 32 mM MgCl_2 in a 10:1 volume ratio at the inlet of the flow chamber and perfused over two procoagulant microspots. The proximal microspot only contained 50 $\mu\text{g/ml}$ (2 μl) Horm type I collagen (Nycomed Pharma, Munich, Germany), the distal microspot additionally contained 500 pM (2 μl) recombinant human TF. The TF level in our flow assay (applied as dried micro spot) cannot be compared with that in CAT, TEG, and fibrin generation-clot lysis test (TF in solution). During blood perfusion, brightfield and two-color fluorescence images were recorded every 2 min using two equivalent EVOS-FL microscopes (Life Technologies, Bleiswijk, The Netherlands), equipped with red fluorescent protein and Cy5 light-emitting diodes and dichroic cubes, an Olympus UPLSAPO 60x oil-immersion objective, and a sensitive CCD camera (1360×1024 pixels). Duplicate runs were performed simultaneously by two laboratory workers at standard settings of illumination and camera chip sensitivity. Time to fibrin formation was determined by visual inspection. Image analysis was performed using semiautomated scripts in the open-access Fiji image analysis software and by visual scoring of unprocessed brightfield and fluorescence images. The analysis of the images provided five outcome parameters: V1, platelet surface area coverage (% SAC); V2, PS exposure (% SAC); V3, fibrin formation (% SAC); V4, thrombus morphological score (scale 0–5; 0, no or few adhered platelets; 1, multiple single adhered platelets; 2, extensive coverage of single adhered platelets; 3, small platelet aggregates; 4, medium-size aggregates and thrombi; 5, large aggregates and thrombi); and V5, fibrin score (scale 0–3; 0, no fibrin; 1, minor fibrin formation; 2, considerable fibrin formation; 3, complete area covered with fibrin).^{44,45}

2.7 | Statistical analysis

Statistical significance of differences between two independent groups was determined using the Mann-Whitney test. Multiple comparison between the mean of matched groups showing a normal distribution was according to the one-way ANOVA with post-hoc

Dunnnett multiple comparisons test. Differences with p values <0.05 were considered significant.

3 | RESULTS

3.1 | Factor V supplementation supports the correction of DOAC-inhibited thrombin generation in plasma by PCC

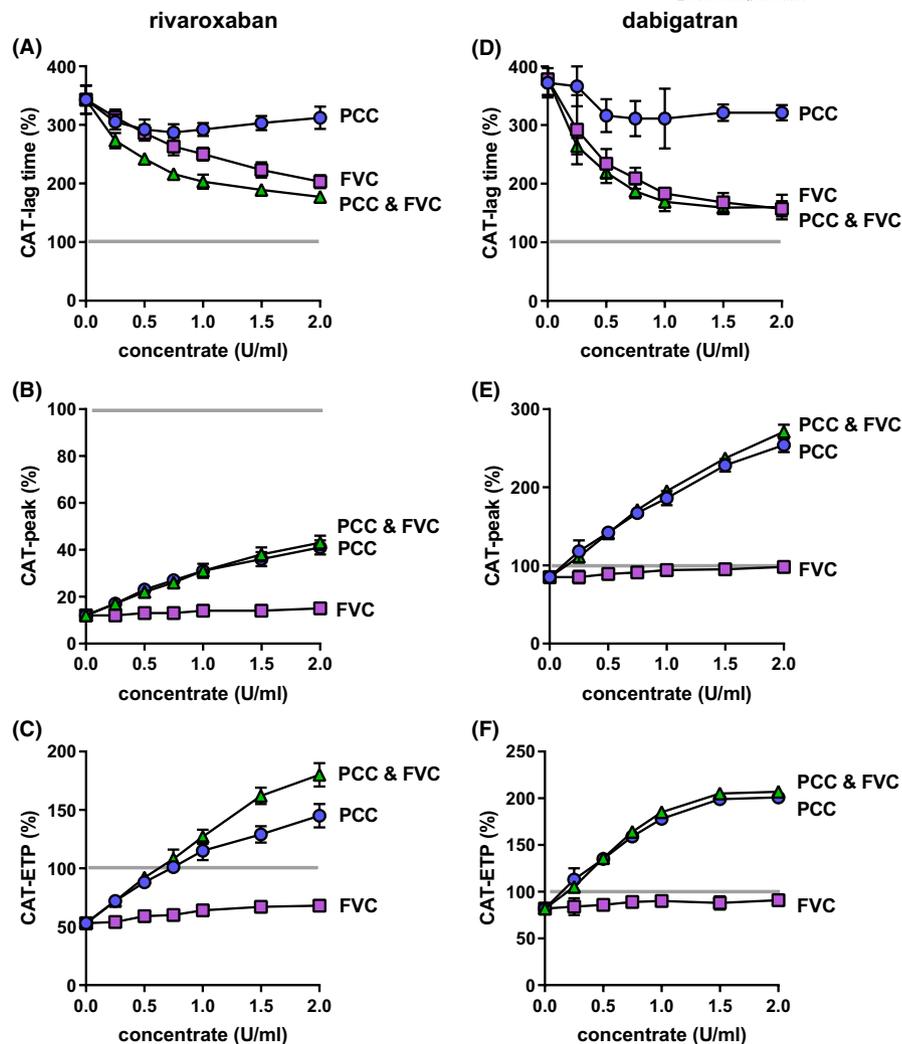
The thrombin generation assay is a generally accepted laboratory method to assess the reversal of DOAC anticoagulation.^{5,29} As a first approach to study this reversal, normal plasma was treated with rivaroxaban to prolong the thrombin generation lag time to 340% (Figure 1A) and to reduce thrombin peak (Figure 1B) and ETP level (Figure 1C) to 12% and 53%, respectively. In a dose-dependent manner, the supplementation of PCC to this rivaroxaban-treated plasma restored the ETP (Figure 1C) and improved the thrombin peak level (Figure 1B), whereas it hardly affected the lag time (Figure 1A). The addition of FVC, on the other hand, improved the lag time (Figure 1A), but not the thrombin peak and ETP level (Figure 1B,C). A synergistic effect of PCC and FVC, when applied at a 1:1 ratio to rivaroxaban-anticoagulated plasma, was only observed for the lag time and the ETP (Figure 1A,C), not for the thrombin peak (Figure 1B). At 1 U/ml PCC and FVC supplementation, a near-maximal lag time reduction was observed with complete normalization of the ETP.

In another approach, normal plasma was treated with 300 ng/ml dabigatran, an anticoagulant that predominantly affected the thrombin generation lag time (Figure 1D) with minimal effects on thrombin peak and ETP levels (Figure 1E,F). The lag time in dabigatran-anticoagulated plasma was prolonged to 370% and it only marginally improved by PCC, whereas a clear dose-dependent correction was observed with FVC, either alone or in combination with PCC at a 1:1 ratio (Figure 1D). Maximal effects were observed at a FVC dosing of ≥ 1 U/ml. By itself, FVC had no effect on the thrombin peak or ETP in dabigatran-anticoagulated plasma, whereas PCC, either alone or in combination with FVC in a 1:1 ratio, increased the thrombin peak and ETP to above normal values (Figure 1E,F).

3.2 | Factor V supplementation supports the correction of DOAC-inhibited fibrin generation and clot stability in plasma by PCC

The plasma CT is obtained from a traditional clotting assay, such as the fibrin generation clot lysis test. The CT (similarly to the lag time of thrombin generation measurements) was prolonged in DOAC-anticoagulated plasma (Figure 2A,C). Again, the plasma was treated with rivaroxaban or dabigatran at a concentration of 300 ng/ml, which resulted in a 3.8- and 8-fold CT prolongation, respectively. The subsequent supplementation of FVC and PCC at a 1:1 ratio or FVC alone synergistically improved CT, whereas PCC alone had a much lower corrective effect, regardless of the DOAC

FIGURE 1 Correction of thrombin generation in DOAC-anticoagulated plasma by PCC, FVC, or PCC+FVC. Normal plasma was spiked with 300 ng/ml rivaroxaban (A–C) or dabigatran (D–F). Samples were supplemented with different concentrations of PCC, FVC, or PCC+FVC. Samples were subjected to CAT. Data are presented as % of control and represent the means \pm SD of three separately performed experiments with the same normal plasma pool. Gray horizontal line: 100% control. The y-axis scaling of panels B and E (CAT-peak) and panels C and F (CAT-ETP) were adjusted to achieve the best possible resolution of the data. CAT, calibrated automated thrombography; DOAC, direct oral anticoagulant; ETP, endogenous thrombin potential; FVC, factor V concentrate; PCC, prothrombin complex concentrate



used (Figure 2A,C). A maximal CT correction in rivaroxaban- or dabigatran-anticoagulated plasma was observed with PCC+FVC at doses of ≥ 1 and ≥ 1.5 U/ml, respectively.

Treatment with DOAC may reduce clot stability, a condition known to be reversed by PCC.¹⁶ We indeed found that the clot stability was reduced in rivaroxaban- or dabigatran-anticoagulated plasma samples, as illustrated by a shortened CLT in the fibrin generation clot lysis test (Figure 2B,D). Supplementation of FVC and PCC synergistically improved the CLT to above-normal values in either rivaroxaban- or dabigatran-anticoagulated plasma. Complete correction of clot stability was observed at 1 U/ml PCC+FVC for both types of DOAC. Supplementation of FVC alone did not have a corrective effect on CLT in rivaroxaban plasma (Figure 2B), whereas it corrected the CLT in dabigatran plasma (Figure 2D).

3.3 | Factor V addition supports the correction of clot formation and stability by PCC in DOAC-treated whole blood

The TEG method, in contrast to the conventional thrombin generation or fibrin generation clot lysis assays, can be carried out in

recalcified citrated whole blood. Based on the experimental results so far, we performed TEG studies with 300 ng/ml DOAC and 1 U/ml PCC and/or 1 U/ml FVC. Figure 3 shows representative TEG traces of effects of PCC and FVC, alone or in combination, to whole blood samples treated with rivaroxaban (Figure 3A) or dabigatran (Figure 3E). The treatment of blood with either rivaroxaban or dabigatran resulted in a 2- to 3.5-fold prolongation of the time to clot formation (reaction time, R) (Figure 3B,F) and a 30%–45% reduced clot strength (maximal amplitude, MA) (Figure 3C,G). The reduction of clot stability (CLT) was evident, but less pronounced (Figure 3D,H). Added PCC or FVC separately showed a partial correction of R and MA, whereas the effect was more evident for the combination of PCC and FVC (Figure 3B,C,F,G). Addition of PCC alone or in combination with FVC, but not FVC alone, increased clot stability to above normal (Figure 3D,H).

3.4 | Factor V with PCC corrects the anticoagulant effects of DOAC inhibition in whole blood under flow

The thrombus forming and coagulant potential of blood to fibrin clot formation under flow can be accurately studied in vitro by perfusion

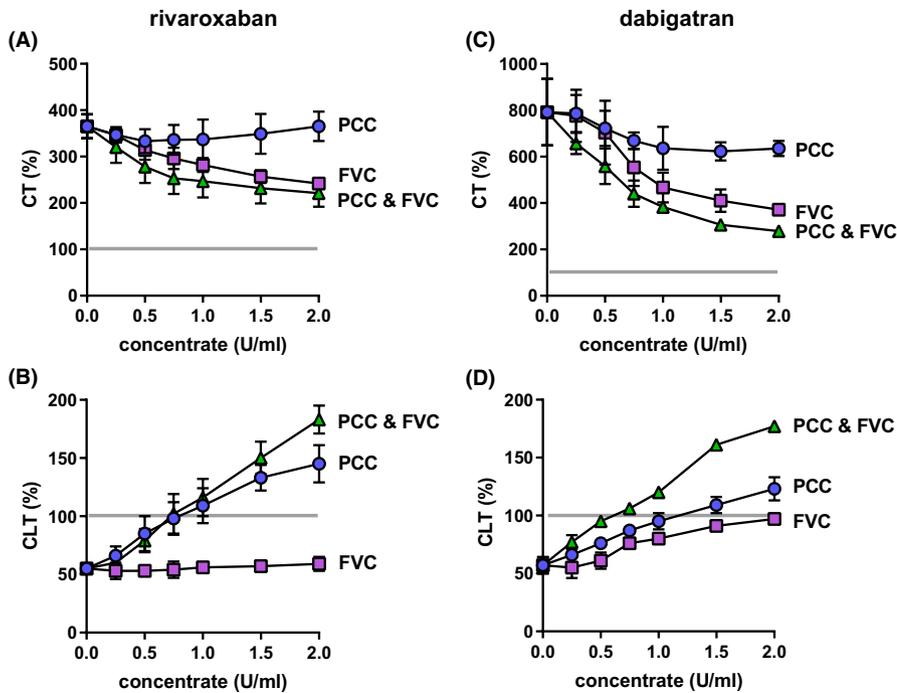


FIGURE 2 Correction of clot formation and lysis in DOAC-anticoagulated plasma by PCC, FVC, or PCC+FVC. Normal plasma was spiked with 300 ng/ml rivaroxaban (A, B) or dabigatran (C, D). Samples were supplemented with different concentrations PCC, FVC, or PCC+FVC. Prepared samples were subjected to the fibrin generation clot lysis assay in the presence of tPA. Data are presented as % of control and represent the means \pm SD of three separate experiments with the same normal plasma pool. Gray horizontal line: 100% control. CT, clotting time; CLT, clot lysis time; DOAC, direct oral anticoagulant; FVC, factor V concentrate; PCC, prothrombin complex concentrate. Please note dissimilar y-axis scaling for CT panels A and C

of recalcified whole blood over a collagen/TF surface using an established microfluidic device.⁴⁶ For these experiments, we lowered the DOAC concentration to 50 ng/ml, thus enabling completion of the measurements within the time limit of the experimental setup (10 min, 1 ml blood). The spiking of blood samples with this concentration of rivaroxaban or dabigatran resulted in a 2.9-fold and 2.1-fold prolonged time-to-fibrin formation, respectively. Addition of 1 U/ml PCC or 1 U/ml FVC alone to rivaroxaban or dabigatran anticoagulated blood only partly corrected the time to fibrin. Interestingly, combined treatment with PCC and FVC completely restored time to fibrin to normal values (Figure 4A,B).

Measurement of the surface area coverage can be used to assess platelet adhesion (from brightfield microscopic images), platelet activation (images of AF568-annexin A5 binding, measuring phosphatidylserine exposure), and the total amount of fluorescent AF746-fibrin formed. Platelet deposition and phosphatidylserine exposure were not significantly affected in dabigatran-treated blood (Figure 5E,F). In contrast, both platelet deposition and phosphatidylserine staining were reduced in rivaroxaban anticoagulated blood. Addition of PCC and FVC alone or in combination corrected this rivaroxaban effect (Figure 5B,C). Markedly, fibrin formation was almost abolished in the presence of either rivaroxaban or dabigatran and was partly corrected by the factor concentrates with the most pronounced effect of combined PCC and FVC (Figure 5D,G). In the absence of TF on the collagen-coated matrix, platelet deposition and phosphatidylserine exposure were lower than in the presence of TF (Figure S1, parameters V1 and V2). In the absence of TF, platelet deposition was inhibited as well by rivaroxaban, an effect that was corrected by both PCC and FVC. No fibrin formation was detectable in the absence of TF (Figure S1, parameter V3).

Thrombus morphology and fibrin scores can be used as semi-quantitative measures of platelet aggregation and fibrin formation,

respectively.⁴⁶ Using rivaroxaban-treated blood on both TF- and non-TF-coated surfaces, platelet aggregate formation was decreased (Figure S1, parameter V4). The reduced platelet aggregation could largely be restored by addition of PCC, FVC, or the combination of both. Effects of dabigatran on platelet aggregate formation were essentially absent. On the other hand, as also shown for fibrin formation kinetics in Figure 5, the amount of fibrin formed was decreased after both rivaroxaban and dabigatran and could again only partially be restored by the addition of PCC, FVC, or the combination.

4 | DISCUSSION

As a pro-hemostatic additive, PCC was originally intended to treat and prevent bleeds associated with a VKCF deficiency, as in patients receiving vitamin K antagonists. Nowadays, PCC is more and more used off-label to treat emergency bleeds of any cause including trauma and prior treatment with DOAC. Preparations of PCC are rich in VKCF, but essentially lack other coagulation proteins. Although this composition is ideal to treat vitamin K antagonist-associated bleeds, it may be suboptimal for the off-label applications. The results from the present in vitro study demonstrate a beneficial effect of the combined use of PCC and FVC in the reversal of rivaroxaban as well as dabigatran anticoagulation regarding thrombin generation, fibrin formation, and clot stability.

Dabigatran and rivaroxaban are small synthetic drugs that specifically target the active sites of respectively thrombin and FXa.^{47,48} Dabigatran has a clear, profound effect on coagulation by extending the initiation phase of coagulation with a concomitant delay in fibrin clot formation, as was noticed in the thrombin generation and fibrin clot formation assays under static as well as flow

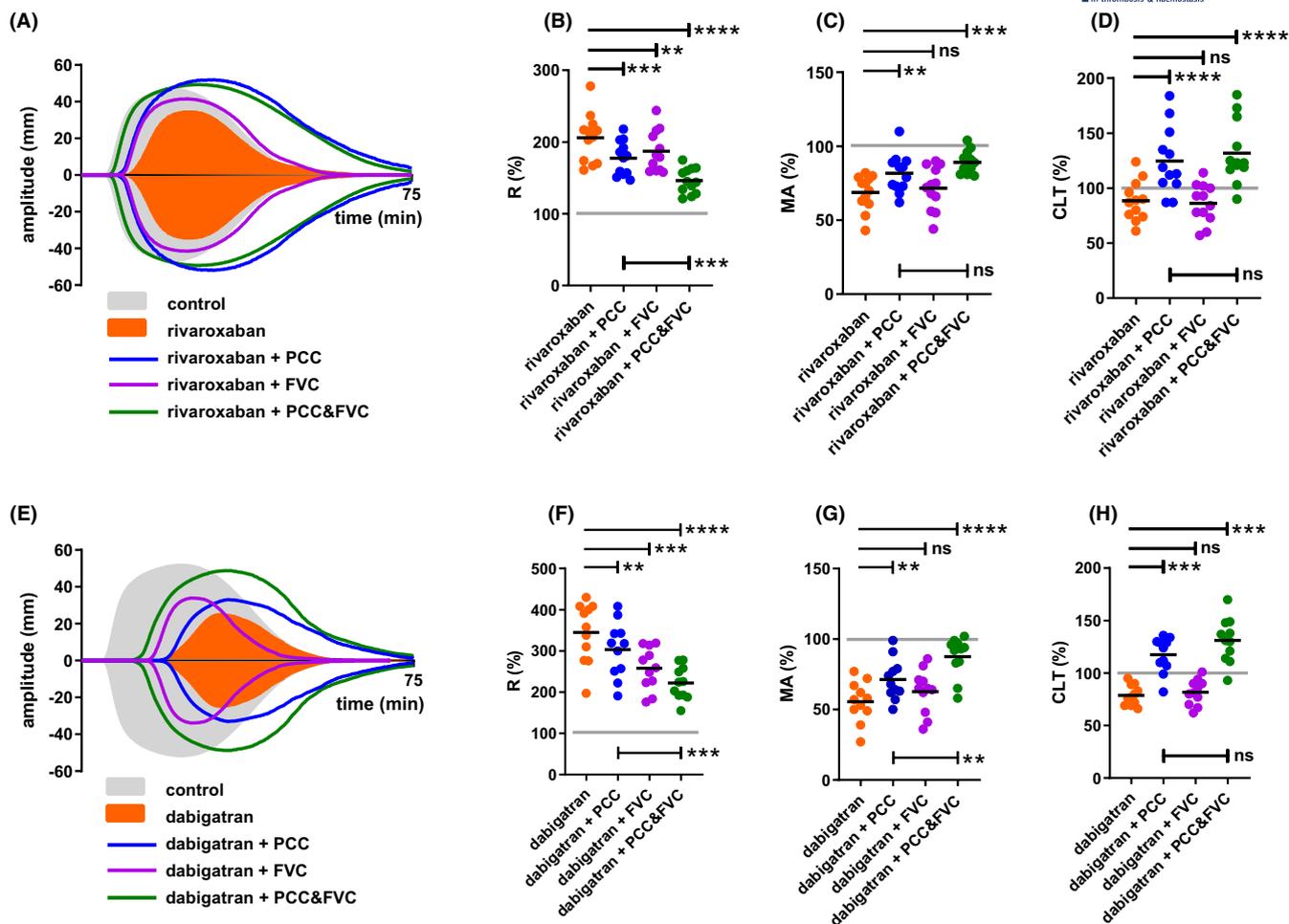


FIGURE 3 Correction of clot formation and clot lysis in DOAC-anticoagulated whole blood by PCC, FVC, or PCC+FVC. Normal citrated whole blood was spiked with 300 ng/ml rivaroxaban (A–D) or dabigatran (E–H). Samples were supplemented with PCC (1 U/ml), FVC (1 U/ml), or PCC+FVC (both 1 U/ml). Concentrations of DOACs and factor concentrate are given for the plasma compartment at a calculated hematocrit of 40%. Prepared samples were subjected to thromboelastography (TEG) in the presence of tPA. Shown TEG tracings (A, E) are representative for 11 (dabigatran) and 12 (rivaroxaban) complete sets of experiments with different donors. Data presented in scatter dot plots (B–D, E–H) show % of control incubations (not containing DOAC or factor concentrate). Each dot represents a separate donor. Population median is shown as a small horizontal black line. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns, not significant. Please note that y-axis scaling of panels B and F (TEG-R values) are dissimilar for optimal data resolution. DOAC, direct oral anticoagulant; FVC, factor V concentrate; PCC, prothrombin complex concentrate; tPA, tissue plasminogen activator

conditions. During the initiation phase of coagulation, thrombin generation is primarily dependent on the TF/FVIIa complex that converts FX zymogen into its active counterpart FXa.⁴⁹ Activation of FV is another crucial event in the initiation of thrombin generation.^{31,32} Although FV and FVII can be activated by both FXa and thrombin, the anticoagulant dabigatran hinders entry into the thrombin generation propagation phase, in which the FXa/FVa complex formation is enhanced for a burst in thrombin generation. On the other hand, rivaroxaban not only prolongs the initiation phase of thrombin generation, but also efficiently reduces the thrombin burst in the propagation phase, as evidenced by a marked reduction in thrombin peak and ETP in the thrombin generation assay. This is explained by the fact that its target enzyme FXa is crucial in the initiation phase as a component of the extrinsic tenase complex (TF-FVIIa-FXa), operates as a feedback

activator of FV and FVII, and is rate limiting in the propagation phase by determining the concentration of the prothrombinase complex (FXa/FVa).^{32,49}

When used as a reversal agent for DOACs, PCC exerts its procoagulant effect predominantly in the propagation phase of thrombin generation. During this phase, thrombin generation is primarily dependent on the availability of FXa for sufficient FXa/FVa complex assembly.⁴⁹ Hence, by adding PCC, the concentrations of VKCF including that of FX will increase. This results in more FXa available for the FVa/FXa complex assembly with a concomitant increase in thrombin peak and ETP, as was noticed in the present and previously published experiments.^{17,36} At a limited reduction of thrombin peak and ETP (e.g., as seen with dabigatran), PCC increases the thrombin generation parameters to above-normal values, as was also seen previously.¹⁷

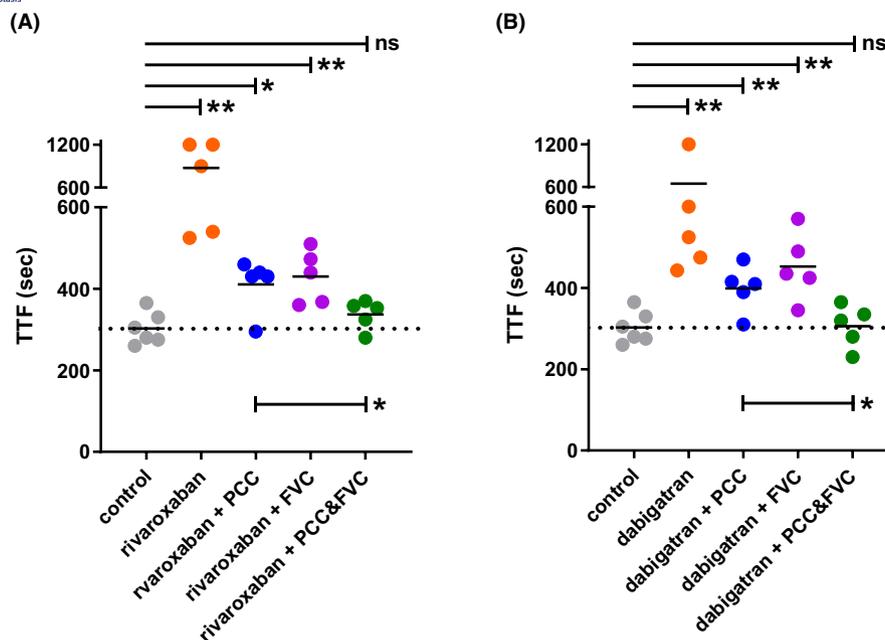


FIGURE 4 Time to fibrin formation under flow is normalized by dual addition of PCC and FV to DOAC-anticoagulated whole blood. Citrated whole blood, spiked with rivaroxaban or dabigatran and supplemented with PCC (1 U/ml), FVC (1 U/ml), or PCC+FVC (both 1 U/ml), was perfused under recalcification conditions over collagen/TF microspots for 10 min at a wall shear rate of 1000 s^{-1} . To be able to complete measurements within the experimental time limit, we chose a lower DOAC concentration of 50 ng/ml instead of the 300 ng/ml as in Figures 1-3. Shown is the quantified effect of rivaroxaban (A) and dabigatran (B), and presence of PCC and/or FVC on time to fibrin formation (TTF), compared with control. Each dot represents the average of duplicate runs performed simultaneously by two laboratory workers. TTF was arbitrarily set at 1200 s, in case no fibrin formation was observed during the experimental time span. Dotted line represents the average time to fibrin of the controls. Data are represented as dot plots with means ($n = 5-6$), * $p < 0.05$, ** $p < 0.01$, ns, not significant. DOAC, direct oral anticoagulant; FVC, factor V concentrate; PCC, prothrombin complex concentrate

Application of PCC was unable to fully reverse the DOAC-inhibited thrombin generation initiation phase, as indicated by the observed only-partial correction of the lag time and a concomitant partial correction of time required for initial fibrin clot formation in the fibrin generation tests and TEG; these observations have also been made before.^{16,17,36} It is known that the activation of FV is of paramount importance in initiating the coagulation system.^{31,32} Consequently, the lag time of thrombin generation and the plasma clotting time are more sensitive to FV levels than the thrombin peak and ETP levels.^{34,50,51} This FV dependency may also apply to DOAC anticoagulated plasma. Results from a previously reported in vitro study comparing FVa and FEIBA (PCC with activated FVII) for reversal of the direct thrombin inhibitor melagatran show that, in particular, the lag time is sensitive to levels of activated FV.⁵² We observed with dabigatran- as well rivaroxaban-anticoagulated plasma that FVC alone had a pronounced corrective effect on the lag time while hardly affecting the thrombin peak and ETP. Similarly, FVC alone improved the clotting time in DOAC-anticoagulated plasma and blood, as noticed in the fibrin generation tests under static (CT, TEG-R) and flow conditions (time to fibrin [TTF]). We observed a clear synergistic effect when FVC was combined with PCC, especially in the tests using minimally altered whole blood (TEG, flow assay). In control plasma samples, thrombin generation parameters and clotting times are influenced by FV levels below 25%.^{34,50,51,53} This is in contrast to our experiments with DOAC-spiked plasmas and added FVC, in which an influence of FV on these

test outcomes was even observed at FVC levels exceeding 1 U/ml. It should be noted, however, that transfusing PCC may lead to a relative deficiency of coagulation factors not present in PCC. In normal plasma with factor levels of 1 U/ml, FX is present at a 5-fold molar excess over FV (160 nM and 30 nM, respectively).⁵⁴ This molar excess of FX over FV is even further increased to 10-fold upon addition of 1.25 U/ml PCC (corresponding to 1.0 U/ml FX). Considering this high PCC dose requirement for optimal DOAC reversal (1.25 U/ml \cong 50 U/kg), it is not surprising that a high FVC dose is also required for complete reversal.

In the microfluidic perfusion assay, the clotting time correction (TTF) by PCC was clearly more pronounced compared with the clotting time parameters that we obtained by thromboelastography (TEG-R) and the fibrin generation assay (CT). The increased sensitivity of whole-blood flow assays for DOAC reversal by PCC relates to the lower DOAC concentration (50 ng/ml compared with 300 ng/ml in other assays) and the only local TF availability.^{17,36,55} Our flow-perfusion testing was also effective in demonstrating a supporting role for FVC in the reversal of DOAC anticoagulation by PCC. Under flow over collagen/TF microspots, the combined use of PCC and FVC completely normalized the TTF formation in cases of anticoagulation with rivaroxaban or dabigatran. Flow may render the initiation phase more sensitive to feedback activation of FVII and probably also FV by FXa.⁵⁶ In addition, the whole-blood flow conditions have proved to be sensitive to FXa formation levels.⁵⁵ Another established advantage of our perfusion model is the simultaneous measurement

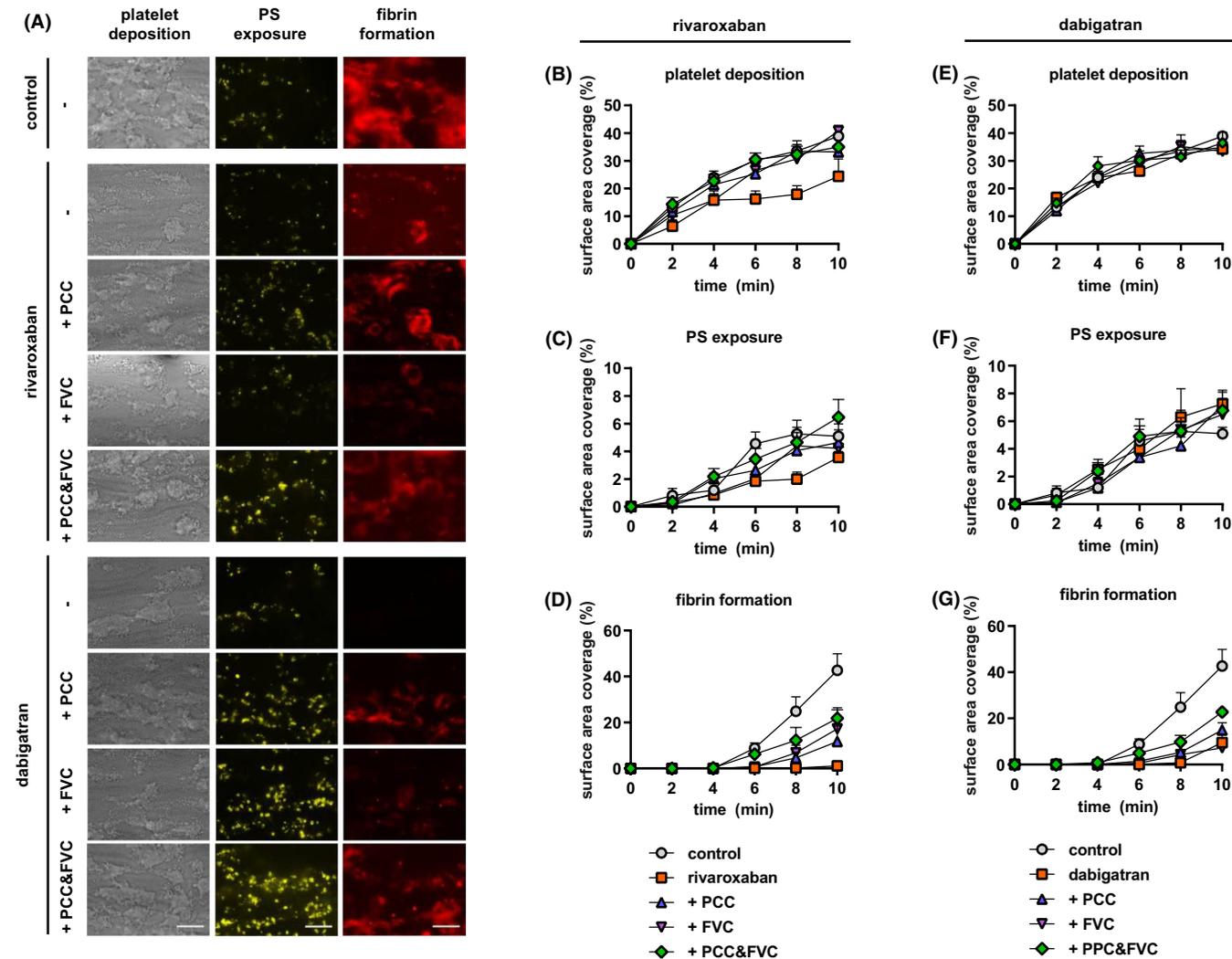


FIGURE 5 Kinetic effects of rivaroxaban or dabigatran in the presence of PCC and FV on platelet-fibrin thrombus formation under flow. Citrated whole blood, spiked with rivaroxaban or dabigatran and supplemented with PCC, FVC, or PCC&FVC, was perfused under recalcification conditions over collagen/TF microspots for 10 min at a wall shear rate of 1000 s^{-1} . Brightfield and fluorescence images were taken every 2 min. Shown are (A) representative brightfield and fluorescence images from collagen/TF microspots after 10 min of flow. Graphs provide time-dependent information on the surface area covered over time of platelet deposition (brightfield), PS exposure (AF568-labeled annexin A5), and fibrin formation (AF647-labeled fibrinogen) for treatment with (B–D) rivaroxaban and with (E–G) dabigatran in combination with PCC, FVC, or PCC and FVC. Data are presented as means + SD ($n = 5$). Bars represent $30 \mu\text{m}$. DOAC, direct oral anticoagulant; FVC, factor V concentrate; PCC, prothrombin complex concentrate

of fibrin generation, platelet deposition, and platelet activation (as determined by phosphatidylserine exposure).⁵⁷ We observed that platelet deposition and phosphatidylserine exposure were not significantly affected in dabigatran-treated blood; therefore, it appears that the initiation of coagulation that triggers platelet adhesion might not be blocked by dabigatran. In contrast, we observed a reduction in platelet deposition and activation with rivaroxaban that could be normalized by PCC and FVC. This might relate to the observation by others showing that rivaroxaban exerts an antiplatelet effect by attenuating FXa-induced platelet activation and aggregation.^{58,59}

Effective bleeding correction relies not only on the time it takes to form a fibrin clot, but also on the clot strength and stability.^{60,61} The maximal amplitude reached in TEG is a measure of clot strength, whereas the clot lysis time initiated by tPA reflects clot

stability. Dense fibrin clots produced in the presence of high thrombin concentrations are composed of thin fibers that are relatively resistant to fibrinolysis. In contrast, loose clots formed in conditions with lower thrombin concentrations are composed of thick fibrin fibers that are more prone to fibrinolysis.⁶² Accordingly, clot strength and stability are reduced in the cases of rivaroxaban and dabigatran anticoagulation. Our results revealed a clear and profound corrective effect of PCC on the lowered clot strength and stability caused by DOAC anticoagulation. We observed that PCC not only partly corrected reduced clot strength in the presence of rivaroxaban and dabigatran, but even improved the clot stability to above normal. This overnormalization with rivaroxaban is in agreement with a previously published study with patients receiving PCC as a treatment for FXa inhibitor-related major bleeding

events.²⁸ An enhanced thrombin activatable fibrinolysis inhibitor activation may contribute to clot stability improvement by PCC.¹⁶ Supplementation of FVC had a potentiating effect on the improvement of clot strength and stability by PCC, especially in the case of dabigatran anticoagulation. In thrombin generation, however, FVC had no effect on the dabigatran-reduced thrombin peak (maximal thrombin concentration reached) and ETP (area under the thrombin generation curve) regardless of the presence of PCC. The lack of a direct link between such thrombin generation measurements and fibrin structure and stability measurements has also been noticed by others and warrants additional in-depth research on this important topic.⁶³

Taken together, our in vitro findings provide a proof of concept for the beneficial effect of the combined use of PCC and FVC in the reversal of rivaroxaban or dabigatran anticoagulation under static as well as flow conditions. Although PCC alone mainly exerted its DOAC reversal effect by increasing clot strength and stability, the combination of PCC and FVC also corrected clotting time. Collectively, our results are indicative for a critical role of FV in correcting reduced thrombin generation and hampered fibrin clot formation resulting from DOACs in vitro and point toward a potential clinical application of FVC. Some limitations may apply to the present study. First, we used normal plasma that was spiked with dabigatran or rivaroxaban and that does not reflect actual patients with thrombophilia or bleeding diathesis. Second, used tests were optimized to measure in vitro reversal of DOAC anticoagulation and may not be representative for in vivo hemostasis. Further studies in experimental animal bleeding models are warranted to follow-up our in vitro study. This offers potential to study also a possible beneficial effect of PCC and FVC in other off-label applications.

RELATIONSHIP DISCLOSURE

H. J. M. Brinkman, M. Zuurveld, and J. C. M. Meijers are employed by Sanquin, until 2020 manufacturer of PCC (Cofact). F. Swieringa, A. Veninga, S. L. N. Brouns, and J. W. M. Heemskerk declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Herm Jan M. Brinkman conceived and supervised the project, designed experiments, analyzed and interpreted data, and wrote the first draft of the manuscript. Frauke Swieringa designed and performed flow experiments, acquired, analyzed, and interpreted data, and cowrote the manuscript. Marleen Zuurveld designed and performed thrombin generation and clotting experiments, acquired and analyzed data. Alicia Veninga and Sanne L. N. Brouns performed flow experiments, acquired and analyzed data. Johan W. M. Heemskerk and Joost C. M. Meijers conceived and supervised the project and edited the manuscript. All authors read and approved the final manuscript.

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SUPPORTING INFORMATION

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