Vascular haemostasis is achieved by complex physical and biochemical reactions of platelets and coagulation factors at the site of injury. In the presence of blood flow, platelet activation and coagulation processes are dynamically affected by local influx and efflux of platelets, coagulation factors, and inhibitors. However, in vitro clinical evaluation of haemostatic function is performed under static conditions (shear rates < 0.1 s⁻¹). Isolated measurements of platelet count, plasma-based prothrombin time (PT), activated partial thromboplastin time (aPTT), and fibrinogen level cannot be inferred to reflect overall haemostatic function in vivo. Platelet aggregation is normally tested in response to a single agonist in anticoagulated plasma or whole blood. Therefore, complex interactions of multiple platelet agonists and coagulation factors with platelets in vivo cannot be assessed with conventional PT/aPTT or platelet aggregometry. In perioperative bleeding, the assessment of coagulopathy is further complicated by haemodilution, which strongly influences dynamic interactions of erythrocytes, platelets, and coagulation factors under flow conditions.

We hypothesized that overall haemostatic functions are differently influenced by haemodilution between low and high blood flow patterns (e.g. arteriole and artery). Therefore, we evaluated the effects of haemodilution and the procoagulant activities of various factor concentrates on thrombus formation in human whole blood using a microchip-based flow-chamber system in parallel with rotational thromboelastometry.

**Methods**

Human plasma derived von Willebrand factor (vWF; Humate P), prothrombin complex concentrate (PCC; Beriplex), and...
fibrinogen concentrate (RioStap) were all obtained from CSL Behring (Marburg, Germany). The stock solution of Humate P contained factor VIII (FVIII) 70.0 U ml⁻¹ and vWF 154.8 U ml⁻¹, and was used in our in vitro experiments to simulate the release of FVIII/vWF from endothelium that normally occurs in vivo during surgery and trauma.²¹ ²² Further, PCC (0.3 U ml⁻¹) and fibrinogen concentrates (1–2 g litre⁻¹) were added to diluted whole blood samples to model the replacement of vitamin K-dependent clotting factors and fibrinogen, respectively.²³

Blood samples

The study was conducted according to a protocol approved by the local institutional review board (Emory University, Atlanta, Georgia, USA). For the first part of the study, blood samples were collected from 12 healthy volunteers (4 females and 8 males, age 27–61 yr) after informed written consents were obtained. These subjects had not taken medication(s) that affect platelet function or coagulation in the preceding 2 weeks. Whole blood samples were collected in 3.2% sodium citrate (Venocjet II® 4.5 ml; Terumo, Japan) with corn trypsin inhibitor (CTI; final concentration; 50 μg ml⁻¹). They were immediately used for perfusion experiments. The whole blood sample for thromboelastometry was collected in 3.2% sodium citrate (Vacutainer® 4.5 ml; Beckton-Dickinson, Franklin Lakes, NJ, USA). For in vitro modelling of haemodilution, whole blood samples were diluted 6:4 v/v with normal saline (i.e. 40% volume replacement). Some of the 3.2% sodium citrate blood was immediately centrifuged at 2000 × g for 20 min to obtain platelet-poor plasma. The plasma samples were stored at −80°C until batch analysis.

For the second part of the study conducted to evaluate in vivo haemodilution, whole blood samples were collected from 15 consented patients undergoing cardiac surgery with cardiopulmonary bypass (CPB). Inclusion criteria were age >18 yr, normal PT/aPTT and normal platelet count (150–400 × 10⁹ litre⁻¹). Patients on warfarin or clopidogrel within 5 days before surgery, and those with pre-existing hepatic dysfunction (elevated aspartate aminotransferase or alanine aminotransferase >40 IU ml⁻¹) or renal insufficiency (serum creatinine ≥1.5 mg dl⁻¹), were excluded. Patients included in the study were given heparin 400 U kg⁻¹ to achieve activating clotting time (ACT) >400 s before instituting CPB. Per clinical protocol, tranexamic acid (Cyclokapron; Pfizer, New York, NY, USA) was administered i.v. at 15 mg kg⁻¹ as a loading dose, followed by infusion at 7.5 mg kg⁻¹ h⁻¹. Intraoperative red blood cell salvage (Cell Saver 5, Haemonetics, Braintree, MA, USA) was used in all cases. Red blood cells were transfused to maintain haematocrit 21%. Heparin anticoagulation was reversed after CPB with 200–250 mg of protamine sulphate. Blood samples were obtained before heparin administration (baseline), and after protamine administration before any haemostatic intervention (post-CPB). Thereafter, if microvascular bleeding persisted, haemostatic interventions (fresh-frozen plasma, cryoprecipitate, or platelets) were administered at the discretion of the attending anesthesiologist.

Laboratory measurements

Haematocrit (%) and platelet count were measured using the Coulter AcTanlyser (Coulter Corporation, Miami, FL, USA). Measurement of vWF, fibrinogen, PT, aPTT, prothrombin (FII), FVIII, and antithrombin (AT) levels was performed on a coagulation analyser (STA Compact, Diagnostica Stago, Parsippany, NJ, USA) using manufacturer’s kits and directions. Fibrinogen concentrations were determined using the modified Clauss method.

Flow-chamber assay

Thrombus formation under flow was evaluated using the Total Thrombus Formation Analysis System (T-TAS; Fujimori Kagyo, Tokyo, Japan).²⁸ ²⁹ This newly developed microchip-based flow-chamber system is equipped with a rectangular capillary (width 300, depth 60 μm, and length 15 mm), pneumatic pump, video microscope, and flow pressure sensor (Fig. 1). Recalculated whole blood is perfused at 37°C through the microcapillary chamber pre-coated with tissue factor and collagen. The flow pressure changes are monitored by the pressure transducer located upstream in the microcapillary. Thrombus formation or breakdown within the microcapillary alters resistance causing the pressure to increase or decrease, respectively. In the experiments under flow rates of 10 and 3 ml min⁻¹, initial shear rates against the microcapillary wall are calculated to be 1100 and 330 s⁻¹, respectively. These shear rates correspond to normal shear rate in arterioles and medium-sized arteries.²⁶ Whole blood was perfused at a flow rate of 10 or 3 ml min⁻¹ before and after 40% dilution. For the latter, perfusion experiments were repeated after in vitro addition of (i) vWF, 1.5 U ml⁻¹; (ii) PCC, 0.3 U ml⁻¹; (iii) fibrinogen, 2 g litre⁻¹; or (iv) combination of PCC, 0.3 U ml⁻¹ and fibrinogen, 1 g litre⁻¹ (all final concentrations).

Thrombus formation was monitored qualitatively by video microscopy, and was also quantified by the flow pressure changes in the capillary. The endpoint parameters of the flow pressure included the onset of thrombus formation (Ton; the onset of thrombus formation defined as the time to flow pressure of 5 kPa), and the time to 80 kPa (T₈₀; a parameter that reflects thrombus growth rate).²⁸ ²⁹

Thromboelastometry

Changes in viscoelastic properties of whole blood clotting were evaluated using rotational thromboelastometry (TEM Systems, Inc., Durham, NC, USA). All measurements were performed at 37°C. Coagulation was triggered in recalcified whole blood (300 μl mixed with 20 μl of 0.2 M CaCl₂) using tissue factor (EXTEM, TEM Systems, Inc.). The following thromboelastometric parameters were collected: clotting time (CT, s), which corresponds to the lag time before clotting; clot formation time (CFT, s), which reflects the initial rate of clot formation; maximum clot firmness (MCF, mm) as the maximal tensile strength of clot.¹⁰ EXTEM measurements were obtained before and after 40% dilution. For the latter, EXTEM measurements were repeated after in vitro addition of (i) vWF, 1.5 U ml⁻¹; (ii) PCC, 0.3 U ml⁻¹; (iii) fibrinogen, 2 g litre⁻¹; or (iv) combination of PCC, 0.3 U ml⁻¹ and fibrinogen, 1 g litre⁻¹.
Analysis of thrombus by confocal microscopy

The composition of thrombus formed in the flow chamber before and after the dilution was evaluated using immunostaining and confocal laser scanning microscopy. After completion of each perfusion, thrombus was carefully removed from the mid-segment of the capillary, washed three times with phosphate-buffered saline, and then incubated with fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD41 (platelet glycoprotein IIb) IgG (1:6 dilution) at room temperature for 15 min in the dark. FITC-conjugated mouse IgG was used as a control. After three washes with Tris-buffered saline containing 0.1% Triton X-100 (TBST), thrombus specimen was immobilized with OptiLyse C (Beckman Coulter, France) for 15 min at room temperature. After additional three washes with TBST, the sample was blocked for 1 h at room temperature with Block Ace (Yukijirushi, Japan) containing 1 mg ml⁻¹ of normal goat IgG. Thereafter, the sample was incubated with rabbit anti-human fibrinogen IgG (1:99 dilution) labelled with Alexa 594 at room temperature for 30 min in the dark. Alexa 594-conjugated rabbit IgG was used as a control. The final specimens were examined using an LSM700 confocal microscope (Zeiss, Germany).

Western blot analysis

At the end of perfusion experiment, thrombus was recovered from the microchip, dissolved in 100 ml of 62.5 mM Tris–HCl, pH 6.8, 2.15% sodium dodecyl sulphate (SDS), 15% glycerol, 0.005% bromophenol blue, and boiled for 1 min. The sample was subjected to 10–20% SDS–polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred to nitrocellulose membrane. The membrane was then incubated with a sheep anti-human thrombin (Affinity Biologicals, Ancaster, ON, Canada) or a rabbit anti-human fibrinogen antibody (AssayPro, St Charles, MO, USA). It was then washed extensively, and incubated with a horseradish peroxidase-linked secondary antibody. The signals were detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA).

Flow-chamber assay and thromboelastometry in cardiac surgery patients

Additional flow-chamber experiments were performed to confirm our in vitro dilution model using whole blood from cardiac surgical patients. Perfusion experiments were performed in recalcified whole blood at baseline, and after CPB. PCC and fibrinogen concentrates represent major haemostatic interventions after CPB. Plasma vWF level tends to remain normal or supra-normal during surgery. The flow rate of 3 μl min⁻¹ was thus chosen to evaluate procoagulant effects of PCC and fibrinogen, which are preferably demonstrated at a lower shear rate. PCC and fibrinogen were added in vitro to post-CPB blood as follows: (i) PCC, 0.3 U ml⁻¹; (ii) fibrinogen, 2 g litre⁻¹; or (iii) combination of PCC, 0.3 U ml⁻¹ and fibrinogen, 1 g litre⁻¹ (all in final concentrations).

For thromboelastometry, EXTEM was measured at baseline and post-CPB. In post-CPB samples, EXTEM measurements were repeated after in vitro addition of PCC, fibrinogen, or their combination as in the above perfusion experiment. FIBTEM was also used for determining fibrin-specific clot firmness after fibrinogen replacement by inhibiting platelet-fibrin(ogen) interactions using cytochalasin D.
Statistical analysis
Data are expressed as median (IQR) according to non-normal distributions by the Kolmogorov–Smirnov test. The statistical significance of the difference among the groups were assessed by Wilcoxon’s rank-sum test, and non-parametric Friedman analysis of variance as appropriate. A P-value of <0.05 was considered significant. All analyses were performed using Graph-Pad Prism, Version 5.0 (Graph-Pad Software, Inc., San Diego, CA, USA).

Results
Thrombus formation in whole blood obtained from healthy volunteers
In healthy volunteers, baseline values [median (IQR)] of haematocrit and platelet count were 44.8 (38.3–47.3)%, and 222 (205–257) × 10^9 litre^-1, respectively. The vWF and fibrinogen levels were in normal ranges [81.0 (57.0–113)%, and 2.1 (1.8–2.3) g litre^-1, respectively].

Morphological evaluation of thrombus formation
The qualitative analysis of normal thrombus formation by video microscopy (×200) demonstrated a time-dependent process that proceeds in both the capillary wall and the centre of the capillary (Fig. 2). During perfusion at 10 µl min^-1, a small amount of thrombus was deposited over the capillary within 3–4 min. The development of intra-luminal thrombus was rapidly seen, and the capillary was occluded within 1–2 min after the initial appearance of thrombus (Fig. 2a, control). Deposition of thrombus during perfusion at 3 µl min^-1 occurred similarly to perfusion at 10 µl min^-1, but general expansion of thrombus was observed more slowly (Fig. 2a, control). After 40% dilution of whole blood, initial appearance of thrombus was delayed for the perfusion at 10 µl min^-1, while it was not affected at 3 µl min^-1 (Fig. 2a and b, 40% dilution). Conversely, subsequently growth of thrombus was more affected at 3 µl min^-1 than at 10 µl min^-1. Adding vWF to diluted whole blood accelerated intracapillary thrombus formation with perfusion at 10 µl min^-1, but not 3 µl min^-1 (Fig. 2a and b, vWF). PCC seemed to increase thrombus growth particularly near the capillary wall (i.e. mural thrombus) at both flow rates (Fig. 2a and b, PCC). Addition of fibrinogen concentrate also led to thicker mural thrombus formation, but did not cause complete capillary occlusion (Fig. 2a and b, Fgn). The thickness of mural thrombus was further increased with fibrinogen in combination with PCC at both flow rates, but was more clearly demonstrated at 3 µl min^-1 (Fig. 2a and b, PCC and Fgn).

The immunostained thrombus examined by confocal microscopy demonstrated that the thrombus was composed of platelets (in green) containing abundant fibrin fibres (in red) (Fig. 3). Although abundant platelets and fibrin deposition were confirmed in control samples at both flow rates, thick fibrin fibres were frequently observed at 10 µl min^-1 relative to 3 µl min^-1 (Fig. 3a and b, control). In addition, fibrin fibres and platelet thrombus formed at 10 µl min^-1 seemed to follow the direction of flow. After 40% dilution with saline, thinner fewer fibrin fibres were seen at both flow rates, but the decrease was more obvious at 3 µl min^-1 (Fig. 3a and b, 40% dilution). Adding vWF increased platelets in the thrombus at 10 µl min^-1, not at 3 µl min^-1 (Fig. 3a and b, vWF) in agreement with the results of video microscopy (Fig. 2). On the other hand, images revealed that there was an abundance of fibrin fibres after PCC or fibrinogen intervention (Fig. 3a and b, PCC and Fgn, respectively). Increased deposition of thick fibrin fibres was observed after fibrinogen, whereas densely packed fibrin fibres were increased after PCC. These changes were more clearly shown at 3 µl min^-1 than at 10 µl min^-1.

Western blot analysis of the thrombus formed in control blood showed considerable amounts of thrombin inside the thrombus at both 10 and 3 µl min^-1 (Fig. 4a). After 40% dilution, decreased thrombin was observed at both flow rates. Addition of PCC to diluted blood enhanced the amount of thrombin in the thrombus at both flow rates, while there were no significant changes after supplementing vWF. There were more abundant thrombin-catalysed fibrin species (α, β, γ-chains and γ-dimers) in thrombus formed at 3 µl min^-1 compared with 10 µl min^-1 (Fig. 4b). After dilution, these fibrin chains were decreased at both flow rates. The decreases in fibrin after dilution were reversible with the addition of fibrinogen at 3 µl min^-1. The combination of PCC and fibrinogen increased both thrombin and fibrinogen contents at both flow rates.

Flow pressure changes during thrombus formation
Representative flow pressure changes in the capillary are shown in Figure 5a. Both onset (Ton) and growth (T80) of thrombus formation took longer at 3 µl min^-1 relative to 10 µl min^-1 in undiluted control blood. After 40% dilution, both Ton and T80 were prolonged at both flow rates (P<0.05) (Fig. 5a and b), but the changes of Ton at T80 relative to baseline values were greater at 10 µl min^-1 than at 3 µl min^-1 (+96 vs +40% for Ton; +85 vs +50% for T80). In diluted whole blood, Ton was shortened by 38% with vWF at 10 µl min^-1 (P<0.05 vs 40% dilution), but not at 3 µl min^-1. Conversely, T80, but not Ton, was improved by PCC only at the lower flow rate. After adding fibrinogen, T80 was prolonged at both flow rates (P<0.05 vs 40% dilution). Prolonged T80 with fibrinogen replacement at both flow rates was partially restored by the combination of PCC and fibrinogen. In particular, Ton at 3 µl min^-1 was shortened by 45% after adding PCC to fibrinogen (P<0.05 vs fibrinogen).

Thromboelastometry
After 40% dilution, MCF was decreased (Table 1) (P<0.05). Compared with the results from flow assays, the interventions with vWF and PCC in diluted blood did not improve any thromboelastometric parameters. On the other hand, fibrinogen and the combination of PCC and fibrinogen improved MCF (P<0.05, respectively, vs 40% dilution).
**Fig 2** Video microscopic images of thrombus formation under flow conditions. (a) During perfusion at 10 μl min\(^{-1}\), small thrombus was deposited on the capillary within 3–4 min. Rapid growth of thrombus was observed, occupying the capillary just 1–2 min after the appearance of thrombus. (b) The initial appearance of thrombus at 3 μl min\(^{-1}\) was similar to perfusion at 10 μl min\(^{-1}\), but subsequent growth of thrombus was slower at 3 μl min\(^{-1}\). *Occlusion occurred in the capillary. After 40% dilution of whole blood, the initial appearance of thrombus was prolonged for perfusion at 10 μl min\(^{-1}\), while it was not affected at 3 μl min\(^{-1}\). Conversely, subsequent growth of thrombus was delayed at 3 μl min\(^{-1}\) more than at 10 μl min\(^{-1}\). Addition of vWF to diluted whole blood accelerated appearance of thrombus at 10 μl min\(^{-1}\). Adding PCC increased both intra-luminal and mural thrombus formation at both flow rates. Addition of fibrinogen (Fgn) concentrate increased the thickness of mural thrombus, but did not cause the complete capillary occlusion.
Thrombus formation in whole blood from patients undergoing cardiac surgery

Blood cell counts and other coagulation values from cardiac surgical patients are summarized in Table 2. Baseline laboratory values were within normal ranges. After CPB, haematocrit, platelet count, fibrinogen, prothrombin, and AT were significantly decreased from baseline ($P < 0.05$). PT and aPTT were prolonged to $\sim 1.6$- to $1.8$-fold from baseline ($P < 0.05$, respectively). On the other hand, FVIII levels were maintained, and vWF levels were above the baseline after CPB.

Thrombus formation under flow conditions before and after CPB

The results of video microscopic evaluation and flow pressure changes in whole blood from cardiac surgery patients were in agreement with results in the diluted healthy volunteer blood. After CPB, both deposition and subsequent growth of thrombi on the capillary at $3 \mu l \text{ min}^{-1}$ were prolonged compared with baseline. This prolonged thrombus formation was shortened by adding PCC. Addition of fibrinogen concentrate to post-CPB samples increased the thickness of mural thrombus, but did not cause complete capillary occlusion. In combination with PCC, the thickness of thrombus was further increased with fibrinogen at $3 \mu l \text{ min}^{-1}$.

For flow pressure changes, $T_{on}$ and $T_{80}$ after CPB were prolonged by $102$ and $78\%$ from baseline, respectively ($P < 0.05$) (Fig. 6). Addition of PCC shortened both $T_{on}$ and $T_{80}$ at $3 \mu l \text{ min}^{-1}$ ($P < 0.05$ vs post-CPB), while addition of fibrinogen prolonged these parameters. Prolongations of $T_{on}$ and $T_{80}$ were decreased by adding PCC to fibrinogen.

Fig 3 Analysis of thrombus by confocal laser scanning microscopy. Blood was perfused from left to right for all panels. For each panel, fibrin stained by anti-Fibrinogen-Alexa594 (left panel), platelets stained by anti-CD41-FITC (middle panel), and merged platelet and fibrin staining (right panel) are shown. The white bar represents 100 $\mu m$. The thrombus formed in the mid-segment of the microchip capillary was composed of platelets (in green) containing fibrin strands (in red). Fgn, fibrinogen.
Thromboelastometry

In post-CPB samples, both CT\textsubscript{TE} and CF\textsubscript{TE} tended to be prolonged compared with baseline (Table 3). In addition, both MCF\textsubscript{TE} and MCFF\textsubscript{IB} were decreased after CPB compared with baseline ($P < 0.05$, respectively). In contrast to the results of flow experiments, addition of PCC alone at the end of CPB did not improve EXTEM parameters. On the other hand, improvements of parameters were shown in thromboelastometry clearly compared with the flow assay after adding fibrinogen. Fibrinogen not only shortened CT\textsubscript{TE} and CF\textsubscript{TE}, but also increased MCF\textsubscript{TE} and MCFF\textsubscript{IB} ($P < 0.05$, respectively).

Discussion

We demonstrated the influence of blood flow rates on the composition of thrombus (platelets, thrombin, fibrin, etc.), and procoagulant responses to different haemostatic components (vWF, PCC, and fibrinogen concentrate) added to diluted blood. Normal thrombus formation in vivo initially depends on vWF-mediated platelet adhesion in arterial flow.\textsuperscript{26} Lower platelet count and vWF delayed the onset of thrombus formation after 40% in vitro haemodilution. The delay was more pronounced at 10 compared with 3 $\mu l\ min^{-1}$. Further, delayed thrombus formation after dilution was restored by adding vWF at 10 $\mu l\ min^{-1}$, but not at 3 $\mu l\ min^{-1}$. Ruggeri and colleagues\textsuperscript{26} previously demonstrated that impaired collagen-induced platelet thrombus formation in washed platelets (without plasma) was restored by exogenously added vWF at a shear rate of 1500 $s^{-1}$, but not at 300 $s^{-1}$. An effect of vWF on thromboelastometry was not evident under low shear condition (0.1 $s^{-1}$). Clot strength on thromboelastometry reflects thrombin-activated platelet interactions with fibrin(ogen) via glycoprotein IIb/IIIa receptors,\textsuperscript{27} but platelet adhesion involving glycoprotein Ib/IX receptors and vWF cannot be evaluated.\textsuperscript{28} However, vWF activity is less prone to perioperative decreases compared with other coagulation factors including fibrinogen and prothrombin. In diluted blood, increasing vitamin K-dependent coagulation factors using PCC led to improved mural thrombus growth, which was
more pronounced at $3 \mu l \text{min}^{-1}$. These findings were corroborated by increased amounts of thrombin and fibrin formation within the thrombus on confocal microscopy and western blot analysis. Thus, PCC seems to increase the amount of thrombus-bound thrombin and mural fibrin formation, but intra-luminal thrombus growth is limited when blood flow (shear rate) is high ($10 \mu l \text{min}^{-1}$). Increased mural thrombus formation could be the mechanism responsible for improved haemostasis after PCC administration, which has been documented in vivo using porcine dilutional coagulopathy models.29–31

Maintaining plasma fibrinogen is considered to be important for perioperative haemostasis.32,33 In our study, video microscopic data revealed thickened thrombus along capillary walls at $3 \mu l \text{min}^{-1}$ after supplementing fibrinogen in diluted whole blood from healthy volunteers. In addition, western blot analysis showed that 40% dilution with saline caused decrease in fibrin $\alpha$- and $\beta$-chains, and cross-linked fibrin ({$\gamma$}-dimer) at the lower flow rate relative to the higher flow rate. Fibrin $\alpha$- and $\beta$-chains were restored after fibrinogen replacement at $3 \mu l \text{min}^{-1}$. In our flow-chamber system, intra-luminal thrombus development is reflected in the rate of pressure increase. Interestingly, fibrinogen supplementation in both diluted volunteer blood and post-CPB blood resulted in delayed capillary occlusion, which was more extensive at the higher flow rate compared with the lower rate. Paradoxical

Table 1  Thromboelastometric parameters in vitro haemodilution. Values are median (IQR). *P < 0.05 vs control and **P < 0.05 vs 40% dilution. vWF, von Willebrand factor; PCC, prothrombin complex concentrate; CT, clotting time; CFT, clot formation time; MCF, maximum clot firmness

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>40% dilution</th>
<th>+vWF 1.5 U ml$^{-1}$</th>
<th>+PCC 0.3 U ml$^{-1}$</th>
<th>+Fibrinogen 2 g litre$^{-1}$</th>
<th>+PCC 0.3 U ml$^{-1}$ + fibrinogen 1 g litre$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT$_{\text{EXTEM}}$ (s)</td>
<td>93 (78–99)</td>
<td>61 (50–69)</td>
<td>63 (49–69)</td>
<td>67 (65–70)</td>
<td>44 (38–46)</td>
<td>43 (40–47)</td>
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<tr>
<td>CF$_{\text{EXTEM}}$ (s)</td>
<td>155 (131–180)</td>
<td>166 (141–172)</td>
<td>156 (146–168)</td>
<td>170 (146–186)</td>
<td>76 (67–91)**</td>
<td>125 (113–131)</td>
</tr>
<tr>
<td>MCF$_{\text{EXTEM}}$ (mm)</td>
<td>53 (52–54)</td>
<td>47 (45–48)*</td>
<td>47 (46–49)</td>
<td>47 (46–48)</td>
<td>58 (56–60)**</td>
<td>53 (51–54)**</td>
</tr>
</tbody>
</table>

Fig 5  Effects of haemodilution and haemostatic components in whole blood from. (a) Representative flow pressure patterns. At $10 \mu l \text{min}^{-1}$, onset of thrombus formation (Ton) was more affected than capillary occlusion (T80) by haemodilution and corrective haemostatic interventions. On the other hand, T80 was more affected than Ton at $3 \mu l \text{min}^{-1}$. (b) Onset of thrombus formation (Ton), and (c) time to capillary occlusion (T80). After 40% dilution, both Ton and T80 were prolonged at both flow rates. Addition of vWF shortened Ton at $10 \mu l \text{min}^{-1}$, but not at $3 \mu l \text{min}^{-1}$; PCC shortened T80 at $3 \mu l \text{min}^{-1}$. However, addition of fibrinogen to diluted whole blood prolonged Ton at $10 \mu l \text{min}^{-1}$ and T80 at both flow rates. After addition of both fibrinogen and PCC, Ton at $3 \mu l \text{min}^{-1}$ was shortened by 46% compared with Ton after 40% dilution. *P < 0.05 vs control; **P < 0.05 vs 40% dilution. Fgn, fibrinogen.
findings (i.e., increased thickness of mural thrombus with delayed pressure increase) are presumably because of reduced intra-luminal extension of thrombus after supplementing fibrinogen. Indeed, Remijn and colleagues previously observed loosely packed thrombi over collagen in the absence of fibrinogen under a shear rate of 1600 s⁻¹, which became more densely packed after fibrinogen was added. A rapid adsorption of thrombin to fibrin strands seems to be important in localizing thrombin activity and limiting intravascular thrombus extension.

After severe haemodilution, thrombus formation is progressively disturbed because of the loss of multiple procoagulant factors and platelets. Therefore, combined therapies using different haemostatic products can be reasonable and efficacious interventions. In our study, procoagulant effects of combined PCC and fibrinogen were differently shown by the flow experiments and thromboelastometry. In the flow assay, increased thickness of mural thrombus by fibrinogen plus PCC was demonstrated at both flow rates. On the other hand, increased fibrin polymerization was strongly reflected on thromboelastometry as fibrinogen replacement improved clot formation parameters more than combined PCC and fibrinogen alone (Tables 1 and 3). Indeed, Weber and colleagues have recently demonstrated that point-of-care test-guided transfusion of fibrinogen concentrate, PCC, and platelets for post-CPB bleeding can be superior to the conventional coagulation testing in terms of haemostatic efficacy and cost-effectiveness.

There are several advantages of our microchip-based flow-chamber system compared with conventional assays. The advantage of testing whole blood is the inclusion of rheological effects on haemostasis exerted by red blood cells. Turitto and Weiss demonstrated that the extent of platelet adhesion is inversely proportional to haematocrit. We have also demonstrated that 20% volume replacement of warfarin-treated whole blood with fresh-frozen plasma caused the dilution of erythrocytes and platelets, and paradoxically obtunded the onset of thrombus formation under flow conditions. In this regard, flow-chamber testing can be considered more physiologically as it includes dynamic interactions of both cellular and soluble elements of haemostasis.

The limitations of our study are as follows. First, the flow-chamber system does not reflect natural exposures of collagen, tissue factor, or both, nor does it include organ-specific endothelial responses (e.g., vasoconstriction). Secondly, CTI is used to inhibit FXIIa and to prevent premature clotting of blood in the reservoir. It is plausible that CTI reduces thrombus formation by interfering with platelet-mediated FXIIa activation. Finally, we used normal saline as the diluent of whole blood, and thus coagulation disturbances might have been more extensive than with other buffered colloids. In addition, albumin was the only type of colloid used

### Table 2: Haematologic parameters in patients undergoing CPB.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>Post-CPB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haematocrit (%)</td>
<td>37.7 (36.1–39.6)</td>
<td>24.9 (23.4–27.7), [−36.2%]</td>
</tr>
<tr>
<td>Platelet (× 10⁹ litre⁻¹)</td>
<td>185 (157–210)</td>
<td>95 (73–108), [−40.3%]</td>
</tr>
<tr>
<td>vWF (%)</td>
<td>117 (106–158)</td>
<td>178 (157–194), [+50.0%]</td>
</tr>
<tr>
<td>Fibrinogen (g litre⁻¹)</td>
<td>2.9 (2.6–3.1)</td>
<td>1.7 (1.6–1.8), [−42.0%]</td>
</tr>
<tr>
<td>PT (s)</td>
<td>11.9 (11.6–12.4)</td>
<td>18.7 (17.5–20.7), [+57.7%]</td>
</tr>
<tr>
<td>aPTT (s)</td>
<td>33.8 (32.6–34.8)</td>
<td>58.0 (49.0–68.3), [+65.6%]</td>
</tr>
<tr>
<td>Prothrombin (%)</td>
<td>104 (89.0–111)</td>
<td>45 (29–48), [−60.9%]</td>
</tr>
<tr>
<td>FVIII (%)</td>
<td>106 (102–134)</td>
<td>105 (99–115), [−4.5%]</td>
</tr>
<tr>
<td>AT (%)</td>
<td>89 (84–91)</td>
<td>36 (33–45), [−56.7%]</td>
</tr>
</tbody>
</table>

*P < 0.05 vs baseline; **P < 0.05 vs post-CPB.

### Table 3: Thromboelastometric parameters in patients undergoing CPB.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>Post-CPB</th>
<th>+ PCC 0.3 U ml⁻¹</th>
<th>+ Fibrinogen 2 g litre⁻¹</th>
<th>+ PCC 0.3 U ml⁻¹ + fibrinogen 1 g litre⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTEXTEM (s)</td>
<td>61 (52–75)</td>
<td>77 (78–95)</td>
<td>73 (62–83)</td>
<td>65 (59–78)**</td>
<td>62 (48–67)**</td>
</tr>
<tr>
<td>CTFEXTEM (s)</td>
<td>94 (74–113)</td>
<td>114 (97–163)</td>
<td>129 (97–193)</td>
<td>71 (61–74)**</td>
<td>106 (94–138)</td>
</tr>
<tr>
<td>MCFEXTEM (mm)</td>
<td>65 (60–68)</td>
<td>57 (50–61)*</td>
<td>52 (47–59)</td>
<td>65 (62–69)**</td>
<td>55 (53–61)</td>
</tr>
<tr>
<td>MCFFIBTEM (mm)</td>
<td>21 (18–25)</td>
<td>10 (8.5–13)*</td>
<td>–</td>
<td>30 (28–31)**</td>
<td>–</td>
</tr>
</tbody>
</table>
in the clinical part of the study. Further studies are thus needed to address the effects of different crystalloids and colloids on coagulation under flow conditions.

In summary, our study highlights the contribution of blood flow to thrombus formation after haemodilution and subsequent haemostatic component interventions. Fibrin deposition was more extensively decreased by haemodilution under the lower flow rate compared with the higher flow rate. The amount of thrombin in the thrombus was decreased at both flow rates. Replacement of vWF accelerated intra-luminal thrombus formation at the higher flow rate, whereas fibrinogen concentrate was more efficient at the lower flow rate. The addition of PCC improved mural thrombus formation at both flow rates. Incorporating blood flow conditions is potentially useful in evaluating therapeutic efficacy of haemostatic agents (e.g. vWF and PCC) that involve platelet and plasma factor interactions.

Authors’ contributions
S.O., K.H., and K.A.T. have designed the study protocol. S.O., F.S., and E.P.C. enrolled subjects. T.O. and F.S. conducted coagulation assays. All the authors contributed to the analysis and preparation of the manuscript.

Declaration of interest
K.H. and T.O. are employees of Fujimori Kogyo, Co. Ltd. K.A.T. has previously received honoraria for lectures related to factor concentrates (CSL Behring, Marburg, Germany), and ROTEM (TEM Innovations, Munich, Germany); neither company was involved in the planning or analysis of the present study.

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