Journal of Thrombosis and Haemostasis, **14**: 1453–1461 DOI: 10.1111/jth.13354

ORIGINAL ARTICLE

Compaction of fibrin clots reveals the antifibrinolytic effect of factor XIII

D. C. RIJKEN, S. ABDUL, J. J. M. C. MALFLIET, F. W. G. LEEBEEK and S. UITTE DE WILLIGE
Department of Hematology, Erasmus University Medical Center, Rotterdam, the Netherlands

To cite this article: Rijken DC, Abdul S, Malfliet JJMC, Leebeek FWG, Uitte de Willige S. Compaction of fibrin clots reveals the antifibrinolytic effect of factor XIII. *J Thromb Haemost* 2016; **14**: 1453–61.

Essentials

- Factor XIIIa inhibits fibrinolysis by forming fibrin-fibrin and fibrin-inhibitor cross-links.
- Conflicting studies about magnitude and mechanisms of inhibition have been reported.
- Factor XIIIa most strongly inhibits lysis of mechanically compacted or retracted plasma clots.
- Cross-links of α2-antiplasmin to fibrin prevent the inhibitor from being expelled from the clot.

Summary. *Background:* Although insights into the underlying mechanisms of the effect of factor XIII on fibrinolysis have improved considerably in the last few decades, in particular with the discovery that activated FXIII (FXIIIa) cross-links α_2-antiplasmin to fibrin, the topic remains a matter of debate. *Objective:* To elucidate the mechanisms of the antifibrinolytic effect of FXIII. *Methods and Results:* Platelet-poor plasma clot lysis, induced by the addition of tissue-type plasminogen activator, was measured in the presence or absence of a specific FXIIIa inhibitor. Both in a turbidity assay and in a fluorescence assay, the FXIIIa inhibitor had only a small inhibitory effect: 1.6-fold less tissue-type plasminogen activator was required for 50% clot lysis in the presence of the FXIIIa inhibitor. However, when the plasma clot was compacted by centrifugation, the FXIIIa inhibitor had a strong inhibitory effect, with 7.7-fold less tissue-type plasminogen activator being required for 50% clot lysis in the presence of the FXIIIa inhibitor. In both experiments, the effects of the FXIIIa inhibitor were entirely dependent on the cross-linking of α_2-antiplasmin to fibrin. The FXIIIa inhibitor reduced the amount of α_2-antiplasmin present in the compacted clots from approximately 30% to < 4%. The results were confirmed with experiments in which compaction was achieved by platelet-mediated clot retraction. *Conclusions:* Compaction or retraction of fibrin clots reveals the strong antifibrinolytic effect of FXIII. This is explained by the cross-linking of α_2-antiplasmin to fibrin by FXIIIa, which prevents the plasmin inhibitor from being fully expelled from the clot during compaction/retraction.

Keywords: clot retraction; factor XIII; fibrinolysis; hemostasis; thrombosis.

Correspondence: Dingeman C. Rijken, Department of Hematology, Office Nb-845, Erasmus University Medical Center Rotterdam, P.O. Box 2040, 3000 CA Rotterdam, the Netherlands.
Tel.: +31 10 70 31 197; fax +31 10 70 44 745.
E-mail: d.rijken@erasmusmc.nl

Received 3 November 2015
Manuscript handled by: T. Lisman
Final decision: P. H. Reitsma, 15 April 2016

Introduction

Coagulation factor XIII strongly affects fibrin clot structure and function, and therefore plays a role in the pathophysiology of arterial and venous thromboembolic diseases [1]. FXIII is converted by thrombin into activated FXIII (FXIIIa), which is a transglutaminase that forms ε-(γ-glutamyl)lysyl cross-links between two polypeptide chains [2]. The enzyme stabilizes fibrin by the formation of primarily γ-chain dimers and α-chain polymers. Cross-linking makes fibrin clots insoluble in weak acid or urea [3], and modifies their mechanical properties by increasing the rigidity and elasticity [4,5]. This does not originate from alterations in network morphology, but most likely from stiffening of the individual fibers [6]. FXIII-mediated cross-linking probably tightens the coupling between the protofibrils within a fibrin fiber [7], which is associated with slightly thinner fibers and a higher fiber density in a clot [8].

Cross-linking might also affect the sensitivity of fibrin clots to fibrinolysis, a process that prevents blood clots from growing and occluding vessels by using the plasminogen–plasmin system [9]. However, this is a topic for which there has been a longlasting debate about both the magnitude of the effect of FXIIIa and the mechanisms involved. When we take a look at the literature, it is useful to distinguish studies that used purified fibrin clots

and studies that used plasma or whole blood clots. In purified systems, some authors found no effect of fibrin cross-linking on clot lysis [5,10], whereas other authors reported that the molecular cross-links producing α-chain polymers, in particular the very high molecular weight α-chain polymers obtained with elevated FXIIIa concentrations (possible provided by platelets), are associated with the inhibition of fibrinolysis [11]. However, others claimed that resistance to fibrinolysis was not induced by α-chain polymer formation or γ-chain dimer formation, but by γ-chain multimer formation, which only occurs over a period of hours to days [12]. In studies showing the inhibition of fibrinolysis by cross-linking, the effect is often small [8].

When plasma or whole blood is used, FXIIIa produces not only intramolecular cross-links within fibrin, but also intermolecular cross-links between fibrin and α_2-antiplasmin (α_2AP) [13]. This results in a significant inhibition of physiologically occurring fibrinolysis [14]. The relative contributions of fibrin–fibrin cross-links and fibrin–α_2AP cross-links to the inhibition of lysis of plasma or whole blood clots by FXIIIa are not yet clear. Jansen *et al*. [15] reported that fibrin–fibrin cross-linking does not contribute significantly to the resistance of blood clots to fibrinolysis, but that cross-linking of α_2AP to fibrin is essential. This was recently confirmed by Fraser *et al*. [16], who showed that the antifibrinolytic function of FXIII in plasma clots is exclusively expressed through α_2AP cross-linking to fibrin. In contrast, Reed *et al*. [17] reported that both fibrin–fibrin and α_2AP–fibrin cross-linking cause the resistance of experimental pulmonary emboli to fibrinolysis.

Another source of the varying results between different studies is the design of the clot lysis experiments [18]. However, it is still unclear why and how the expression of the inhibition of clot lysis by FXIIIa varies so strongly in different models and assays. Mutch *et al*. [19] showed that model thrombi formed under flow in a Chandler loop clearly reveal the effect of FXIII on fibrinolysis, but the essential feature of this model for the effect is still unknown. The present study deals with the mechanisms of the inhibition of clot lysis by FXIII, and shows that mechanical clot compaction, which is a model for physiologic clot retraction, is essential for fully revealing the inhibition of plasma clot lysis.

Materials and methods

Materials

Purified α_2AP was obtained from Calbiochem (San Diego, CA, USA), human thrombin, bovine serum albumin (BSA) and cytochalasin D (5 mg mL^{-1} in dimethylsulfoxide [DMSO]) were obtained from Sigma-Aldrich (St Louis, MO, USA), tissue factor (Innovin) was obtained from Dade Behring (Marburg, Germany), and recombinant tissue-type plasminogen activator (t-PA; Actilyse) was obtained from Boehringer Ingelheim (Ingelheim am Rein, Germany). The active site-directed transglutaminase/FXIIIa inhibitor 1,3-dimethyl-2-[(2-oxo-propyl)thio]imidazolium chloride (FXIIIa inhibitor) was obtained from Zedira (Darmstadt, Germany) (catalog number D003). The inhibitor was dissolved in 20% DMSO at a concentration of 900 mM, further diluted with water to 40 mM, and stored frozen in aliquots.

Human fibrinogen (depleted of plasminogen, von Willebrand factor, and fibronectin) was obtained from Enzyme Research Laboratories (South Bend, IN, USA), and labeled with fluorescein isothiocyanate (FITC) (Sigma-Aldrich), as described elsewhere [20]. The molar fluorescein/fibrinogen ratio was 6.6.

Pooled normal plasma was prepared from citrated platelet-poor plasma from 10 apparently healthy donors, and was always used when plasma or normal plasma is specified in this article. Frozen α_2AP-deficient plasma was obtained from Affinity Biologicals (Ancaster, Ontario, Canada). Platelet-rich plasma was prepared by centrifugation (265 × *g* for 10 min) of citrated blood from three apparently healthy donors, and each plasma was used within 1.5 h after preparation. The platelet count ranged from 346×10^9 L^{-1} to 399×10^9 L^{-1}.

Turbidity assay of plasma clot lysis

The turbidity assay was performed essentially as described previously [21]. However, clotting was induced by thrombin rather than by tissue factor and phospholipid vesicles. In short, plasma was diluted 1.7-fold in assay buffer (25 mM Hepes, 137 mM NaCl, 3.5 mM KCl, 1% [w/v] BSA, pH 7.4) at room temperature. The diluted plasma (85 μL) was added to the wells of a microtiter plate containing 15 μL of a reaction mixture, consisting of thrombin (0.2, 1.0 or 3.3 NIH U mL^{-1}), $CaCl_2$ (17 mM), t-PA (30 ng mL^{-1}) and FXIIIa inhibitor (0 mM or 1 mM) in assay buffer. The concentrations refer to the final concentrations in the 100-μL reaction volume. After mixing, each well was covered with 50 μL of paraffin oil, and the microtiter plate was placed into the preheated chamber of a microplate reader (Victor^3; PerkinElmer, Waltham, MA, USA), and the turbidity was measured as OD at 405 nm every minute for 120 min at 37 °C. The clot lysis time (CLT) was calculated as the time from the midpoint of minimum turbidity to maximum turbidity, which represents clot formation, to the midpoint of maximum turbidity to minimum turbidity, which represents clot lysis. When the clot formation was too rapid, the clotting time was roughly estimated. The assays were performed in triplicate.

Plasma clot lysis assay with and without clot compaction

Aliquots of 80 μL of plasma supplemented with FITC-labeled fibrinogen (100 μg mL^{-1}) were mixed in Eppendorf

tubes with 20 μL of a reaction mixture, consisting of tissue factor (Innovin, diluted 1000-fold), $CaCl_2$ (20 mM), t-PA (0–100 ng mL^{-1}) and FXIIIa inhibitor (0 mM or 1 mM) in turbidity assay buffer. The concentrations refer to the final concentrations in the 100-μL reaction volume. After incubation for 0.5 h at 37 °C, the clots were disconnected from the tube wall, and compacted by centrifugation for 1 min at 21 100 × *g*. The incubation was then continued for 2.5 h at 37 °C with shaking. In the plasma clot lysis assay without clot compaction, the clots were not centrifuged after 0.5 h, but incubated for 3 h at 37 °C. After the incubation, all clots were centrifuged for 1 min at 21 100 × *g*. Aliquots of 20 μL of the supernatants were mixed with 80 μL of Tris-buffered saline containing 0.01% Tween-20 (in triplicate), and the released fluorescence of FITC-labeled fibrin degradation products was determined in the microplate reader with 485 nm and 535 nm as the excitation and the emission wavelengths, respectively, to calculate the extent of lysis. The signal of fully lysed clots was set at 100% lysis. This signal was 13% higher than the signal of the corresponding amount of FITC-labeled fibrinogen, indicating that internal quenching of the fluorescence in FITC-labeled fibrin was decreased in FITC-labeled fibrin degradation products.

The assays were additionally performed with α_2AP-deficient plasma instead of normal plasma. In these experiments, the t-PA concentration range was 0–25 ng mL^{-1} (final concentrations in the 100-μL reaction volume). In control experiments, α_2AP-deficient plasma was reconstituted with 45 μg mL^{-1} purified α_2AP.

Plasma clot lysis assay with and without platelet-mediated clot retraction

Eppendorf tubes were treated with 1% Tween-20 in water for 5 min to prevent fibrin sticking to the wall. Aliquots of 80 μL of platelet-rich plasma supplemented with FITC-labeled fibrinogen (100 μg mL^{-1}) were mixed in these tubes with 20 μL of a reaction mixture, consisting of tissue factor (Innovin, diluted 1000-fold), $CaCl_2$ (20 mM), t-PA (0–100 ng mL^{-1}), cytochalasin D (0 μg mL^{-1} or 10 μg mL^{-1}) and FXIIIa inhibitor (0 mM or 1 mM) in turbidity assay buffer. The concentrations refer to the final concentrations in the 100-μL reaction volume. After incubation for 0.5 h at 37 °C, the incubation of the retracted clots (those without cytochalasin D) was continued for 2.5 h at 37 °C with shaking. The incubation of the non-retracted clots (those with cytochalasin D) was continued for 2.5 h at 37 °C without shaking. The extent of lysis was determined as described above for the compacted and non-compacted clots.

Cross-linking of α_2AP to fibrin

Clots were prepared essentially as in the plasma clot lysis assay with clot compaction, but with a two-fold increase in size. In more detail, aliquots of 160 μL of plasma were mixed in Eppendorf tubes with 40 μL of a reaction mixture, consisting of tissue factor (Innovin, diluted 1000-fold), $CaCl_2$ (20 mM) and FXIIIa inhibitor (0 mM or 1 mM) in turbidity assay buffer. The concentrations refer to the final concentrations in the 200-μL reaction volume. Controls did not contain tissue factor and $CaCl_2$. After incubation for 0.5 h at 37 °C, the clots were disconnected from the tube wall, and compacted by centrifugation for 1 min at 21 100 × *g*. The extents of compaction, as determined by weight analyses, amounted to 91.1% and 94.7%, respectively, for 0 mM and 1 mM FXIIIa inhibitor. The distribution of α_2AP after compaction was determined by measuring α_2AP activity in the clot supernatants, and by performing SDS-PAGE and western blotting of the compacted clots. To this end, the clots were washed five times with 500 μL of Tris-buffered saline containing 0.01% Tween-20 and 1 mM FXIIIa inhibitor, and dissolved in 160 μL of SDS sample buffer (Laemli) containing dithiothreitol for 15 min at 95 °C. SDS-PAGE was performed on 7.5% Criterion Tris-HCl gels (Biorad Laboratories, Hercules, CA, USA), and blots were stained with goat anti-human α_2AP IgG–horseradish peroxidase (Affinity Biologicals) and BM blue POD substrate (Sigma-Aldrich).

Miscellaneous

α_2AP activity was determined with a chromogenic substrate method (Berichrom α_2AP kit; Siemens Healthcare, Erlangen, Germany). Statistical analyses were performed with SPSS for Windows, version 17.0 (SPSS, Chicago, IL, USA), with the independent samples *t*-test. *P*-values of < 0.05 were considered to be statistically significant.

Results

Effect of FXIII in a turbidity assay of plasma clot lysis

The effect of FXIII on plasma clot lysis was assessed by using the transglutaminase/FXIIIa inhibitor 1,3-dimethyl-2-[(2-oxo-propyl)thio]imidazolium chloride (FXIIIa inhibitor). Citrated plasma was supplemented with t-PA, and clotted with calcium and three different concentrations of thrombin (0.2, 1.0 and 3.3 NIH U mL^{-1}) in the presence or absence of 1 mM FXIIIa inhibitor. Each thrombin concentration yielded a somewhat different but reproducible clot lysis profile (Fig. 1). FXIIIa inhibitor reduced the CLT from 43.9 ± 0.3 min to 37.8 ± 1.1 min (mean ± standard deviation, $n = 3$, $P = 0.001$; 14% reduction) at 0.2 NIH U mL^{-1} thrombin, from 70.2 ± 15.4 min to 55.2 ± 4.8 min ($P = 0.18$; 21% reduction) at 1.0 NIH U mL^{-1} thrombin, and from 75.3 ± 5.6 min to 61.8 ± 1.8 min ($P = 0.017$, 18% reduction) at 3.3 NIH U mL^{-1} thrombin. These results showed that FXIIIa inhibitor slightly accelerated clot lysis under all conditions, implying

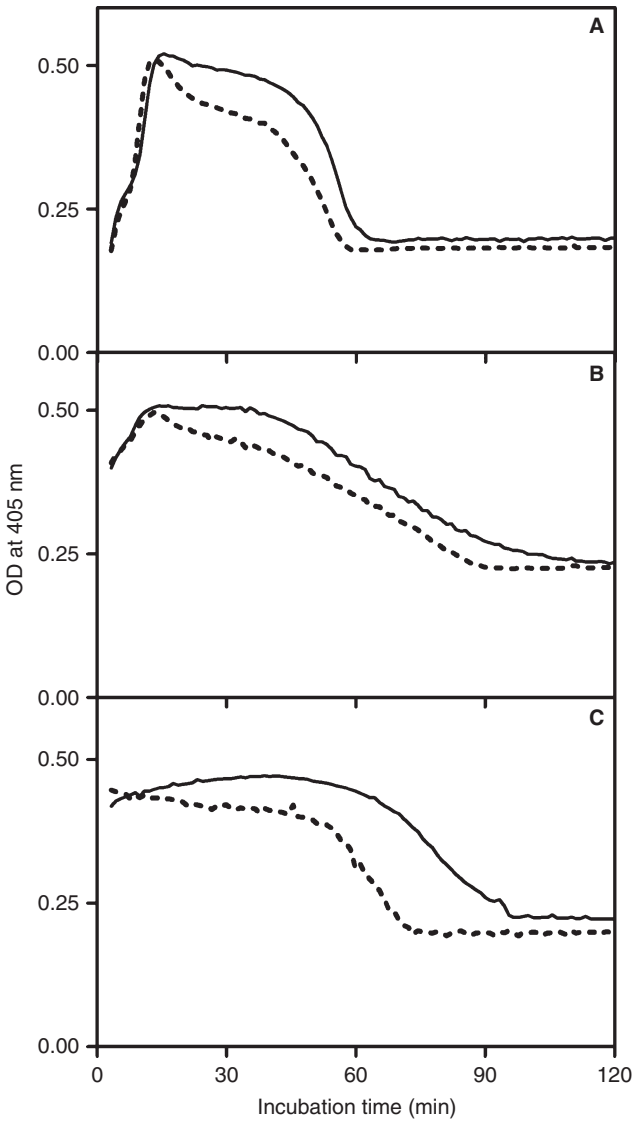

Fig. 1. Effect of activated factor XIII (FXIIIa) inhibitor on plasma clot lysis in a turbidity assay. Three different thrombin concentrations were used to clot plasma containing 30 ng mL^{-1} tissue-type plasminogen activator: (A) 0.2 NIH U mL^{-1}; (B) 1.0 NIH U mL^{-1}; (C) 3.3 NIH U mL^{-1}). The clot lysis profiles were monitored by measuring the OD at 405 nm during an incubation period of 120 min. Representative examples of the experiments are shown. Solid lines: in the absence of FXIIIa inhibitor. Dotted lines: in the presence of 1 mM FXIIIa inhibitor.

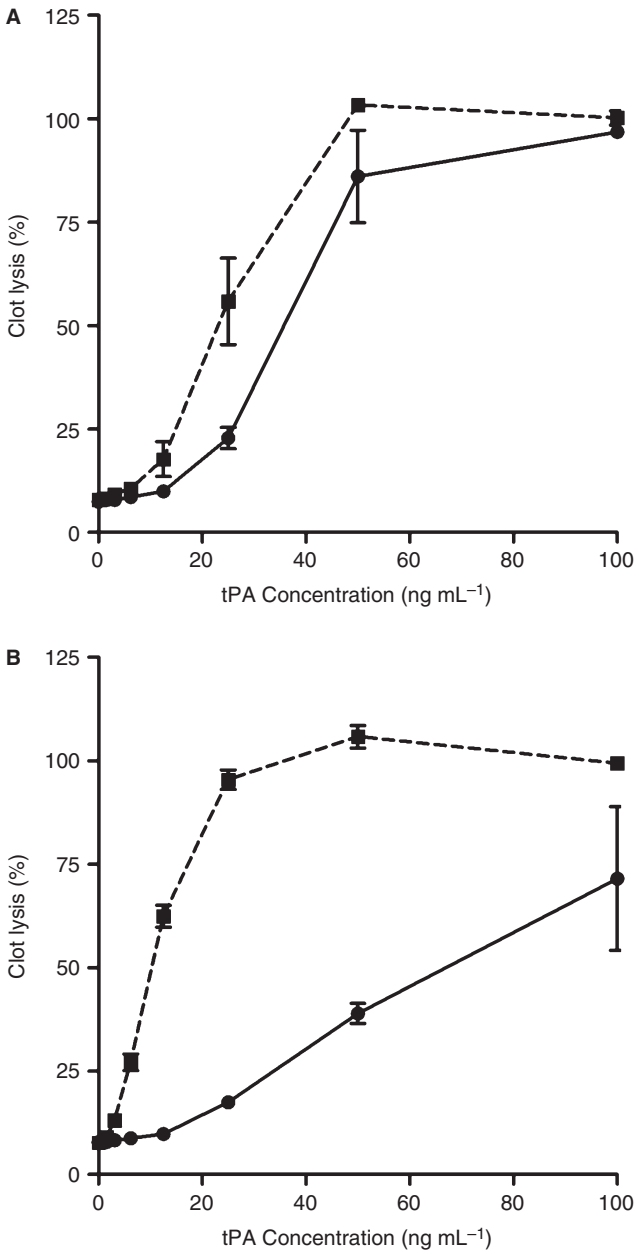

Fig. 2. Effect of activated factor XIII (FXIIIa) inhibitor on plasma clot lysis in a fluorometric assay. Plasma supplemented with fluorescein isothiocyanate-labeled fibrinogen was clotted in the presence of varying concentrations of tissue-type plasminogen activator (t-PA), and incubated for 3 h without (A) or with (B) mechanical compaction of the clot after 30 min. The experiments were carried out in triplicate by performing them on three different days, and clot lysis is shown as mean ± standard error of the mean. Solid lines: in the absence of FXIIIa inhibitor. Dotted lines: in the presence of 1 mM FXIIIa inhibitor.

that FXIII inhibited fibrinolysis to a small extent in this assay.

Effect of FXIII on plasma clot lysis with and without clot compaction

The effect of FXIII on plasma clot lysis was additionally assessed in an assay that allowed experiments with and without mechanical clot compaction. Without clot compaction, 50% clot lysis was obtained at 36.8 ± 3.5 ng mL^{-1} t-PA in the absence of FXIIIa inhibitor and at 23.6 ± 5.8 ng mL^{-1} t-PA, i.e. 1.6-fold less t-PA, in the presence of FXIIIa inhibitor (Fig. 2A; Table 1; $P = 0.028$). These results showed that FXIIIa inhibitor slightly but significantly accelerated clot lysis, implying that FXIII inhibited fibrinolysis only to a small extent in

Table 1 Plasma clot composition and tissue-type plasminogen activator (t-PA) concentrations required for 50% clot lysis, derived from the experiments shown in Figs 2–4

Plasma clot composition				t-PA concentration required for 50% lysis		
Plasma	Clot compaction/ retraction	FXIIIa inhibitor	Number of experiments	Mean ± SD (ng mL^{-1})	Ratio (− inh/+ inh)	*P*-value (− inh versus + inh)
Normal	−	−	3	36.8 ± 3.5	1.6	0.028*
Normal	−	+	3	23.6 ± 5.8		
Normal	+	−	3	80.0 ± 22.6	7.7	0.006*
Normal	+	+	3	10.4 ± 1.9		
α_2AP-deficient	−	−	4	5.2 ± 2.2	0.9	0.853
α_2AP-deficient	−	+	4	5.5 ± 2.1		
α_2AP-deficient	+	−	4	3.0 ± 0.1	1.0	0.898
α_2AP-deficient	+	+	4	2.9 ± 1.1		
Platelet-rich	−	−	3	72.0 ± 14.7	1.3	0.172
Platelet-rich	−	+	3	55.1 ± 9.6		
Platelet-rich	+	−	3	59.3 ± 12.1	4.2	0.004*
Platelet-rich	+	+	3	14.3 ± 4.1		

α_2AP, α_2-antiplasmin; FXIIIa, activated factor XIIIa; inh, FXIIIa inhibitor; SD, standard deviation. *Statistically significant difference.

this assay without compaction, similarly as in the turbidity assay.

With clot compaction, 50% clot lysis was obtained at 80.0 ± 22.6 ng mL^{-1} t-PA in the absence of FXIIIa inhibitor and at 10.4 ± 1.9 ng mL^{-1} t-PA, i.e. 7.7-fold less t-PA, in the presence of FXIII inhibitor (Fig. 2B; Table 1; $P = 0.006$). These results showed that FXIIIa inhibitor strongly accelerated clot lysis, implying that FXIII effectively inhibited fibrinolysis in this assay with clot compaction, far more than in the assay without clot compaction.

Role of α_2AP

In order to determine to what extent the cross-linking of α_2AP to fibrin was responsible for the inhibition of plasma clot lysis by FXIIIa, assays similar to those shown in Fig. 2A,B were performed with α_2AP-deficient plasma rather than normal plasma.

Without clot compaction, 50% clot lysis was obtained at 5.2 ± 2.2 ng mL^{-1} t-PA in the absence of FXIIIa inhibitor and at 5.5 ± 2.1 ng mL^{-1} t-PA in the presence of FXIIIa inhibitor (Fig. 3A; Table 1; $P = 0.853$). With clot compaction, 50% clot lysis was obtained at 3.0 ± 0.1 ng mL^{-1} t-PA in the absence of FXIIIa inhibitor and at 2.9 ± 1.1 ng mL^{-1} t-PA in the presence of FXIII inhibitor (Fig. 3B; Table 1; $P = 0.898$). These results showed that FXIIIa inhibitor had no significant effects on clot lysis under these conditions, either with or without clot compaction.

To prove that the absence of an effect of FXIIIa inhibitor was really attributable to the deficiency of α_2AP and not to other variations in the plasma, α_2AP-deficient plasma was supplemented with 45 μg mL^{-1} purified α_2AP, and plasma clot lysis was measured with clot compaction. In the presence of FXIIIa inhibitor, 50% clot lysis was obtained at a 6.0-fold lower t-PA concentration than in the absence of FXIIIa inhibitor (data not shown), which is close to the factor of 7.7 obtained with normal plasma (Table 1).

These results indicated that the inhibitory effects of FXIII on fibrinolysis can be fully ascribed to cross-linking of α_2AP to fibrin, both in the assay with clot compaction and in the assay without clot compaction.

Effect of FXIII on plasma clot lysis with and without platelet-mediated clot retraction

The effect of FXIII on plasma clot lysis was also assessed with platelet-rich plasma samples from three different donors. Clot retraction occurred within 20 min. Control clots that did not retract were prepared in the presence of cytochalasin D. With these non-retracted clots, 50% clot lysis was obtained at 72.0 ± 14.7 ng mL^{-1} t-PA in the absence of FXIIIa inhibitor and at 55.1 ± 9.6 ng mL^{-1} t-PA, i.e. 1.3-fold less t-PA, in the presence of FXIIIa inhibitor (Fig. 4A; Table 1; $P = 0.172$). These results showed that FXIIIa inhibitor slightly accelerated clot lysis in each individual donor, although statistical significance was not reached.

With clot retraction, 50% clot lysis was obtained at 59.3 ± 12.1 ng mL^{-1} t-PA in the absence of FXIIIa inhibitor and at 14.3 ± 4.1 ng mL^{-1} t-PA, i.e. 4.2-fold less t-PA, in the presence of FXIII inhibitor (Fig. 4B; Table 1; $P = 0.004$). These results showed that FXIIIa inhibitor strongly accelerated clot lysis, implying that FXIII effectively inhibited fibrinolysis in this assay with clot retraction, far more than in the assay without clot retraction.

Distribution of α_2AP after clot compaction

To obtain a better understanding of why compacted clots fully revealed the antifibrinolytic and α_2AP-dependent effect of FXIII, the distribution of α_2AP after clot

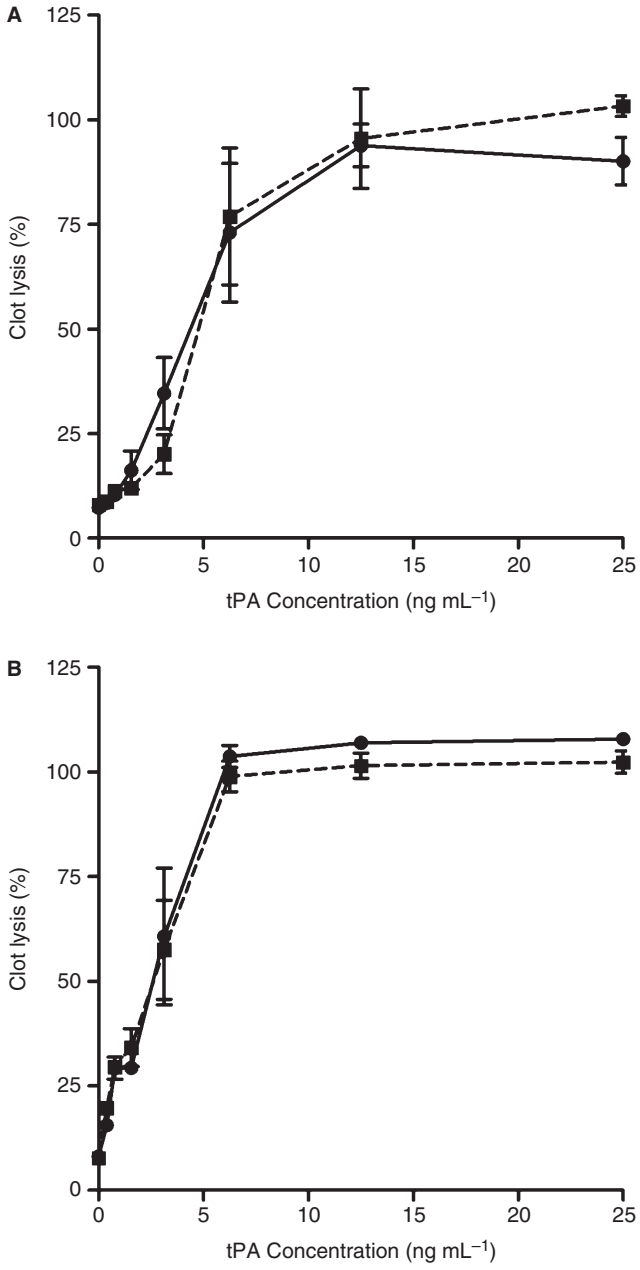

Fig. 3. Effect of activated factor XIII (FXIIIa) inhibitor on plasma clot lysis without α_2-antiplasmin (α_2AP) in a fluorometric assay. α_2AP-deficient plasma supplemented with fluorescein isothiocyanate-labeled fibrinogen was clotted in the presence of varying concentrations of tissue-type plasminogen activator (t-PA), and incubated for 3 h without (A) or with (B) mechanical compaction of the clot after 30 min. The experiments were carried out in quadruplicate by performing them on four different days, and clot lysis is shown as mean ± standard error of the mean. Solid lines: in the absence of FXIIIa inhibitor. Dotted lines: in the presence of 1 mM FXIIIa inhibitor.

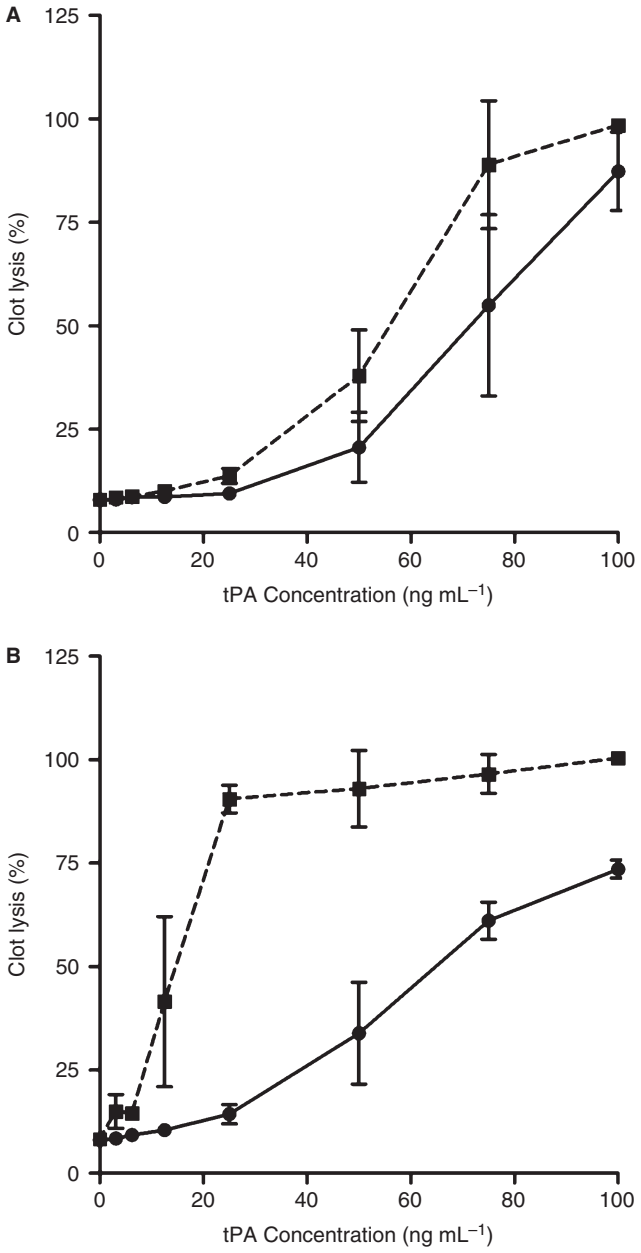

Fig. 4. Effect of activated factor XIII (FXIIIa) inhibitor on platelet-rich plasma clot lysis in a fluorometric assay. Platelet-rich plasma supplemented with fluorescein isothiocyanate-labeled fibrinogen was clotted in the presence of varying concentrations of tissue-type plasminogen activator (t-PA), and incubated for 3 h with (A) or without (B) cytochalasin D, which prevents clot retraction. The experiments were carried out in triplicate by performing them on three different days with platelet-rich plasma from three different donors, and clot lysis is shown as mean ± standard error of the mean. Solid lines: in the absence of FXIIIa inhibitor. Dotted lines: in the presence of 1 mM FXIIIa inhibitor.

compaction was determined. As shown in Fig. 5A, the supernatant of a compacted plasma clot contained 68.8% α_2AP of the unclotted control plasma, indicating that 31.2% remained in the clot. In contrast, in the presence of FXIIIa inhibitor, the supernatant contained 96.1%, indicating that only 3.9% remained in the clot, and that nearly all α_2AP was expelled from the clot during compaction. In other words, FXIII ensures that a significant amount of α_2AP remains in the clot during compaction. This was confirmed in the experiment shown in Fig. 5B.

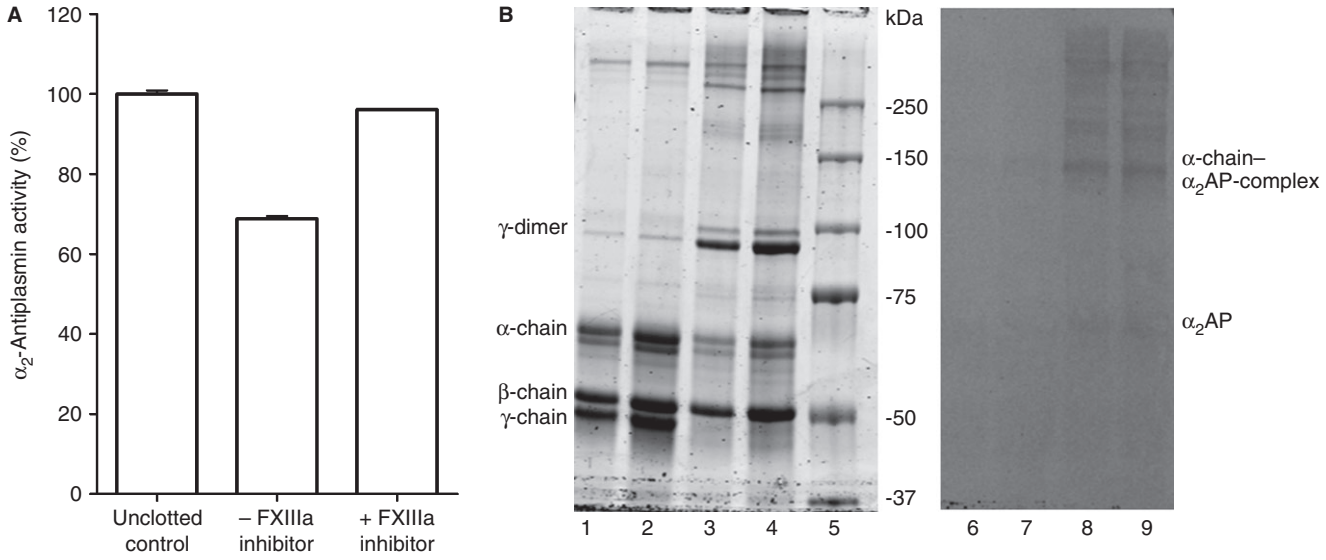

Fig. 5. Effect of activated factor XIII (FXIIIa) inhibitor on α_2-antiplasmin (α_2AP) during clot compaction. Plasma was clotted for 30 min in the absence or presence of 1 mM FXIIIa inhibitor, and the resulting clots were compacted by centrifugation. (A) α_2AP activity was measured in the supernatants, and compared with the activity in the unclotted plasma control, which amounted to 0.91 U mL^{-1} (100%). The experiments were performed in triplicate, and the results are shown as mean ± standard deviation. The standard deviations were small, and are hardly visible in the bars. (B) The compacted clots were washed and dissolved, and then analysed by SDS-PAGE (lanes 1–5) and western blotting with antibodies against α_2AP (lanes 6–9). Lanes 1 and 2: 2 μL and 4 μL of dissolved clot with FXIIIa inhibitor. Lanes 3 and 4: 2 μL and 4 μL of dissolved clot without FXIIIa inhibitor. Lane 5: protein standards. Lanes 6 and 7: 10 μL and 20 μL of dissolved clot with FXIIIa inhibitor. Lanes 8 and 9: 10 μL and 20 μL of dissolved clot without FXIII inhibitor. The estimated positions of the α-chain (doublet), β-chain and γ-chain of fibrin on the gel are indicated, as are the estimated positions of α-chain–α_2AP-complex and free α_2AP on the blot.

Lanes 1–4 showed that, in the absence of FXIIIa inhibitor, the fibrin γ-chain was fully converted into γ-dimer, and the fibrin α-chain partially into α-polymers (> 150 kDa). Lanes 6–9 showed no α_2AP in the clots prepared in the presence of FXIIIa inhibitor, but significant amounts of α_2AP in complex with α-chain and α-polymers in the clots prepared in the absence of FXIIIa inhibitor. These experiments also showed that the concentration of FXIIIa inhibitor (1 mM) was sufficiently high to block FXIIIa, and that there was hardly any clot binding or incorporation of α_2AP in the absence of cross-linking.

Discussion

This study further elucidates the mechanisms of the antifibrinolytic effect of FXIII, and clarifies the literature on this topic. Lysis of plasma clots without compaction, as tested in the turbidity assay (Fig. 1) and in the assay with FITC-labeled fibrin (Fig. 2A), was slightly but significantly inhibited by FXIIIa. In the latter assay, a 1.6-fold higher t-PA concentration was required for 50% lysis in the presence of (endogenous) FXIIIa than in the absence of FXIIIa (Table 1). As the inhibition of clot lysis by FXIIIa was absent in clots from α_2AP-deficient plasma (Fig. 3A), the inhibition was caused by cross-linking of α_2AP to fibrin, and not by molecular cross-links within fibrin. Our results imply that fibrin-bound α_2AP is a more efficient inhibitor of t-PA-induced fibrinolysis than free α_2AP in solution. The likely mechanism is that fibrin-bound α_2AP is localized on the fibrin surface, where t-PA-induced plasminogen activation occurs and plasmin has to perform its function. Our results are largely in agreement with those of Sakata *et al*. [14], although this study did not present data for non-compacted clots. Other studies failed to show reproducible effects of FXIII on lysis of non-compacted plasma clots [19], probably because the effects are small.

α_2AP circulates in two forms: a native form with a methionine (Met) at the N-terminus, and a 12-residue smaller form with an asparagine (Asn) at the N-terminus [22–24]. Met-α_2AP becomes cross-linked to fibrin approximately 13 times more slowly than Asn-α_2AP, resulting in a strongly (~ 50%) decreased CLT in a turbidity assay [22]. However, the latter observation seems to be at odds with the small effect of a total blockade of cross-linking with the FXIIIa inhibitor in the turbidity assay of the present study (14–21% decrease), and requires more research.

The most striking result of our study was that lysis of compacted plasma clots was strongly inhibited by FXIIIa. In the presence of (endogenous) FXIIIa, a 7.7-fold higher t-PA concentration was required for 50% lysis than in the absence of FXIIIa (Table 1). Again, as the inhibition was not found in clots from α_2AP-deficient plasma (Fig. 3B), the inhibition was caused by cross-linking of α_2AP to fibrin, and not by molecular cross-links within fibrin. Lysis of compacted clots was strongly inhibited by FXIII, most likely because FXIIIa prevented α_2AP from

being expelled from the clot during compaction. In the presence of FXIIIa, 31.2% of the α_2AP remained associated with the clot, whereas in the absence of FXIIIa only a negligible amount, i.e. 3.9%, of α_2AP remained in the clot (Fig. 5). The very low clot binding or incorporation of α_2AP in the absence of cross-linking does not confirm the strong non-covalent interaction of α_2AP with fibrin as observed with surface plasmon resonance [25]. The present study shows that two mechanisms are involved in the inhibition of lysis of compacted plasma clots by FXIIIa: (i) cross-linked α_2AP is a more efficient inhibitor of plasmin generated by t-PA on the fibrin surface than free α_2AP (small effect); and (ii) cross-linking of α_2AP ensures that a significant proportion of α_2AP remains associated with the clot during compaction (large effect).

These mechanisms are consistent with the observation that a non-cross-linking derivative of α_2AP produced significantly less inhibition of lysis of compacted plasma clots than native α_2AP [14]. The critical role of compaction explains a long-standing controversy between Gaffney and Whitaker [26], who found a strong inhibitory effect of FXIII on lysis of plasma clots harvested by being wound onto a glass rod (i.e. compaction), and Rampling and Flexman [18], who found no inhibitory effect when testing undisturbed plasma clots (i.e. no compaction). The role of compaction could also explain a more recent observation that model thrombi formed under flow in a Chandler loop reveal the inhibitory effect of FXIII on fibrinolysis [19]. Our results indicate that it is not the flow that represents the essential feature, but the compaction of the clots that coincidentally takes place in a Chandler loop. Flow in general is not necessarily associated with compaction, and should not be considered as being essential for the expression of the antifibrinolytic effect of FXIII.

The Chandler thrombus was presented as a model in which the effects of cross-linking on fibrinolysis can be determined quantitatively [19]. This model does indeed seem to be convenient for resolving many remaining questions concerning the role of cross-linking in fibrinolysis. However, for testing large numbers of samples, e.g. from patient studies, the model would be less convenient. For this purpose, mechanical compaction of clots by a simple centrifugation step, as performed in this study, may be more suitable.

As mentioned above, no inhibition of fibrinolysis by FXIIIa could be demonstrated with clots prepared from α_2AP-deficient plasma, either with or without clot compaction. This suggests that the inhibition resulting from the formation of fibrin–fibrin cross-links was too small for detection in this system, and agrees with earlier reports that fibrin–fibrin cross-linking does not contribute significantly to the resistance of clots to fibrinolysis [15,16]. However, we cannot exclude the possibility that stronger cross-linking resulting in very high molecular weight α-chain polymers [11] and/or γ-chain multimers [12] would have resulted in resistance to fibrinolysis independently of α_2AP. The latter structures could also have been responsible for the observed resistance of experimental pulmonary emboli to fibrinolysis by fibrin–fibrin cross-links [17].

This study shows that the inhibition of fibrinolysis by FXIIIa is most pronounced in fibrin clots that are compacted, because, in these clots, FXIIIa determines the amount of α_2AP that remains associated with the clot (Fig. 6). *In vivo* clot compaction occurs by platelet-mediated clot retraction. The clot lysis results of this study were confirmed with experiments in which compaction was achieved by platelet-mediated clot retraction rather than by centrifugation (Fig. 4), suggesting that this study has *in vivo* relevance.

It is anticipated that the timing of α_2AP cross-linking and clot retraction may be important. *In vitro* experiments have shown that α_2AP cross-linking occurs within a few minutes, whereas clot retraction requires dozens of minutes, suggesting that α_2AP cross-linking is nearly complete before clot retraction starts. However, *in vivo* data are limited, particularly in pathologic conditions. In theory, both delayed α_2AP cross-linking and accelerated retraction could result in an α_2AP-poor clot, possibly leading to faster clot lysis and a bleeding tendency. Not only the timing but also the extent of α_2AP cross-linking to fibrin could regulate fibrinolysis. *In vitro* data from previous studies [13,27] and from the present study (Fig. 5A) show that only a proportion of α_2AP is cross-linked to the fibrin clot, suggesting that both increased and decreased cross-linking could occur *in vivo*, potentially leading to a thrombotic tendency and a bleeding tendency, respectively. However, it is still not known how strongly the cross-linking of α_2AP varies among healthy individuals or patients. Only a few preliminary reports have dealt with this topic [28–31], and more clinical research is needed.

Addendum

D. C. Rijken conceived and designed the study, and wrote the manuscript. S. Abdul interpreted the data and

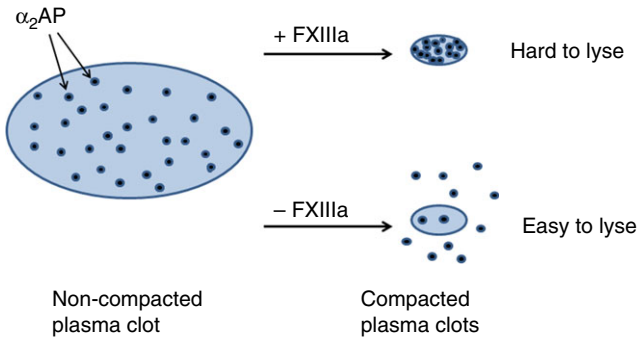

Fig. 6. Activated factor XIII (FXIIIa) regulates the amount of α_2-antiplasmin (α_2AP) in compacted clots, and thereby their lysibility by plasmin.

critically revised the manuscript. J. J. M. C. Malfliet performed the experiments and critically revised the manuscript. F. W. G. Leebeek critically revised the manuscript. S. Uitte de Willige designed the study, interpreted the data, and critically revised the manuscript.

Acknowledgements

This research was supported by a grant from the Dutch Thrombosis Foundation (TSN 2009-1). S. Uitte de Willige was funded by the EHA-ISTH Joint Fellowship 2013.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

References

1 Undas A, Ariens RA. Fibrin clot structure and function: a role in the pathophysiology of arterial and venous thromboembolic diseases. *Arterioscler Thromb Vasc Biol* 2011; **31**: e88–99.
2 Muszbek L, Bereczky Z, Bagoly Z, Komaromi I, Katona E. Factor XIII: a coagulation factor with multiple plasmatic and cellular functions. *Physiol Rev* 2011; **91**: 931–72.
3 Lorand L. Fibrin clots. *Nature* 1950; **166**: 694–5.
4 Shen LL, Hermans J, McDonagh J, McDonagh RP, Carr M. Effects of calcium ion and covalent crosslinking on formation and elasticity of fibrin cells. *Thromb Res* 1975; **6**: 255–65.
5 Gladner JA, Nossal R. Effects of crosslinking on the rigidity and proteolytic susceptibility of human fibrin clots. *Thromb Res* 1983; **30**: 273–88.
6 Ryan EA, Mockros LF, Weisel JW, Lorand L. Structural origins of fibrin clot rheology. *Biophys J* 1999; **77**: 2813–26.
7 Kurniawan NA, Grimbergen J, Koopman J, Koenderink GH. Factor XIII stiffens fibrin clots by causing fiber compaction. *J Thromb Haemost* 2014; **12**: 1687–96.
8 Hethershaw EL, Cilia La Corte AL, Duval C, Ali M, Grant PJ, Ariens RA, Philippou H. The effect of blood coagulation factor XIII on fibrin clot structure and fibrinolysis. *J Thromb Haemost* 2014; **12**: 197–205.
9 Rijken DC, Lijnen HR. New insights into the molecular mechanisms of the fibrinolytic system. *J Thromb Haemost* 2009; **7**: 4–13.
10 Haverkate F. Lysis of crosslinked and non-crosslinked purified fibrin. *Thromb Diath Haemost* 1975; **34**: 584–5.
11 Francis CW, Marder VJ. Increased resistance to plasmic degradation of fibrin with highly crosslinked alpha-polymer chains formed at high factor XIII concentrations. *Blood* 1988; **71**: 1361–5.
12 Siebenlist KR, Mosesson MW. Progressive cross-linking of fibrin gamma chains increases resistance to fibrinolysis. *J Biol Chem* 1994; **269**: 28414–19.
13 Sakata Y, Aoki N. Cross-linking of alpha 2-plasmin inhibitor to fibrin by fibrin-stabilizing factor. *J Clin Invest* 1980; **65**: 290–7.
14 Sakata Y, Aoki N. Significance of cross-linking of alpha 2-plasmin inhibitor to fibrin in inhibition of fibrinolysis and in hemostasis. *J Clin Invest* 1982; **69**: 536–42.
15 Jansen JW, Haverkate F, Koopman J, Nieuwenhuis HK, Kluft C, Boschman TA. Influence of factor XIIIa activity on human whole blood clot lysis in vitro. *Thromb Haemost* 1987; **57**: 171–5.
16 Fraser SR, Booth NA, Mutch NJ. The antifibrinolytic function of factor XIII is exclusively expressed through alpha(2)-antiplasmin cross-linking. *Blood* 2011; **117**: 6371–4.
17 Reed GL, Houng AK. The contribution of activated factor XIII to fibrinolytic resistance in experimental pulmonary embolism. *Circulation* 1999; **99**: 299–304.
18 Rampling MW, Flexman C. Factor XIII cross-linking and fibrinolytic rates. *Thromb Res* 1979; **16**: 877–80.
19 Mutch NJ, Koikkalainen JS, Fraser SR, Duthie KM, Griffin M, Mitchell J, Watson HG, Booth NA. Model thrombi formed under flow reveal the role of factor XIII-mediated cross-linking in resistance to fibrinolysis. *J Thromb Haemost* 2010; **8**: 2017–24.
20 Sakharov DV, Nagelkerke JF, Rijken DC. Rearrangements of the fibrin network and spatial distribution of fibrinolytic components during plasma clot lysis. Study with confocal microscopy. *J Biol Chem* 1996; **271**: 2133–8.
21 Talens S, Malfliet JJ, Rudez G, Spronk HM, Janssen NA, Meijer P, Kluft C, de Maat MP, Rijken DC. Biological variation in tPA-induced plasma clot lysis time. *Thromb Haemost* 2012; **108**: 640–6.
22 Lee KN, Jackson KW, Christiansen VJ, Chung KH, McKee PA. A novel plasma proteinase potentiates alpha2-antiplasmin inhibition of fibrin digestion. *Blood* 2004; **103**: 3783–8.
23 Uitte de Willige S, Malfliet JJ, Deckers JW, Dippel DW, Leebeek FW, Rijken DC. Plasma levels of soluble fibroblast activation protein in arterial thrombosis: determinants and cleavage of its substrate alpha-2-antiplasmin. *Int J Cardiol* 2015; **178**: 105–10.
24 Uitte de Willige S, Malfliet JJ, Janssen HL, Leebeek FW, Rijken DC. Increased N-terminal cleavage of alpha-2-antiplasmin in patients with liver cirrhosis. *J Thromb Haemost* 2013; **11**: 2029–36.
25 Tsurupa G, Yakovlev S, McKee P, Medved L. Noncovalent interaction of alpha(2)-antiplasmin with fibrin(ogen): localization of alpha(2)-antiplasmin-binding sites. *Biochemistry* 2010; **49**: 7643–51.
26 Gaffney PJ, Whitaker AN. Fibrin crosslinks and lysis rates. *Thromb Res* 1979; **14**: 85–94.
27 Tamaki T, Aoki N. Cross-linking of alpha 2-plasmin inhibitor and fibronectin to fibrin by fibrin-stabilizing factor. *Biochim Biophys Acta* 1981; **661**: 280–6.
28 Schroder V, Kohler HP. Effect of factor XIII Val34Leu on alpha2-antiplasmin incorporation into fibrin. *Thromb Haemost* 2000; **84**: 1128–30.
29 Dunn EJ, Philippou H, Ariens RA, Grant PJ. Molecular mechanisms involved in the resistance of fibrin to clot lysis by plasmin in subjects with type 2 diabetes mellitus. *Diabetologia* 2006; **49**: 1071–80.
30 Agren A, Jorneskog G, Elgue G, Henriksson P, Wallen H, Wiman B. Increased incorporation of antiplasmin into the fibrin network in patients with type 1 diabetes. *Diabetes Care* 2014; **37**: 2007–14.
31 Rijken DC, Uitte de Willige S. Comment on Agren et al. Increased incorporation of antiplasmin into the fibrin network in patients with type 1 diabetes. *Diabetes Care* 2014;**37**:e243.2007–2014.