

# Binding of Coagulation Factor XIII Zymogen to Activated Platelet Subpopulations: Roles of Integrin $\alpha_{IIb}\beta_3$ and Fibrinogen

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## Abstract

Factor XIIIa (fXIIIa) is a transglutaminase that plays a crucial role in fibrin clot stabilization and regulation of fibrinolysis. It is known to bind to procoagulant platelets. In contrast, the zymogen fXIII interaction with platelets is not well characterized. We investigated the interaction of zymogen fXIII with activated platelet subpopulations. Confocal microscopy and flow cytometry using fluorescently labelled factors and antibodies. Phosphatidylserine (PS)-positive activated platelets bound 700 to 800 molecules/cell of fXIII at 100 nM, while both PS-negative activated platelets and resting platelets bound 200 to 400 molecules/cell. The binding was reversible, calcium-independent and linear within the fXIII concentration range of up to 1,000 nM. fXIII predominantly bound to the caps of procoagulant platelets and co-localized with fibrinogen. Exogenous fibrinogen promoted fXIII binding by activated PS-negative platelets; this effect was abolished by the integrin  $\alpha_{IIb}\beta_3$  antagonist monafra. The fXIII binding was 1.5- to 3-fold decreased for platelets from four patients with grey platelet syndrome, and was variable for platelets from six patients with Glanzmann's thrombasthenia. Strong platelet stimulation, fibrinogen and  $\alpha_{IIb}\beta_3$  play essential roles in fXIII binding, without any of them fXIII does not bind to platelets. The preferential binding in the cap-like structures might be important for increasing local fXIII concentration in platelet thrombi.

## Keywords

- ▶ factor XIII
- ▶ platelet subpopulations
- ▶ integrin  $\alpha_{IIb}\beta_3$
- ▶ fibrinogen
- ▶ Glanzmann's thrombasthenia

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## Introduction

Coagulation factor XIII (fXIII) is a proenzyme circulating in blood at 14 to 28 µg/mL (~88 nM). Its active form, the transglutaminase fXIIIa, is a critical regulator of fibrinolysis. fXIII is present in two forms: plasma-derived fXIII is a tetramer of two A- and two B-subunits that are non-covalently associated, and platelet-derived fXIII contains only two A-subunits.<sup>1</sup> Plasma-derived fXIII activation proceeds via two steps. Thrombin first cleaves a 4-kD activation peptide from each of the A-subunits, followed by calcium-dependent conformational change leading to dissociation of B-subunits and fXIIIa release.<sup>2</sup> The cellular counterpart is also activated by the combined action of thrombin and Ca<sup>2+</sup> to form the active transglutaminase.<sup>3,4</sup> Active transglutaminase fXIIIa carries out a number of reactions.<sup>5</sup> Of these, the fibrin (ogen) fibrils crosslinking is important for regulation of fibrinolysis<sup>2</sup> and mediating red blood cell retention in venous thrombi.<sup>6</sup>

Platelet-derived fXIIIa constitutes up to 50% of total blood fXIIIa. Two possible functions have been considered for the platelet transglutaminase: stabilization of intracellular motors' interaction with cytoskeleton upon activation,<sup>7</sup> and participation in extracellular anti-fibrinolytic process<sup>8</sup> or even fibrin formation.<sup>9</sup> In addition, platelet fXIIIa may contribute to thrombosis through the formation of procoagulant platelets.<sup>10,11</sup> Together with platelet-derived tissue transglutaminase (tTG), fXIIIa may participate in the retention of α-granule proteins in the 'cap'-like structures<sup>12,13</sup> of the phosphatidylserine (PS)-positive (procoagulant) platelet subpopulation,<sup>14,15</sup> thus contributing to the formation of a procoagulant surface.

Interaction of plasma-derived fXIII, mostly of its activated form fXIIIa, with platelets and fibrin(ogen) was studied by several groups.<sup>2,9,16,17</sup> The reports about the putative binding site for fXIIIa on platelets are not fully in agreement: some of them concluded the essential role of integrin αIIbβ3 and fibrin (ogen)<sup>18</sup>; other considered that integrin αIIbβ3 and platelet-bound fibrin(ogen) were not the binding site for fXIIIa<sup>16</sup>; another has reported that binding of fXIIIa to thrombin-activated platelets was inhibited by plasmin<sup>19</sup>; also, interesting to note that the study of the fXIII binding using antibodies against subunit A showed fXIII significantly increased on thrombin receptor-activating peptide-activated platelets in whole blood compared to unstimulated sample, but not in washed platelets.<sup>17</sup> However, these studies did not take into account the activated platelet heterogeneity, and did not study quantitative binding of zymogen fXIII. The objective of this study was therefore to quantitatively investigate fXIII zymogen binding to the PS-positive and PS-negative subpopulations of activated platelets. These subpopulations were recently shown to be heterogeneously distributed in arterial thrombi<sup>20</sup> and this also raises a question about distribution of fXIII.

## Methods

**Reagents:** The following materials were obtained from the sources shown in parentheses: prostaglandin E1 (MP Bio-

chemicals, Irvine, California, United States); Fura-Red, fluorescein-5-isothiocyanate (FITC), Alexa Fluor 647 (Molecular Probes, Eugene, Oregon, United States); Alexa Fluor 647-conjugated annexin V, FITC-conjugated annexin V, Alexa Fluor 647-conjugated CD62P antibody (Biolegend, San Diego, California, United States); D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) (Calbiochem, San Diego, California, United States); FITC-conjugated anti-human fibrinogen antibody (Labvision, Fremont, California, United States); anti-fXIIIa immunoglobulin G (IgG) (clone AC-1A1) and anti-tTG Ig (clone CUB7402) (Thermo Scientific, Rockford, Illinois, United States); anti-Fibrinogen IgG (clone 15H12) (Abcam, Cambridge, United Kingdom); and R-Phycoerythrin AffiniPure F(ab')<sub>2</sub> Fragment Goat Anti-Mouse IgG (H + L) (Jackson ImmunoResearch, Pennsylvania, United States). Integrin αIIbβ3 antagonist Monafam (F(ab')<sub>2</sub> fragment of a monoclonal antibody that blocks integrin αIIbβ3 activity) was kindly provided by A. V. Mazurov (Russian Cardiology Research and Production Complex, Russia).<sup>21</sup> Collagen-related peptide (CRP) was kindly provided by Prof. R. W. Farndale (University of Cambridge, Cambridge, United Kingdom). Green fluorescent protein (GFP)-conjugated calibration beads were kindly provided by Dr. Alexei L. Drobyshev (Center for Theoretical Problems of Physicochemical Pharmacology, Moscow, Russia). All other reagents including fibrinogen were from Sigma-Aldrich (Sigma-Aldrich, St Louis, Missouri, United States).

**Patients: Dysfibrinogenemia (DF)**—The patient with DF had a homozygous fibrinogen Metz mutation (p.Arg16Cys) in the Aα chain preventing fibrinopeptide A release, so no fibrin clot formation was observed, although fibrinopeptide B was released normally.<sup>13</sup> Platelet aggregation was normal. The patient suffered from severe bleeding and had a history of fibrinogen transfusions; the last transfusion was 3 years before this study.

**Glanzmann thrombasthenia (GT):** Eight GT patients were included in the study. All patients had a history of cutaneous mucosal bleeding. They lacked native (type 1 and 2) or active (type 3) integrin αIIbβ3 as determined by flow cytometry. Two patients (G1 to G2) were recruited in the centre for exploration of bleeding and thrombosis disorders (CHU Timone, Marseille, France). Six patients were recruited in Moscow Children's Center for Hematology (G3 to G8).

**GT 1:** The GT patient, Marseille, lacked integrin αIIbβ3 as determined by flow cytometry (type 1). Her platelets had aggregation with ristocetin, but not with other agonists. She had a history of bleeding during surgery, post-partum and menorrhagia.

**GT 2:** The GT patient, Marseille, lacked integrin αIIbβ3 as determined by flow cytometry (type 1). She possessed two recessive mutations in the ITGA2B exon 28 p.Gly978Val and exon 29np.Trp998X. Her platelets had aggregation with ristocetin, but not with other agonists. The patient had a history of bleeding during surgery, nosebleeds, ecchymoses and mucosal bleeding.

**GT 3:** The GT patient, Moscow, lacked integrin αIIbβ3 as determined by flow cytometry (type 2). His platelets

had aggregation with ristocetin. The patient had a severe bleeding syndrome with frequent haematomas and purpuras.

**GT 4:** The GT patients, Moscow, lacked active integrin  $\alpha$ IIb $\beta$ 3 as determined by flow cytometry (type 3). His platelets had aggregation with ristocetin, but not with other agonists. He had petechiae and bruises.

**GT 5 and 6:** The GT patients, Moscow, lacked active integrin  $\alpha$ IIb $\beta$ 3 as determined by flow cytometry (type 3). They were the members of the same family. Platelets aggregated with ristocetin with some disaggregation. There was no aggregation with other agonists. The patients had a history of nosebleeds, haematomas and purpuras.

**GT 7:** The GT patient, Moscow, lacked integrin  $\alpha$ IIb $\beta$ 3 as determined by flow cytometry (type 2). Platelet aggregation was absent for adenosine diphosphate (ADP) and greatly reduced for collagen. The patient had a severe bleeding syndrome with frequent haematomas and purpuras.

**GT8:** The GT patient, Moscow, lacked active integrin  $\alpha$ IIb $\beta$ 3 as determined by flow cytometry (type 3). Her platelets had aggregation with ristocetin, but not with other agonists. She had menorrhagia, petechiae and bruises.

**Grey platelet syndrome (GPS 1):** The GPS patient, Marseille, lacked alpha-granules as determined by flow cytometry and confirmed by electron microscopy. Sanger sequencing revealed a homozygous mutation in the NBEAL2 gene (c 7202 ins TCCTTCATCAC), which is known to play a role in the formation of the  $\alpha$ -granules.<sup>22</sup>

**GPS 2, 3 and 4:** The alpha-granule deficient patients, Moscow, lacked alpha-granules as determined by flow cytometry and had agranular platelets in smear stain. They were members of the same family. Their platelets had decreased aggregation with ADP.<sup>23</sup>

**Human alpha-thrombin preparation:** Alpha-thrombin was activated from prothrombin by filtration through a column with agarose gel with immobilized ecarin. Prothrombin was isolated from blood plasma during purification of ceruloplasmin by ion exchange chromatography on UNOsphere Q and affinity chromatography on neomycin-agarose.<sup>24</sup> After activation of prothrombin by ecarin, alpha-thrombin was purified by affinity chromatography on benzamidine-agarose.<sup>25</sup> According to sodium dodecyl sulphate (SDS)-electrophoresis data, the resulting alpha-thrombin consisted of 31 and 6 kDa chains and was characterized by a Michaelis constant of 37.5 nM for the specific fluorogenic substrate Boc-Val-Pro-Arg-MCA (100 mM NaCl, 10 mM CaCl<sub>2</sub>, 50 mM Tris-HCl, pH 7.4, 0.66% PEG-6000, 0.16–320 nM Boc-Val-Pro-Arg-MCA, 1  $\mu$ g/mL fIIa).

**FXIII preparations:** FXIII was purchased from Enzyme Research and was either additionally dialyzed prior to experiments to remove glycerol, or isolated from human blood plasma. FXIII was isolated from human blood plasma by a fractional precipitation procedure with ammonium sulphate and subsequent ion exchange chromatography on DEAE ToyoPearl M650.<sup>26</sup> The purity of obtained FXIII was verified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). All experiments were reproduced with the both FXIII

preparations; the results were identical, and are presented indiscriminately in the data shown below.

The level of trace fXIIIa amounts in FXIII preparations was estimated by Laemmli 4–15% gradient SDS-PAGE after cross-linking reaction with fibrinogen. For positive controls, fXIIIa was obtained by 30-minute activation of 200 nM FXIII with 150 nM thrombin followed by 1.15  $\mu$ M PPACK addition. The results in **►Supplementary Fig. S1** (available in the online version) suggest that fXIIIa amount in the used FXIII preparations was negligible: 200 nM FXIII did not result in fibrinogen crosslinking (**►Supplementary Fig. S1**, line 4, available in the online version) in contrast to 2 nM fXIIIa (**►Supplementary Fig. S1**, line 8, available in the online version).

**FXIII and fibrinogen labelling:** Dialyzed FXIII or fibrinogen was supplemented with 0.1 M sodium bicarbonate (pH 9.0), 10 mg/mL FITC (MR = 5) or Alexa Fluor 647 (MR = 5), and incubated with continuous stirring for 2 hours (4°C). The reaction was stopped by 30-minute incubation with 1.5 M hydroxylamine (pH 8.5). The reaction mixture was centrifuged for 1 minute at 16,000  $\times$  g in Sephadex G-25 spin columns to separate the conjugate from unreacted labelling reagent. The degree of labelling was controlled by spectrophotometry.

**Platelet isolation:** Platelets were isolated from freshly drawn blood of healthy adult volunteers or patients as previously described.<sup>12,13,27</sup> Investigations were performed in accordance with the Declaration of Helsinki, and written informed consent was obtained from all patients and control donors, or from their parents. Briefly, blood was collected into 3.8% sodium citrate, pH 5.5, at a 9:1 blood/anticoagulant volume ratio and supplemented with prostaglandin E1 (1  $\mu$ M) and apyrase (0.1 unit/mL). The platelet-rich plasma was obtained by centrifugation at 100  $\times$  g for 8 minutes at room temperature, and further was supplemented with one-fourth volume of 3.8% sodium citrate, pH 5.5. Platelets were pelleted at 400  $\times$  g for 5 minutes, suspended in buffer A (150 mM NaCl, 2.7 mM KCl, 1 mM MgCl<sub>2</sub>, 0.4 mM NaH<sub>2</sub>PO<sub>4</sub>, 20 mM HEPES, 5 mM glucose, 0.5% bovine serum albumin), and gel-filtered on Sepharose CL-2B column.

**Kinetic flow cytometry experiments on FXIII binding/dissociation:** The platelets were activated at 1  $\times$  10<sup>5</sup>/ $\mu$ L for 15 minutes using thrombin (100 nM) or CRP (20  $\mu$ g/mL) in the presence of 2.5 mM CaCl<sub>2</sub>. The thrombin activation was terminated by 10-minute incubation with 500 nM PPACK. FITC-fXIII (144 nM) was incubated with activated platelets at room temperature for up to 30 minutes. The aliquots were taken and analysed by flow cytometry at time intervals of 0, 1, 2, 5, 10, 15, 20, 25 and 30 minutes. Upon reaching the binding saturation, the samples were rapidly diluted 20-fold with buffer A containing CaCl<sub>2</sub> for further monitoring the factor dissociation. The association and dissociation curves for individual experiments were fitted with a single exponent model.

**Equilibrium binding of FXIII with subpopulations of activated platelets:** The platelets were activated at 1  $\times$  10<sup>5</sup>/ $\mu$ L for 15 minutes using thrombin (100 nM), ADP (100  $\mu$ M) or CRP

(20 µg/mL) in the presence of 2.5 mM CaCl<sub>2</sub> and Alexa 647-Annexin V. Non-activated platelets, which were used as a control, were incubated for 15 minutes in buffer A with 2.5 mM CaCl<sub>2</sub>. The thrombin activation was terminated by 10-minute incubation with 500 nM PPACK. FITC-fXIII was added to the activated platelets for 20 minutes. Thereafter, the samples were analysed with Accuri C6 (BD Biosciences, San Jose, California, United States) flow cytometer. The acquired data were processed using a CFlow software (BD Biosciences). Fluorescence intensity of fXIII-FITC was converted to the mean number of molecules per platelet using a calibration curve obtained with GFP-conjugated calibration beads.

**Effect of external fibrinogen and antagonist of integrin αIIbβ3 (monafam) on binding of fXIII to activated platelets:** Platelets at  $1 \times 10^5/\mu\text{L}$  were pre-incubated with or without an antagonist of integrin αIIbβ3 (monafam, 0.2 mg/mL) for 30 minutes at room temperature, and then for 15 minutes stimulated with ADP (100 µM) or thrombin (100 nM) in the presence of 2.5 mM CaCl<sub>2</sub> and Alexa 647-Annexin V. The thrombin activation was terminated by 10-minute incubation with 500 nM PPACK. Fibrinogen (2 mg/mL) was added to some of the samples with monafam and incubated for 5 minutes. FXIII-FITC (144 nM) was added then for 20 minutes, and samples analysed by flow cytometry.

**Distribution of external fXIII on the membrane of activated platelets:** Glass coverslips (24 × 24 mm, Heinz Herenz, Hamburg, Germany) were cleaned with potassium dichromate, rinsed with distilled water and dried. The clean coverslips were coated with Poly-L-Lysine solution 0.01% or fibrinogen 20 µg/mL for 1 hour at room temperature, rinsed with distilled water and then assembled as part of the flow chamber. Platelets were activated at  $1 \times 10^5/\mu\text{L}$  with thrombin (100 nM) in the presence of 2.5 mM CaCl<sub>2</sub> and with R-phycoerythrin-Annexin V. The activation was terminated by 10-minute incubation with 500 nM PPACK, followed by 20-minute incubation with Alexa Fluor 647-fXIII, FITC-fibrinogen or FITC-anti-fibrinogen antibody. Confocal images were acquired with an Axio Observer Z1 microscope (Carl Zeiss, Jena, Germany) with a 100× oil objective.

**Distribution of platelet-derived fXIII-A and tTG on the pro-coagulant platelets:** Glass coverslips were prepared as described above and then assembled as part of the flow chamber. Platelets were activated at  $1 \times 10^5/\mu\text{L}$  with thrombin (100 nM) and CRP (20 µg/mL) in the presence of 2.5 mM CaCl<sub>2</sub> and Alexa 647-Annexin V. After activation, they were incubated for 5 minutes with FITC-anti-fibrinogen, anti-fXIII-A or anti-tTG antibody and for 5 minutes with the corresponding secondary antibody. Then, the samples were fixed with 1.5% formalin in phosphate-buffered saline for 20 minutes, diluted 15-fold and analysed by confocal microscopy.

**Distribution of fXIII in thrombi under the flow condition:** The clean coverslips were coated with collagen at 200 µg/mL for 1 hour at room temperature, rinsed with distilled water and then assembled as part of the flow chamber. Annexin V-FITC and fXIII-Alexa 647 were added to human blood col-

lected into hirudin with or without PPACK. Then, blood was perfused at  $1,000 \text{ s}^{-1}$  through flow chambers coated with collagen. Imaging was performed with an Axio Observer Z1 microscope (Carl Zeiss, Jena, Germany) with a 100× oil objective.

**Statistics:** All experiments were performed at least in triplicate with platelets from different donors except for studies with patients. Comparisons were carried out with the paired Student's *t*-test or Mann-Whitney test or analysis of variance. Statistical significance was set as *p*-value < 0.05. Values are reported as mean ± standard deviation unless specified otherwise.

## Results

**Kinetic and equilibrium exogenous fXIII binding to the activated platelet subpopulations:** To quantitatively characterize binding of zymogen fXIII to platelets in different states, we used flow cytometry. ▶**Fig. 1** shows kinetics of binding and dissociation of fXIII for the PS-positive and PS-negative platelets produced upon activation with thrombin (A) or CRP (B). Both processes are relatively slow, with characteristic timescales of 20 to 40 minutes. Binding follows an exponential curve, and is reversible upon dilution. PS-positive platelets bind two- to fourfold more fXIII than PS-negative ones.

The equilibrium binding experiments (▶**Fig. 1C**) revealed that binding is linear, without apparent saturation within a physiological range of fXIII concentrations. The level achieved at the physiological fXIII concentration of approximately 100 nM is 700 to 800 molecules/cell for the PS-positive platelets produced by thrombin or CRP. For the PS-negative subpopulation, or for ADP-stimulated platelets, or non-stimulated ones, binding was linear and significantly below that for the PS-positive ones.

Experiments on fXIII binding to platelets in the presence of high concentrations of unlabelled fXIII detected no displacement indicating a high-capacity low-affinity binding scheme, which is in line with apparent binding linearity (▶**Supplementary Fig. S2A**, available in the online version). Binding with the both activated platelet subpopulations was calcium-independent (▶**Supplementary Fig. S2B**, available in the online version) which is consistent with a study by Greenberg et al.<sup>2</sup>

**Contribution of integrin αIIbβ3 and fibrinogen to the exogenous fXIII binding to platelets:** The data above (▶**Fig. 1C**) show that washed platelets stimulated with ADP poorly bind zymogen fXIII. On the other hand, several previous studies on active fXIIIa revealed that it can interact with integrin αIIbβ3,<sup>9,28,29</sup> and fibrinogen γ chain was reported by some to be a probable mediator.<sup>18,30</sup> Based on this, we hypothesized that zymogen fXIII binds to platelets via alpha-granule-derived fibrin(ogen), that in turn can be attached to platelets via integrin αIIbβ3 or via another mechanism.<sup>13</sup> With ADP, binding could be absent because alpha-granules are not secreted significantly for this stimulation.

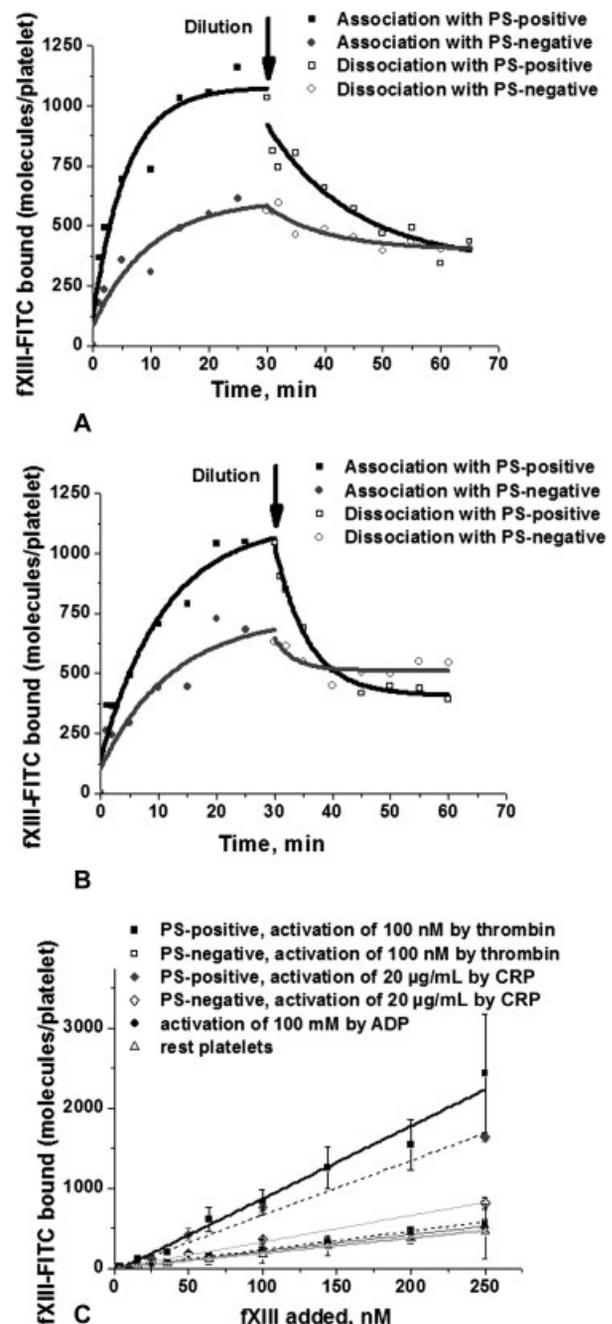
To test this suggestion, we stimulated washed platelets with ADP to induce conformational change in integrin

$\alpha$ IIb $\beta$ 3 but without significant alpha-granules secretion and tested binding of exogenous fXIII in the presence or absence of external fibrinogen or integrin  $\alpha$ IIb $\beta$ 3 inhibitor, monafram ( $\rightarrow$ Fig. 2A). Fibrinogen significantly ( $p < 0.05$ ) promoted fXIII binding, while integrin  $\alpha$ IIb $\beta$ 3 antagonist monafram abolished this increase. When this experiment was performed with thrombin-stimulated platelets in order to induce the platelet subpopulations, PS-negative platelets acted as ADP-stimulated platelets ( $\rightarrow$ Fig. 2B): addition of fibrinogen potentiated binding of fXIII to the PS-negative activated platelets, but not to the PS-positive ones. This improvement was negated by monafram. These data indicate that activated integrin  $\alpha$ IIb $\beta$ 3 indeed is able to bind zymogen fXIII via fibrinogen. We have also shown that gel-filtered platelets in our conditions had no fibrinogen on their surface before activation ( $\rightarrow$ Supplementary Fig. S3, available in the online version). After activation by thrombin (100 nM), PS-positive platelets had higher level of fibrinogen on their membrane than PS-negative ones. The results for the PS-positive platelets suggest that there could be additional mechanisms of interaction of fXIII with platelets that might still be fibrinogen-dependent. Indeed, these platelets have no active integrins  $\alpha$ IIb $\beta$ 3<sup>31</sup> on their membrane and do not bind external fibrinogen<sup>32</sup> and their own fibrinogen/fibrin coverage is not inhibited by monafram.<sup>13</sup> Until now, the mechanisms of formation of the fibrin(ogen) coat on the surface of PS-positive platelets remain unclear. However, it has been shown that fibrin polymerization and transglutaminase-dependent fibrinogen crosslinking are able to retain fibrin(ogen) on the activated PS-positive platelets.<sup>13</sup>

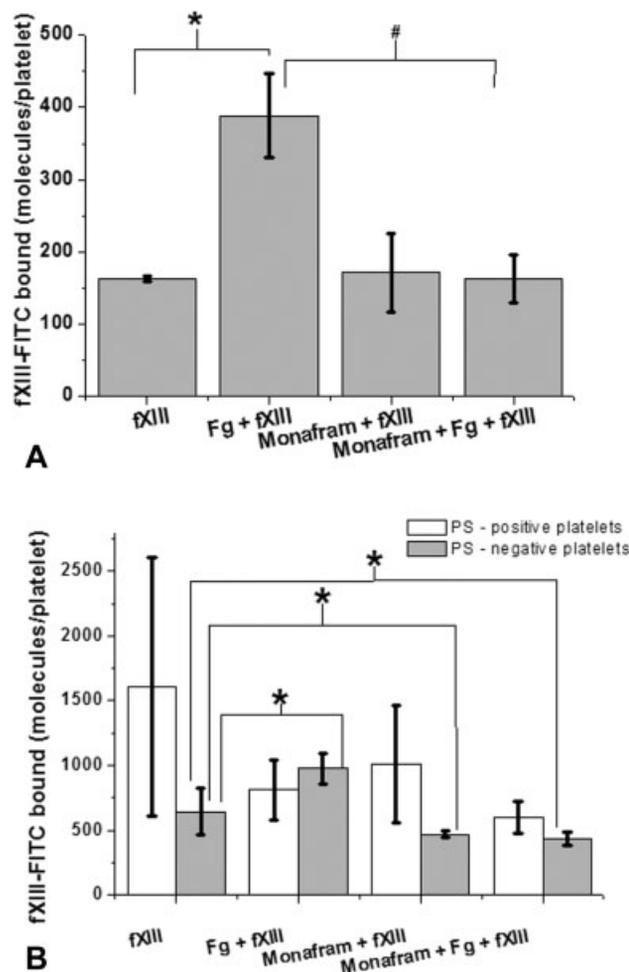
**Binding of fXIII to platelets from patients with GPS or GT:** As an alternative way to look for possible platelet membrane receptors for fXIII binding to platelets, we used platelets from patients with GT ( $\rightarrow$ Fig. 3). Both subpopulations of activated platelets, for patients with GT, bind less fXIII than platelets of health donors. So it is indicated that integrin  $\alpha$ IIb $\beta$ 3 could be involved in the interaction of fXIII to activated platelets.

Besides, we used platelets from patients with GPS. Patients with GPS during activation do not secrete endogenous fibrinogen, which is involved in the fXIII binding, apparently, that can be seen from  $\rightarrow$ Fig. 3. Our data show that binding of fXIII with platelets from these patients was significantly decreased ( $\rightarrow$ Fig. 3) (Mann-Whitney test,  $p < 0.05$ ). So it seems that granular components and integrin  $\alpha$ IIb $\beta$ 3 are both essential.

**Distribution of exogenous fXIII on the surface of activated platelet subpopulations:** We characterized distribution of zymogen fXIII on both platelet subpopulations ( $\rightarrow$ Fig. 4). It was found to interact with both PS-positive and PS-negative subpopulations ( $\rightarrow$ Fig. 4A) but in a different manner. FXIII bound to the 'caps' on the PS-positive procoagulant platelets and co-localized with platelet-derived fibrinogen ( $\rightarrow$ Fig. 4B). The binding of fXIII to the PS-negative platelets was less, and was mostly to the central part of a spread platelet, where majority of its granules is located. It was shown that this area contains higher concentration of integrin  $\alpha$ IIb $\beta$ 3 and



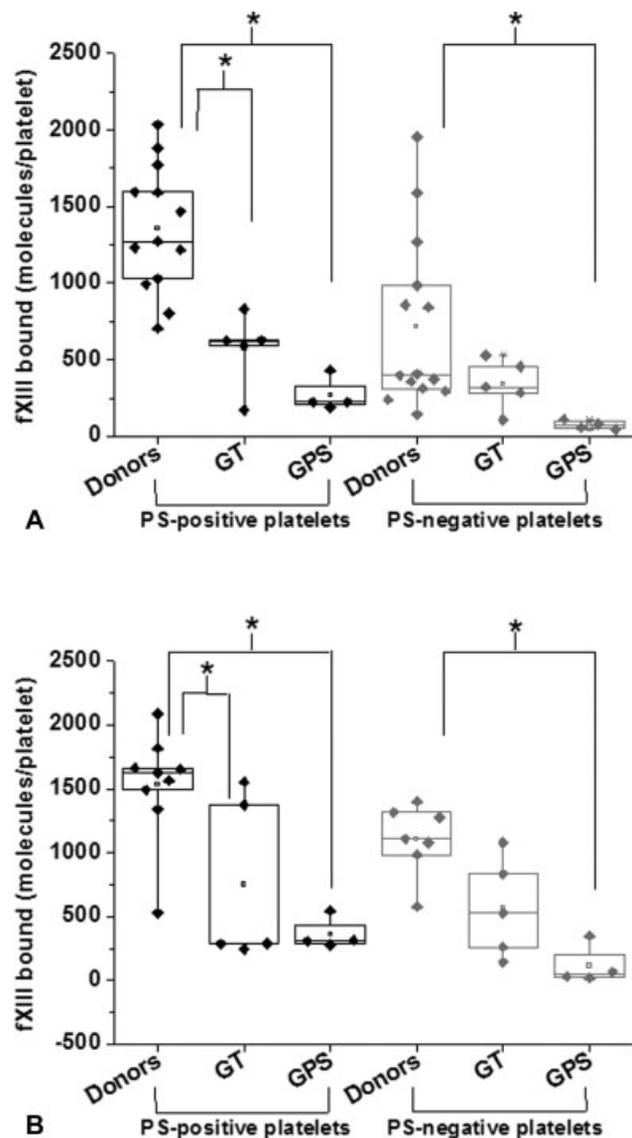
**Fig. 1** Platelet-binding characteristics of factor XIII (fXIII). (A, B) Kinetics of association and dissociation of fXIII to phosphatidylserine (PS)-positive or PS-negative platelets activated by 100 nM thrombin (A) or 20 µg/mL collagen-related peptide (CRP) (B). After addition of fluorescein-5-isothiocyanate (FITC)-fXIII (144 nM), aliquots were collected and analysed in the following time intervals: 0, 1, 2, 5, 10, 15, 20, 25 and 30 minutes. After the sample was diluted 20-fold with buffer A containing 2.5 mM CaCl<sub>2</sub>, measurements were carried out at the same time intervals. A typical experiment out of four healthy donors is shown. The association and dissociation curves for individual experiments were fitted with a single exponent model. (C) Equilibrium binding of fXIII with subpopulations of activated platelets. Platelets at 50000 µL were stimulated with either thrombin (100 nM) or CRP (20 µg/mL) or adenosine 5'-diphosphate (ADP) (100 µM) or non-activated. The thrombin activation was terminated by 10-minute incubation with 500 nM D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK). After 15 minutes, FITC-fXIII was added at indicated concentrations for 20 minutes. The sample was analysed by flow cytometry. Mean values  $\pm$  standard deviation (SD) are shown for  $n = 3$ .



**Fig. 2** Mechanisms of factor XIII (fXIII) binding to activated platelets. (A) Platelets at 50000  $\mu$ L were pre-incubated with or without an integrin  $\alpha$ IIb $\beta$ 3 antagonist (monafram, 0.2 mg/mL) for 0.5 hours, and then stimulated with adenosine 5'-diphosphate (ADP) (100  $\mu$ M). After 15 minutes, fibrinogen (2 mg/mL) was added to some of the samples with monafram and incubated for 5 minutes. fluorescein-5-isothiocyanate (FITC)-fXIII (144 nM) was added then for 20 minutes, and samples analysed by flow cytometry. Mean values  $\pm$  standard deviation (SD) for  $n = 3$  experiments with platelets from three donors are shown. Paired Student's  $t$ -test was used, asterisk indicates  $p < 0.05$ . (B) The experiments were performed as in panel A, with the exception that thrombin at 100 nM was used for platelet stimulation, and annexin V was employed to detect phosphatidylserine (PS). Mean values  $\pm$  SD for  $n = 5$  experiments with platelets from five donors are shown. Analysis of variance (ANOVA), \* $p < 0.05$

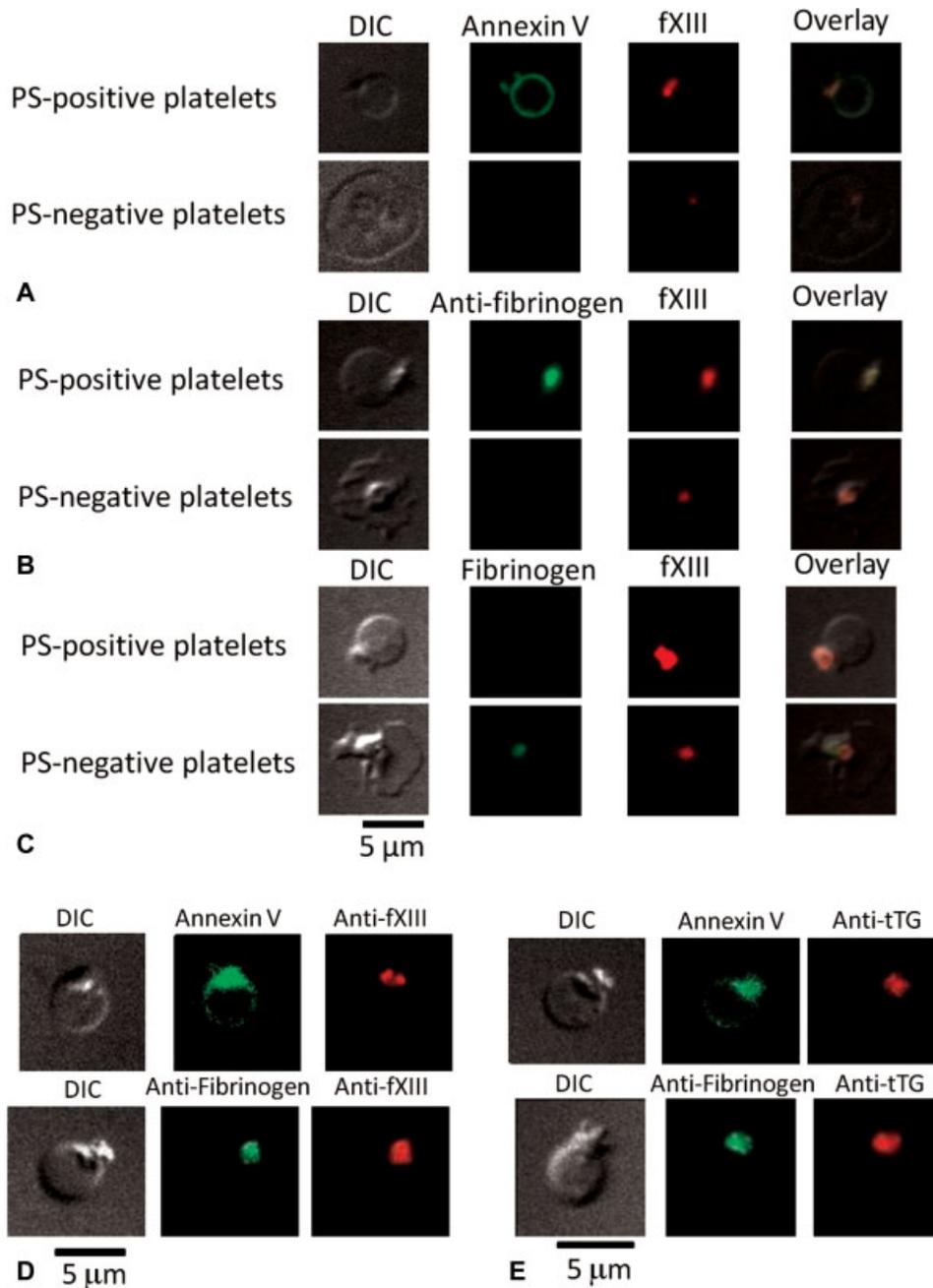
fibrinogen.<sup>33,34</sup> External fibrinogen significantly increases the binding of fXIII with PS-negative subpopulation of platelets ( $\rightarrow$  Fig. 4C).

In order to compare binding of external fXIII with the expression of the platelet-derived transglutaminase enzymes on procoagulant platelets, we characterized distribution of platelet-derived fXIII and tTG using confocal microscopy. Both platelet-derived fXIII and tTG at the specific concave 'cap'-like regions and co-localized with fibrinogen on PS- positive platelets ( $\rightarrow$  Fig. 4D, E) in agreement with Mitchell et al.<sup>8</sup>



**Fig. 3** Binding of factor XIII (fXIII) to platelets from patients with inherited disorders of platelet function. Platelets from health donors or patient with Glanzmann thrombasthenia (GT) or patient with grey platelet syndrome (GPS) at 50000  $\mu$ L were stimulated with either thrombin (100 nM) (A) or collagen-related peptide (CRP) (20 mg/mL) (B). After the activation time (15 minutes), fluorescein-5-isothiocyanate (FITC)-fXIII was added at a concentration of 144 nM. The incubation time was 20 minutes. Thereafter, the sample was analysed by flow cytometry. The boxes depicted left to each dataset include  $\pm$  standard deviation (SD) (the bands inside correspond to 50%); open squares show the average; whiskers show 5 and 95%. Mann-Whitney test, \* $p \leq 0.05$ .

*Distribution of fXIII in thrombi under the flow condition:* To evaluate the distribution of zymogen fXIII in platelet thrombi formed under flow, we carried out experiments on thrombus formation in whole blood perfused over collagen. Annexin V-FITC and fXIII-Alexa 647 were added to whole blood collected into hirudin with or without PPACK before perfusion. The thrombi were visualized with confocal microscopy. In all cases, fXIII was localized in the central part of the thrombi, while the PS-positive platelets were mainly localized on the periphery of the thrombi ( $\rightarrow$  Fig 5). That is, under flow



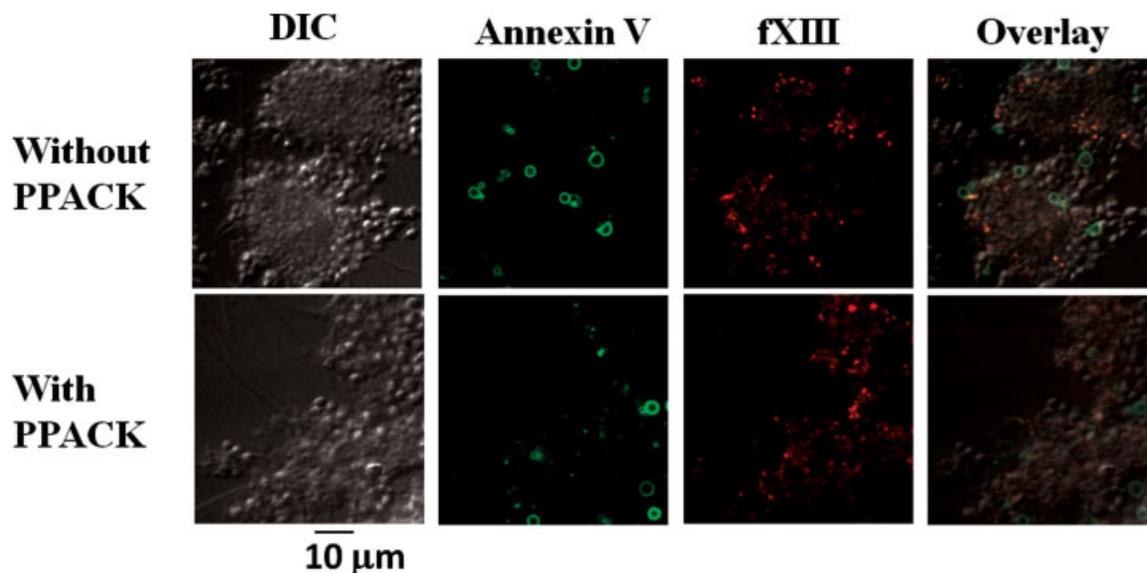
**Fig. 4** Distribution of factor XIII (fXIII) on the surface of activated platelets. Platelets were activated at 50000  $\mu$ L with thrombin in the presence of 2.5 mM  $\text{CaCl}_2$  and with R-phycoerythrin-Annexin V (A). The activation was terminated by 10-minute incubation with 500 nM D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK), followed by 20-minute incubation with Alexa Fluor 647-fXIII, fluorescein-5-isothiocyanate (FITC)-anti-fibrinogen antibody (B) or FITC-fibrinogen (C). Representative differential interference contrast (DIC) and confocal images are shown. (D, E) Platelets were activated at 50000  $\mu$ L with thrombin (100 nM) and collagen-related peptide (CRP) (20  $\mu$ g/mL) in the presence of 2.5 mM  $\text{CaCl}_2$  and Alexa 647-Annexin V. After activation, they were incubated for 5 minutes with FITC-anti-fibrinogen, anti-FXIII-A (D) or anti-tissue transglutaminase (tTG) (E) antibody and for 5 minutes with the corresponding secondary antibody. Then, the samples were fixed with 1.5% formalin in phosphate-buffered saline (PBS) for 20 minutes, diluted 15-fold and analysed by confocal microscopy. Scale bar is 5  $\mu$ m.

conditions, fXIII predominantly interacts with PS-negative platelets than with PS-positive platelets.

## Discussion

The objective of this study was to investigate binding of zymogen fXIII with activated platelet subpopulations. The

main conclusions are: (1) fXIII binds at approximately 700 to 800 molecules per a PS-positive platelets (produced with thrombin or CRP) when 100 nM fXIII is added, with minor binding to PS-negative ones (when produced with thrombin or CRP), ADP-activated or resting platelets; (2) fXIII binding is calcium-independent, linear and non-saturable within a wide range of concentrations for all cases; (3) fXIII interacts



**Fig. 5** Distribution of factor XIII (fXIII) in thrombi under the flow condition. Typical confocal microscopy images of platelet aggregates formed after perfusion of hirudinized human whole blood with or without D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) over immobilized collagen at  $1,000 \text{ s}^{-1}$  for 5 minutes. Platelet aggregates were visualized using differential interference contrast (DIC) and confocal microscopy ( $\times 100$  objective). Representative images of DIC, fluorescein-5-isothiocyanate (FITC)-Annexin V (green) and Alexa Fluor 647-fXIII (red) fluorescence are shown. Scale bar is  $10 \mu\text{m}$ .

with platelets not directly, but through fibrin(ogen) that is in turn bound to integrin  $\alpha\text{IIb}\beta 3$  or retained on platelet surface by crosslinking and polymerization; and (4) exogenous fXIII (as well as platelet-derived fXIIIa and tTG) is predominantly located in the procoagulant platelet 'caps' and co-localized with fibrinogen.

Our results on the biochemistry of zymogen fXIII binding to platelets agree well with the abundance (and, paradoxically, with the diversity) of the reports on its active form fXIIIa. There were several reports on fXIIIa interaction with platelets via integrin  $\alpha\text{IIb}\beta 3$  and  $\gamma$ -chain of fibrinogen<sup>17,18,35</sup>; very recently, zymogen fXIII binding to fibrinogen was well characterized at the molecular level.<sup>36</sup> Here, we observe all these possible scenarios within a single study. Binding of fXIII to the platelets either lacking integrin  $\alpha\text{IIb}\beta 3$  or with inhibited integrins is impaired. Judging from the inefficiency of ADP in inducing fXIII binding sites, its correction by external fibrinogen and failure of grey syndrome platelets to bind zymogen fXIII, the role of alpha-granule secretion and, presumably, of fibrinogen, in mediating zymogen fXIII binding seems significant. This mechanism is in line with what we know about fXIII interactions with fibrinogen: it was previously shown to bind fibrinogen-coated beads in a calcium-independent manner.<sup>2,35</sup> Although we tested the zymogen molecule before binding experiments and found it to be lacking active enzyme form, and although thrombin was inhibited by PPACK in all our experiments to prevent fXIII activation (**Supplementary Fig. S4**, available in the online version), one cannot completely exclude the possibility that some of the fXIII molecules on the platelet surface could get locally activated by some unknown protease.

However, this seems unlikely and does not affect the conclusions on the meaning and extent of fXIII meaning.

Our findings somewhat differ from a previous study,<sup>16</sup> which can be explained by the fact that they used  $^{125}\text{I}$ -fXIIIa (that had much higher numbers of bound molecules), and we used the zymogen form. In addition, they used a 100-fold molar excess unlabelled factor, whereas we have used in our experiments about 15-fold excess of unlabelled fXIII.

Spatial distribution of zymogen fXIII on the activated platelets also seems similar to that reported recently by Mitchell et al for active platelet-derived fXIIIa.<sup>8</sup> Together with platelet-derived tTG (our study thus confirms the view that platelet-derived transglutaminases are somehow at least partially externalized), they are all located in the specific convexities, procoagulant platelet 'caps'. This is natural, as these caps are particularly rich with such adhesive proteins as fibrinogen and thrombospondin.<sup>13</sup> It is also interesting in view of our recent report that these 'caps' are also the primary sites of the membrane-dependent coagulation complexes' assembly and of thrombin generation.<sup>12</sup>

The amount of fXIII (on the order of 700–800 molecules per platelet) bound at a physiological fXIII concentration is not great, but might be significant. In a platelet thrombus, where there are much more platelets than free plasma volume, local concentration of bound fXIII can become quite high. Platelet-bound zymogen, ready to be activated by locally produced thrombin, can thus contribute to thrombus formation and stability. Additional studies are required to elucidate the (patho)physiological significance of zymogen fXIII versus active enzyme fXIIIa binding to platelets.

### What is known about this topic?

- Was showed fXIII significantly increased on TRAP-activated platelets in whole blood compared to unstimulated sample, but not in washed platelets.
- FXIII binding to fibrinogen was well characterized at the molecular level.
- The reports about the putative binding site for active form of fXIIIa on platelets are not fully in agreement: some of them concluded the essential role of integrin  $\alpha$ IIb $\beta$ 3 and fibrin(ogen); other considered that integrin  $\alpha$ IIb $\beta$ 3 and platelet-bound fibrin(ogen) were not the binding site for fXIIIa; another was reported that binding of fXIIIa to thrombin-activated platelets was inhibited by plasmin.

### What does this paper add?

- FXIII binds at approximately 700 to 800 molecules per procoagulant activated platelet (produced with thrombin or CRP) under physiological conditions, with minor binding to PS-negative (when produced with thrombin or CRP), ADP-activated or resting platelets.
- FXIII binding is calcium-independent, linear and non-saturatable within a wide range of concentrations for all cases.
- FXIII interacts with platelets not directly, but through fibrin(ogen) that is in turn bound to integrin  $\alpha$ IIb $\beta$ 3 or retained on platelet surface by crosslinking and polymerization.
- Exogenous fXIII (as well as platelet-derived fXIIIa and tissue transglutaminase) is predominantly located in the procoagulant platelet 'caps' and co-localized with fibrinogen.

#### Authors' Contributions

Y.N.K., N.A.P., S.I.O., E.A.K., A.A.R., A.S.D., M.I.B., M.A.R. and A.V.S. performed experiments and analysed data. H.C., M. A.K. and M.C.A. recruited and characterized patients. M.C. A., F.I.A. and M.A.P. planned research and analysed the data. Y.N.K., N.A.P., M.C.A. and M.A.P. wrote the paper.

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#### Conflict of Interest

None declared.

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