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ORIGINAL ARTICLE

Measuring soluble platelet glycoprotein VI in human plasma by ELISA

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Abstract

Recent experimental evidence demonstrates that the platelet-specific collagen receptor, glycoprotein (GP)VI is essentially all uncleaved on normal circulating platelets, but is shed from the platelet surface in a metalloproteinase-dependent manner in response to GPVI ligands (including collagen), anti-GPVI antibodies or activation at the platelet Fc receptor, FcγRIIa. This raises the question of whether shed ectodomain fragment in plasma could be a useful biomarker of thrombotic risk and/or autoimmune thrombocytopenia. In this study, we developed a sandwich enzyme-linked immunosorbent assay (ELISA) for measuring soluble GPVI in human plasma, using rabbit anti-GPVI polyclonal antibody in the solid-phase, murine anti-GPVI monoclonal antibody (1A12) in the fluid-phase and horseradish peroxidase (HRP)-coupled anti-mouse antibody and enhanced chemiluminescence (ECL) for detection. The ELISA was optimized for sensitivity, reproducibility, inter- and intra-assay precision, addition and recovery and detected GPVI in plasma with a lower detection limit of ~1 ng/mL. Effects of different anti-coagulants (trisodium citrate, acid-citrate-dextrose or EDTA) were negligible. In ten healthy donors, soluble plasma GPVI levels were 18.9 ± 4.1 ng/mL. Treating normal platelet-rich plasma with a GPVI ligand (collagen-related peptide, CRP), calmodulin inhibitor W7 (that induces GPVI shedding without platelet activation) or *N*-ethylmaleimide (that directly activates platelet sheddases), under conditions previously shown to induce GPVI shedding, also increased plasma GPVI levels by up to ~7-fold, compared to previously reported autoimmune (anti-GPVI) patient plasma where soluble GPVI was ~10-fold higher than normal. Characterization of this sensitive ELISA should facilitate analysis of functional/diagnostic role(s) for soluble GPVI in human plasma associated with thrombotic/immune dysfunction.

Keywords: Glycoprotein VI, platelets, ELISA**Introduction**

Binding of platelet glycoprotein (GP) VI to collagen, and/or potentially laminin, is an important trigger for platelet aggregation and activation at the site of vascular injury or disease, where extracellular matrix is exposed [1, 2]. Engagement of GPVI by collagen [1], or other ligands such as collagen-related peptide (CRP) [3], snake toxins (convulxin, alborhagin or crotarhagin) [4, 5] or anti-GPVI antibodies [6] leads to platelet activation and integrin ($\alpha_{IIb}\beta_3$)-dependent aggregation, as well as metalloproteinase-dependent GPVI ectodomain shedding, involving the release of an ~55 kDa soluble ectodomain fragment and

formation of an ~10 kDa remnant fragment that remains platelet-associated [7–10]. GPVI signalling involves Fc receptor γ -chain (FcR γ), that is required for GPVI expression, and contains an immunoreceptor tyrosine-based activation motif (ITAM) within the cytoplasmic domain [11]. In this regard, activation *via* another platelet ITAM-containing receptor, FcγRIIa, also induces GPVI shedding; stimulation of either GPVI or FcγRIIa triggers both GPVI shedding and de-ITAM-ization of FcγRIIa, providing a mechanistic link between thrombotic and immune dysfunction [12].

Western blotting platelets from normal individuals with an antibody against the cytoplasmic tail

(that recognizes both intact GPVI and the ~10 kDa membrane-associated remnant) reveals virtually all of the GPVI on circulating platelets is in an intact, uncleaved form [13]. However, several lines of evidence demonstrate that metalloproteinase-dependent GPVI shedding from platelets can occur in plasma *in vivo*. First, in mouse or monkey models, injection of anti-GPVI antibodies or Fab fragments can result in depletion of surface GPVI by either shedding or internalization pathways [6–14]. Second, when human platelets are injected into NOD/SCID mice, platelet GPVI levels are stable for several days but can be depleted following treatment of the mice with an anti-human GPVI antibody [15]. Third, we showed that a patient with an anti-GPVI autoantibody associated with autoimmune idiopathic thrombocytopenic purpura (ITP) had platelets with lower than normal levels of surface GPVI by flow cytometry, and, in contrast to normal platelets, detectable ~10 kDa membrane-associated remnant fragment of GPVI western blotted by the anti-cytoplasmic tail antibody [16]. We used a newly developed enzyme-linked immunosorbent assay (ELISA) to measure soluble GPVI in this patient, and showed an ~10-fold increase above normal plasma, indicating that soluble plasma GPVI may be a useful platelet-specific biomarker for ITP [16], other platelet-related autoimmune disorders, and possibly thrombotic disorders.

In this study, we characterize the ELISA for measuring soluble GPVI in human plasma, in terms of sample collection, reproducibility and sensitivity. Together, the results suggest that this soluble GPVI ELISA may ultimately provide a useful tool for assessing the diagnostic and/or prognostic value of soluble GPVI in relevant clinical samples.

Materials and methods

All procedures described in this paper were in accordance with the ethical standards of the Monash University Animal Ethics Committee and Monash University Standing Committee for Ethics involving Research into Humans and were in accordance with the Helsinki Declaration of 1975.

Antibodies

Polyclonal rabbit anti-human GPVI antibody was raised against recombinant human GPVI extracellular domain (residues 21–234, excluding the signal sequence) [17] as previously described [16], and affinity-purified on the antigen coupled to Affigel 10/15 using previously published methods [18]. Murine anti-human GPVI monoclonal antibodies, 1G5 and 1A12, have been described elsewhere [7]. The mouse monoclonal antibody, 14A2, that recognizes the platelet receptor, CD151, and activates platelets *via* FcγRIIa, has also been previously

described [19]. The murine monoclonal antibody, 3A2, against human tissue factor was used as a negative control [7]. Polyclonal rabbit anti-mouse HRP-conjugated (immunoabsorbed against human and bovine IgG) was from Dakocytomation (Denmark).

Preparation of human plasma samples

Human plasma from blood of healthy donors was routinely collected into fresh acid-citrate dextrose (ACD; 85 mM trisodium citrate, 78 mM citric acid, 111 mM glucose) anti-coagulant (1.4 mL ACD per 10 mL blood) using a 19-gauge winged infusion kit. Blood was centrifuged at 100g for 20 minutes, and platelet-rich plasma (PRP) was centrifuged at 300g for 15 minutes to produce platelet-poor plasma (PPP). In some experiments, PPP was microfuged at 8000g for 2 minutes then ultracentrifuged at 100 000g for 1 hour to remove platelet microparticles. To assess the effect of different collection methods, blood was collected from the same two donors using 19- or 21-gauge needles, into separate tubes containing ACD, trisodium citrate [0.32% (w/v) final concentration; 1 mL/10 mL blood], or EDTA (10 mM, final concentration). Samples were also collected into a silica-coated coagulation vacutainer (BD Vacutainer SSTTM, Plymouth, UK) to obtain serum from clotted blood.

Preparation of GPVI-depleted plasma

GPVI-depleted plasma used as a diluent in the ELISA was prepared by batch absorption of human plasma (10 mL) with 5 mL of a 1:1 mixture of Affigel-10 and -15 coupled to the anti-GPVI monoclonal antibody, 1G5, as previously described [7]. After mixing overnight at 4°C, the resin was removed by centrifugation at 8000g for 20 minutes and GPVI-depleted plasma was stored at –80°C.

Western blotting of human platelet lysates with anti-GPVI antibodies

Washed platelets at 5×10^8 /mL in Tyrode's buffer were untreated or treated with a final concentration of 2 µg/mL 14A2 (a monoclonal antibody against the platelet receptor, CD151, that activates FcγRIIa, and induces shedding of GPVI) for 2 h at room temperature, electrophoresed on SDS-5–20%-polyacrylamide gels under non-reducing conditions, electrotransferred to nitrocellulose and western blotted with either rabbit polyclonal anti-GPVI IgG or the anti-GPVI monoclonal antibody, 1A12, as previously described [7–16]. Blots were visualized using horseradish-peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibody, respectively, and enhanced chemiluminescence (ECL).

Measurement of soluble GPVI in human plasma by ELISA

Wells of 96-well microtitre plates (Nunc, Roskilde, Denmark) were coated with a rabbit polyclonal anti-GPVI IgG (1 µg/mL) in coating buffer (0.05 M bicarbonate, pH 9.6) for 1 h at room temperature. Wells were washed six times with 100 µL aliquots of 0.01 M sodium phosphate, 0.15 M sodium chloride, pH 7.4 (PBS) containing 0.2% (v/v) Tween-20 (PBS-T), blocked with 1% (w/v) BSA (Sigma, St Louis, MO, USA) in PBS (200 µL) for 1 h at room temperature, then washed six times with PBS-T, before addition of samples in triplicate wells. Each plate routinely included standards consisting of recombinant GPVI ectodomain (0–100 ng/mL, final concentration) in 10% (v/v) GPVI-depleted plasma in PBS. Aliquots of plasma from a single donor were stored at –80°C, and levels of soluble GPVI in this plasma were measured on every plate to serve as an additional internal control. Test samples of plasma were diluted in PBS to be directly comparable to standards. After 1 h at room temperature, wells were washed six times with PBS-T and the fluid-phase primary detecting antibody, murine anti-GPVI monoclonal antibody, 1A12, added at 1 µg/mL (100 µL/well). In some experiments, an isotype-matched control antibody, 3A2 (against human tissue factor), was used as a negative control in parallel samples. After a further 1 h then six washes, HRP-conjugated secondary anti-mouse antibody was added (100 µL/well; 1/500 dilution of stock at 1.3 mg/mL) for 1 h, then after a further six washes, 100 µL of a one-half dilution of SuperSignal ELISA Pico chemiluminescent substrate (Pierce, Rockford, IL, USA) was added and light emission (stable after 1 minute) measured using a Wallac-Victor2 luminescence plate reader (PerkinElmer, Waltham, MA, USA and the Phillum 10s protocol, extra high scale).

To optimize the solid-phase, wells were initially coated with affinity-purified rabbit anti-GPVI IgG at serial dilutions from 1 µg/mL to 0.5 ng/mL, before proceeding with the standard method described above. To optimize the fluid-phase, the primary anti-GPVI antibody was tested over the range, 0.5–1000 ng/mL, or secondary antibody diluted from 1/500 to 1/2 000 000 (at a primary antibody concentration of 1 µg/mL).

Precision and additive capability of the ELISA

To determine the precision of the ELISA, three representative concentrations of recombinant GPVI (low, 0.8 ng/mL; medium, 25 ng/mL; high, 100 ng/mL) were assayed using the standard protocol described above. The mean reading (RLU) was calculated with STDE (standard deviation) and coefficient of variability (CV%). The CV% of the inter-assay variability (triplicate) and the intra-assay

variability (15 different days) were measured by dividing the STDE on the mean with CV% below 10%, indicating acceptable precision. To assess the additive capability of the ELISA, human plasma samples were analysed with or without the addition of a known amount of recombinant soluble GPVI (100 ng/mL, final concentration).

Results

Optimization of the ELISA for measuring soluble GPVI in human plasma

In order to establish a method for analysing soluble GPVI in human plasma using a sandwich ELISA approach, we initially raised and characterized a rabbit polyclonal anti-GPVI IgG and a panel of mouse monoclonal anti-GPVI antibodies using a recombinant human GPVI ectodomain (encompassing the N-terminal 213 residues) as antigen [16, 17]. First, to confirm specificity of the polyclonal IgG, lysates of untreated human platelets or platelets treated with the anti-platelet (CD151) monoclonal antibody, 14A2 (2 µg/mL) for 2 h to induce FcγRIIa-dependent proteolysis of GPVI [12], were western blotted with rabbit anti-GPVI polyclonal antibody or the murine anti-GPVI monoclonal antibody, 1A12 (Figure 1). Both antibodies detected full-length GPVI (~62 kDa) in the untreated platelet

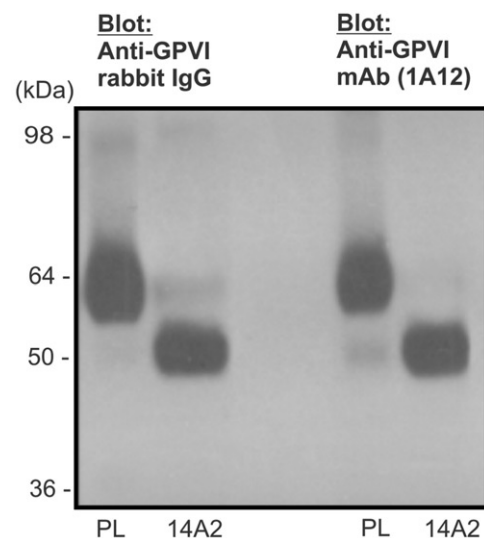


Figure 1. Western blotting of GPVI. Lysates of untreated human platelets or platelets treated with the anti-CD151 monoclonal antibody, 14A2 (2 µg/mL) for 2 h to induce FcγRIIa-dependent proteolysis of GPVI, were western blotted with rabbit anti-GPVI polyclonal antibody or the murine anti-GPVI monoclonal antibody, 1A12. Both antibodies detected full-length GPVI (~62 kDa) in untreated platelet lysates (PL), and the ectodomain fragment of GPVI (~55 kDa) in 14A2-treated platelets. Samples were analysed on SDS-5-20%-polyacrylamide gels under non-reducing conditions, electrotransferred to nitrocellulose and visualized using an HRP-conjugated anti-mouse or anti-rabbit secondary antibody and ECL chemiluminescence.

lysates and the ectodomain fragment of GPVI (~55 kDa) in 14A2-treated platelets.

We subsequently used rabbit anti-GPVI IgG as coating antibody and mouse monoclonal antibody, 1A12, for detection (*via* an HRP-conjugated secondary anti-mouse IgG and chemiluminescence). Optimization of reagent concentrations and washing steps were performed to maximize signal production with lowest background (Figure 2; and data not shown). For production of a standard curve, plates were coated with rabbit anti-GPVI polyclonal antibody at 1 µg/mL, followed by addition of serial dilutions of soluble recombinant GPVI ectodomain (0.4–200 ng/mL) in GPVI-depleted plasma (Figure 2). The average \pm STDE was calculated and converted into dose response curve using linear fit. The dose response curve of the ELISA (Figure 2) illustrates the specificity of the assay as reflected by dose-dependent increase in light emission, with a lower detection limit of ~1 ng/mL, and a linear response ~100 ng/mL, and background light emission within an acceptable range.

To determine the precision of the ELISA, three different concentrations of recombinant GPVI were used (Table I). The coefficient of variability (CV%) of the inter-assay variability (triplicate) and

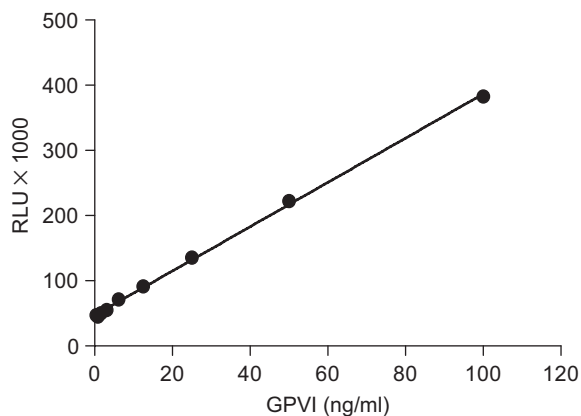


Figure 2. Dose response curve for ELISA. Measurement of recombinant GPVI ectodomain fragment in GPVI-depleted plasma using the standard ELISA. The average $RLU \pm$ STDE was determined in duplicate. The standard curve was generated using prism linear regression ($r^2 = 0.998$).

the intra-assay variability (15 different days) were <9% of the mean. Five normal plasma samples measured in triplicates on five different days showed inter-assay variability <10%, and intra-assay variability <14% (Table II). The additive ability of the assay was tested by adding a known concentration of recombinant GPVI (100 ng/mL) to human plasma samples. As shown in Table II, the recovery rate ranged between 90–110% indicating the assay is additive over the range 0.5–500 ng/mL.

Effect of blood collection and processing on plasma soluble GPVI levels

Blood from the same donor was collected into different anti-coagulant (ACD, citrate, EDTA) using a 21-gauge needle and vacutainer. There was no significant difference in soluble plasma GPVI levels between the different anti-coagulants ($P = 0.8$; one way ANOVA). When blood was collected using the same anti-coagulant (ACD) but with different sized needles, the 21-gauge appeared to increase GPVI levels although with no significant difference ($P = 0.08$ unpaired *t*-test). The 19-gauge needle was used routinely for ELISA measurements, as for other platelet function studies. Finally, ultracentrifugation of a plasma sample at 100 000g for 1 hour showed no significant difference in the level of soluble GPVI, suggesting GPVI measured in the assay was not dependent upon microparticles.

Table II. The inter-assay and intra-assay variability, and recovery rate of the ELISA. Soluble GPVI was measured in five healthy donors on five different days using the standard ELISA, and the average \pm STDE was calculated. The recovery rate was determined by adding a known concentration of recombinant GPVI ectodomain fragment (100 ng/mL) to samples.

	GPVI, ng/mL	Inter-assay CV %	Intra-assay CV %	Recovery (%)
Donor 1	19.4	5.5	9.7	96.0
Donor 2	18.9	4	11	90.5
Donor 3	15.8	10	12.5	95.6
Donor 4	33.7	1.1	13.3	103
Donor 5	13	1.4	11.5	97.5

Table I. ELISA precision as reflected by inter-assay and intra-assay variability. Three concentrations of recombinant GPVI ectodomain fragment diluted in GPVI-depleted plasma were assayed in triplicate on 15 different days using the standard ELISA. The mean reading (RLU) with standard deviation (STDE) and coefficient of variability (CV%) was calculated.

Added soluble GPVI	Inter-assay variability			Intra-assay variability		
	RLU (mean)	STDE	CV %	RLU (mean)	STDE	CV %
100 ng/mL	920773.5	35001.5	3.8	834632.7	69060.53	8.2
25 ng/mL	349613	23190	6.6	343024.1	25999.37	7.5
0.8 ng/mL	55260	1032	1.8	62754.97	5073.243	8.0

However, collecting blood into a silica-coated coagulation tube and analysing serum obtained from clotted blood showed elevated soluble GPVI levels (123.45 ± 4.4 ng/mL compared to standard anti-coagulated blood with ACD 29.05 ± 2.9 ng/mL). This indicates that care must be taken in blood collection to avoid coagulation when measuring soluble GPVI. Depleting the serum of GPVI by immunoabsorption, as described above for plasma, confirmed that the serum did contain elevated soluble GPVI, and that the elevated read-out in the assay was not due to some unknown artefact associated with clotting (soluble GPVI in clot activator tube before GPVI depletion was 132.02 ng/mL, reduced to 0 ng/mL after GPVI depletion using 1G5-coupled resin).

Normal range of GPVI in plasma and effect of inducing GPVI shedding

Blood plasma samples collected from 10 non-smoking, healthy donors aged 20–40 (five male, five female) revealed soluble GPVI levels of 18.90 ± 4.14 ng/mL in the range of 11–24 ng/mL. Treatment of human citrated PRP with the GPVI-selective ligand, CRP (Figure 3a), with the

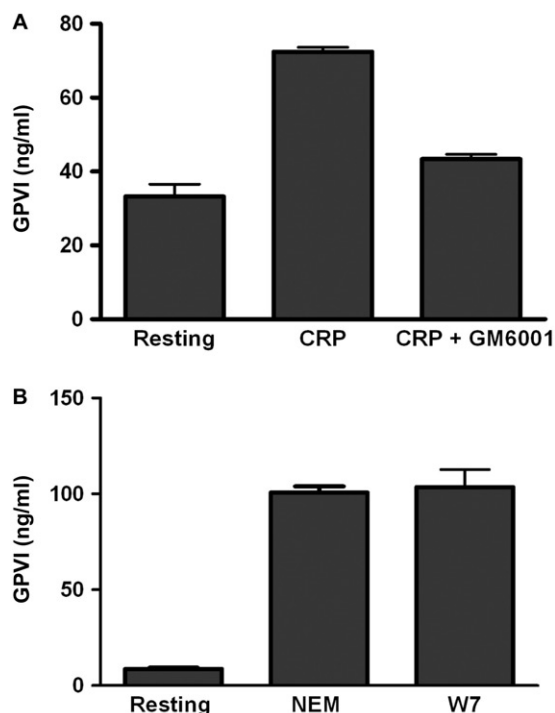


Figure 3. The effect of inducing shedding of platelet GPVI on levels of soluble GPVI in plasma. Human citrated PRP was either untreated or treated with (a) the GPVI ligand CRP ($20 \mu\text{g/mL}$) for 60 minutes in the absence or presence of the metalloproteinase inhibitor, GM6001 ($100 \mu\text{M}$) or (b) with either NEM (5 mM) for 15 minutes or the calmodulin inhibitor W7 ($300 \mu\text{M}$) for 60 minutes. After treatment, plasma was isolated by centrifugation to remove platelets and soluble GPVI was assayed in triplicate using the standard ELISA.

calmodulin inhibitor W7 or the thiol-modifying reagent, NEM, that directly activates platelet shed-dases (Figure 3b), treatments all previously shown to induce metalloproteinase-dependent GPVI shedding from human platelets [9, 10], increased the soluble GPVI levels in isolated plasma. This provides additional evidence that GPVI shedding can occur in human plasma and demonstrates that the assay can detect different levels of GPVI in plasma where platelets are exposed to prothrombotic conditions (GPVI ligands) or conditions where GPVI shedding is induced in the absence of platelet activation (W7 or NEM).

Discussion

Development of a sensitive ELISA for measuring soluble GPVI in human plasma should provide a useful tool for measuring GPVI levels in normal controls compared to disease states including autoimmune disease like ITP patients, and thrombotic disease including acute myocardial events where GPVI levels would be expected to be elevated, based both on experimental results [7, 9, 12] and on initial clinical studies [20] and potentially provide insights into other factors related to platelet GPVI shedding, including platelet aging [21, 22]. Bigalke *et al.* have reported elevated platelet GPVI expression levels in patients with acute coronary syndrome [20, 23], but plasma levels were not evaluated. For analysis of plasma proteins however, properties of both the plasma marker and the assay used for its detection must be within useful limits. In this study, we show that the lower detection limit of a new ECL-based ELISA for soluble GPVI in human plasma is ~ 1 ng/mL; that the assay is specific and sensitive up to 100 ng/mL and with acceptable precision and reproducibility as reflected by inter- and intra-assay variability of either known concentrations of recombinant GPVI or with normal control plasma samples; and the additive ability of the assay yielded a recovery rate of 90–110%. Measurement of soluble GPVI in plasma was essentially independent of the collection methods or anti-coagulant used; however markedly elevated soluble GPVI present in serum from clotted blood indicates coagulation of samples can compromise analysis. In this study, we did not investigate the mechanism by which clotting induces GPVI shedding from platelets.

Previous studies have analysed GPVI in plasma. First, Boylan *et al.* used a semi-quantitative western blot approach to detect plasma GPVI in normal plasma in pull-down experiments where soluble GPVI was separated from plasma by pull-down using beads coated with the GPVI ligand, convulxin [24]. Two preliminary reports (in abstract form) using an ELISA – one involving a single-chain anti-GPVI antibody, the other using a biotin-labelled anti-GPVI monoclonal antibody – suggest a normal

range of soluble GPVI levels from 18–114 pM (1–6 ng/mL) or 6–8 ng/mL, respectively [25, 26]. These values are lower, but not inconsistent, with our range of soluble GPVI in normal plasma (10 donors) of 11–24 ng/mL. Another recent published report uses an ELISA to measure soluble GPVI in 30 Alzheimer's disease patients and 20 age-matched controls, reporting an average value in normal plasma of 0.75 ± 0.43 ng/mL (*cf.* Alzheimer's patients, 0.55 ± 0.18 ng/mL), with a detection range for the assay of 0.08–5 ng/mL [27]. The reason for these differences is unclear but could be due to differences in sample preparation, antibodies, standards, or other factors. A value of ~15 ng/mL soluble GPVI in plasma corresponds to ~3% of total platelet GPVI, estimated from a copy number of ~5000 GPVI molecules per platelet and 200×10^9 platelets/mL, and consistent with our earlier observations that essentially all of the GPVI on circulating platelets is intact. In contrast, glyco-calicin, the soluble ectodomain of another platelet receptor, GPIb α (the ligand-binding subunit of the platelet GPIb-IX-V complex), is found in normal plasma at ~1–3 μ g/mL [28].

Finally, the soluble ectodomain of another platelet receptor, GPV, a component of the GPIb-IX-V complex, is present in human plasma, and circulating levels have recently been analysed using a GPV-specific ELISA [29]. Soluble GPV is potentially generated by thrombin (platelet GPV is a thrombin substrate), or by metalloproteinase-mediated ectodomain shedding, depending on the thrombin concentration and distinguishable by western blotting platelets with an anti-GPV cytoplasmic domain antibody [13]. In healthy individuals, GPV is present at 27.5 ng/mL (a range of 23.5–34.4 ng/mL) [30]. GPV, of the leucine-rich repeat receptor family, has been reported as a collagen-binding protein on platelets [31] although its precise pathophysiological role has not been established. However, soluble GPV is increased in patients with athero-thrombotic disease [30, 32] and like GPVI, may be a useful platelet-specific biomarker.

In conclusion, GPVI is stable on the resting platelet surface unless shedding is induced, and plasma levels are relatively low. Development of a sensitive and well characterized ELISA for measuring soluble GPVI provides a quantitative tool for measuring GPVI in human plasma, and should allow further assessment of GPVI levels in normal and disease states, and facilitate further characterization of the clinical importance of the shed fragment of GPVI as a biomarker.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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