

## **Platelet heterogeneity in activation-induced glycoprotein shedding: functional effects**

*Online data supplement*

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*Running title:* Platelet heterogeneity in glycoprotein shedding

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## **Supplemental Materials and Methods:**

### *Materials*

2-aminoethyldiphenylborinate (2APB), apyrase, bovine serum albumin (BSA), carbonyl cyanide 3-chlorophenylhydrazone (CCCP), dimethylsulfoxide, phorbol myristate acetate (PMA) and  $\alpha$ -thrombin were obtained from Sigma (St. Louis, MO, USA). Collagen related peptide cross-linked (CRP-XL) was purchased from Prof. Richard Farndale (University of Cambridge, UK) and convulxin was obtained from Stago BNL (Leiden, the Netherlands). ABT-737, 2MeS-ADP and thapsigargin were from SantaCruz Biotechnology (Santa Cruz, CA). Fluorescein isothiocyanate (FITC)-labeled monoclonal anti-GPIb $\alpha$  antibody, recognizing the extracellular domain, was acquired from Sanquin (Clone CLB-MB45), (Amsterdam, the Netherlands), fluorescent Megamix calibration beads (3.0, 0.9, 0.5  $\mu$ m) and phycoerythrin (PE)-labeled monoclonal anti-GPVI antibody, recognizing the ligand-binding ectodomain domain (residues 21-234), from Biocytex (Marseille, France), PE-labeled monoclonal anti-GPIb $\alpha$  antibody, recognizing GPIb $\alpha$  and glyocalicin, from DAKO (Heverlee, Belgium), FITC-labeled anti-VWF antibody from Affinity Biologicals (Ancaster, Canada), and FITC-labeled monoclonal anti-GPIX antibody from eBioscience (San Diego, CA, USA). Fab fragment 5G6 against GPIb $\alpha$ , blocking the ADAM17-mediated cleavage of this chain was purified, as described before.<sup>1</sup> Annexin A5 Alexa Fluor (AF)647-conjugated was from Invitrogen (Bleiswijk, the Netherlands), collagen type I from Nycomed Pharma (Munich, Germany). QVD-Oph, Ro318425 and ionomycin were from Calbiochem (San Diego, CA, USA). MDL-28170 and GI254023X (specificity ADAM10>ADAM17)<sup>2</sup> were purchased from Tocris (Bristol, UK); 3,3'-dihexyloxacarbocyanine iodide (DiOC<sub>6</sub>) was from Anaspec (Fremont, CA, USA); GW280264X (ADAM10/17 inhibitor)<sup>2</sup> was from Aobious (Gloucester, MA, USA); dimethyl-BAPTA acetoxymethyl ester (dm-BAPTA AM) was from Molecular Probes (Waltham, MA, USA). TAPI-0 (tumor necrosis factor  $\alpha$  protease inhibitor-0 = ADAM17/TACE inhibitor) was a kind gift from Prof. Dr. A. Ludwig (Aachen, Germany). Human prothrombin and factor Xa were purified and specifically active-site labeled with

Oregon Green (OG)-488, as described.<sup>3-5</sup> Both active-site labeled coagulation factors had retained normal binding properties, while protease activity was fully blocked.<sup>3,4</sup> AF488-labeled factor Va was prepared as described.<sup>6</sup> Factor Xa was isolated from bovine plasma, as reported before.<sup>7</sup>

#### *Platelet isolation*

PRP was prepared by centrifugation at 260 g for 15 min. After a second centrifugation step, platelets were resuspended in HEPES buffer pH 6.6 (10 mM HEPES, 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl<sub>2</sub>, 5 mM glucose and 0.1% BSA). Platelet suspensions were re-centrifuged in the presence of 1:15 ACD and 1 U/mL apyrase and subsequently resuspended into HEPES buffer pH 7.45 (10 mM HEPES, 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl<sub>2</sub>, 5 mM glucose and 0.1% BSA).

#### *Flow cytometric analyses*

Pre-incubations of washed platelets ( $1 \times 10^8$ /mL) with inhibitor or vehicle were performed for 15 min at 37°C. After addition of 2 mM CaCl<sub>2</sub>, platelets were stimulated for indicated times with (combinations of) the following agonists at near-saturating doses: ABT-737 (10 μM), ionomycin (10 μM), convulxin (100 ng/mL), CRP-XL (5 μg/mL), thrombin (4 nM), SFLLRN (15 μM), factor Xa (10 μg/mL), 2MeS-ADP (10 μM), CCCP (100 μM) or PMA (100 nM). Surface expression of GPIIb/IIIa, GPVI or GPIX was measured in subsamples, after labeling with FITC anti-GPIIb/IIIa mAb (10 μg/mL), PE anti-GPVI mAb (5 μg/mL) or FITC anti-GPIX mAb (2.5 μg/mL), respectively; AF647-annexin A5 (5 μg/mL) was added to measure PS exposure. Binding of VWF was analyzed after incubation with 2.5 μg/mL VWF and 0.5 mg/mL ristocetin, followed by labeling with FITC anti-VWF Ab (25 μg/mL). Binding of coagulation factors was determined using OG488-prothrombin, OG488-factor Xa or AF488-factor Va (5 μg/mL).<sup>5,6</sup> Analysis was with an Accuri C6 flow cytometer and software.<sup>8</sup>

*Glycoprotein shedding and microparticle formation*

Using standard flow cytometry settings, forward/side scatter (FSC/SSC) profiles of resting and ionomycin activated platelets in suspension were compared with Megamix fluorescent calibration beads (0.5, 0.9 and 3  $\mu\text{m}$  diameter). Size measurements using nanoparticle tracking analysis and electron microscopy have indicated that approximately 75% of the microparticles from ionomycin-stimulated platelets are smaller than 0.8  $\mu\text{m}$ .<sup>9,10</sup>

*Whole blood thrombus formation with platelet activation*

Washed coverslips were coated with microspots of collagen type I (2  $\mu\text{L}$ , 50  $\mu\text{g}/\text{mL}$ ) and blocked with HEPES buffer pH 7.45 containing 1% BSA. Coated coverslips were mounted in a transparent parallel plate flow chamber (50  $\mu\text{m}$  depth, 3 mm width, 20 mm length).<sup>11</sup> Thrombi with activated platelets were formed on collagen by a 4-min whole blood perfusion (1000  $\text{s}^{-1}$ ), essentially as described elsewhere,<sup>12</sup> but with modifications. In brief, citrated blood was mixed during flow by 1:10 (vol./vol.) co-perfusion with recalcification buffer (64 mM  $\text{CaCl}_2$ , 32 mM  $\text{MgCl}_2$  in HEPES buffer pH 7.45), prior to reaching the flow chamber. Blood perfusion was halted after thrombus contraction, but before visual appearance of fibrin fibers. Thrombi were then rinsed for 5 min with HEPES buffer pH 7.45 containing 25 nM thrombin and stained for GPIIb/IIIa (FITC mAb, 2  $\mu\text{g}/\text{mL}$ ), GPVI (PE mAb, 1  $\mu\text{g}/\text{mL}$ ), and/or PS exposure (AF647 annexin A5, 5  $\mu\text{g}/\text{mL}$ ). Where indicated, the platelet thrombi were post-incubated with ADAM inhibitor GW280264X (5  $\mu\text{M}$ )<sup>2,13</sup> or vehicle (DMSO) in HEPES buffer pH 7.45 at 37°C.

In an alternative protocol, to exclude thrombin generation, citrated blood was recalcified in the presence of 40  $\mu\text{M}$  PPACK,<sup>14</sup> and perfused over collagen as above, after which the thrombi were rinsed for 5 min with HEPES buffer pH 7.45 containing 15  $\mu\text{M}$  SFLLRN. Subsequently, thrombi with SFLLRN-stimulated platelets were stained for binding of VWF (FITC mAb, 25  $\mu\text{g}/\text{mL}$ ) or prothrombin (10  $\mu\text{g}/\text{mL}$ ) and PS exposure (AF647-annexin A5, 5  $\mu\text{g}/\text{mL}$ ). To measure the coagulant capacity of thrombi, thrombin generation was measured. Pre-formed thrombi were allowed to shed receptors by 1 h incubation at 37°C,



and were then perfused with anrod plasma supplemented with fluorogenic thrombin substrate Z-Gly-Gly-Arg-AMC (0.5 mM, Thrombinoscope, Maastricht, The Netherlands) in the presence of CaCl<sub>2</sub>. Fluorescence accumulation due to substrate cleavage was measured in real-time, by recording fluorescence images every 30 sec during a period of 8 min, as described previously.<sup>12</sup>

#### *Fluorescence microscopy*

Differential interference contrast (DIC) and multi-color fluorescence images were captured with a fast line-scanning Zeiss LSM7 system equipped with a 63x oil-immersion objective (Carl Zeiss, Oberkochen, Germany). Fluorescence was recorded at excitation (emission) wavelengths of 488 (505-610), 532 (540-625) and 635 (>655) nm. Where indicated, brightfield and non-confocal fluorescence images (8 bit, 1360x1024 pixels, 142x107 μm) were captured using an EVOS fluorescence microscope (Life Technologies, Bleiswijk, The Netherlands) equipped with an Olympus APLAN 60x oil objective.<sup>14</sup> Image analysis was performed using Fiji image analysis software.<sup>15</sup>

#### *Fibrin formation under flow conditions*

To measure the platelet fibrin-forming potential,<sup>16</sup> citrated whole blood containing tirofiban (5 μg/mL) was perfused over collagen for 5 min (1000 s<sup>-1</sup>), resulting in a single layer of adhered platelets. Platelets were post-perfused with 10 μM ionomycin and 5 mM CaCl<sub>2</sub> in Hepes buffer pH 7.45 for 5 min in the presence or absence of GW280264X. Fibrin formation was continuously monitored by fluorescence microscopy during flow of citrated plasma containing AF647-fibrinogen (15 μg/mL), co-perfused 1:10 with recalcification buffer, at a final shear rate of 250 s<sup>-1</sup>.

**Supplemental Table:**

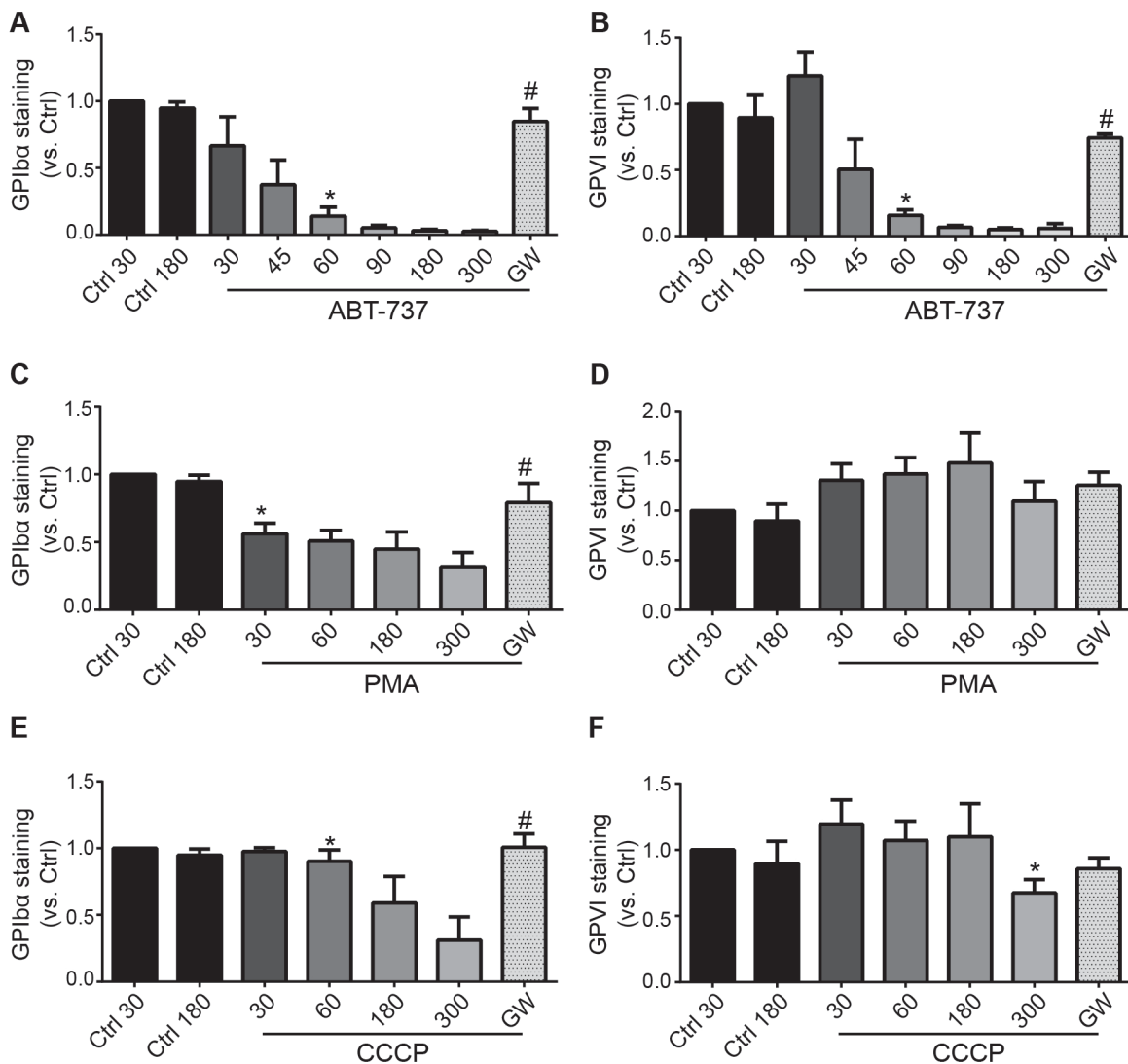
**Table S1: Combined effects of agonists and inhibitors on glycoprotein shedding.**

Washed platelets were pre-treated for 15 min with ADAM10/17 inhibitor GW280264X (5  $\mu$ M),<sup>13</sup> the calpain inhibitor MDL-28170 (200  $\mu$ M),<sup>17</sup> the pan-caspase inhibitor QVD-OPh (10  $\mu$ M),<sup>8</sup> the general PKC inhibitor Ro-318425 (10  $\mu$ M),<sup>18</sup> the calcium chelator dm-BAPTA AM (20  $\mu$ M),<sup>8</sup> or the Ca<sup>2+</sup> entry inhibitor 2APB (100  $\mu$ M).<sup>19</sup> Platelet samples were then stimulated with ionomycin/CaCl<sub>2</sub> (10  $\mu$ M/2 mM), CRP-XL/thrombin (5  $\mu$ g/mL/4 nM), PMA (100 nM), ABT-737 (10  $\mu$ M) or CCCP (100  $\mu$ M), as indicated. For the whole platelet population, surface expression of GPIIb $\alpha$  (mean fluorescence intensity) was measured after stimulation for 60 min (ionomycin, ABT-737) or 180 min (CRP-XL/thrombin, PMA, CCCP). Data were normalized to the values of unstimulated platelets without any inhibitors. Means  $\pm$  SD, n = 3-5; \* $p$ <0.05 vs. no inhibitor.

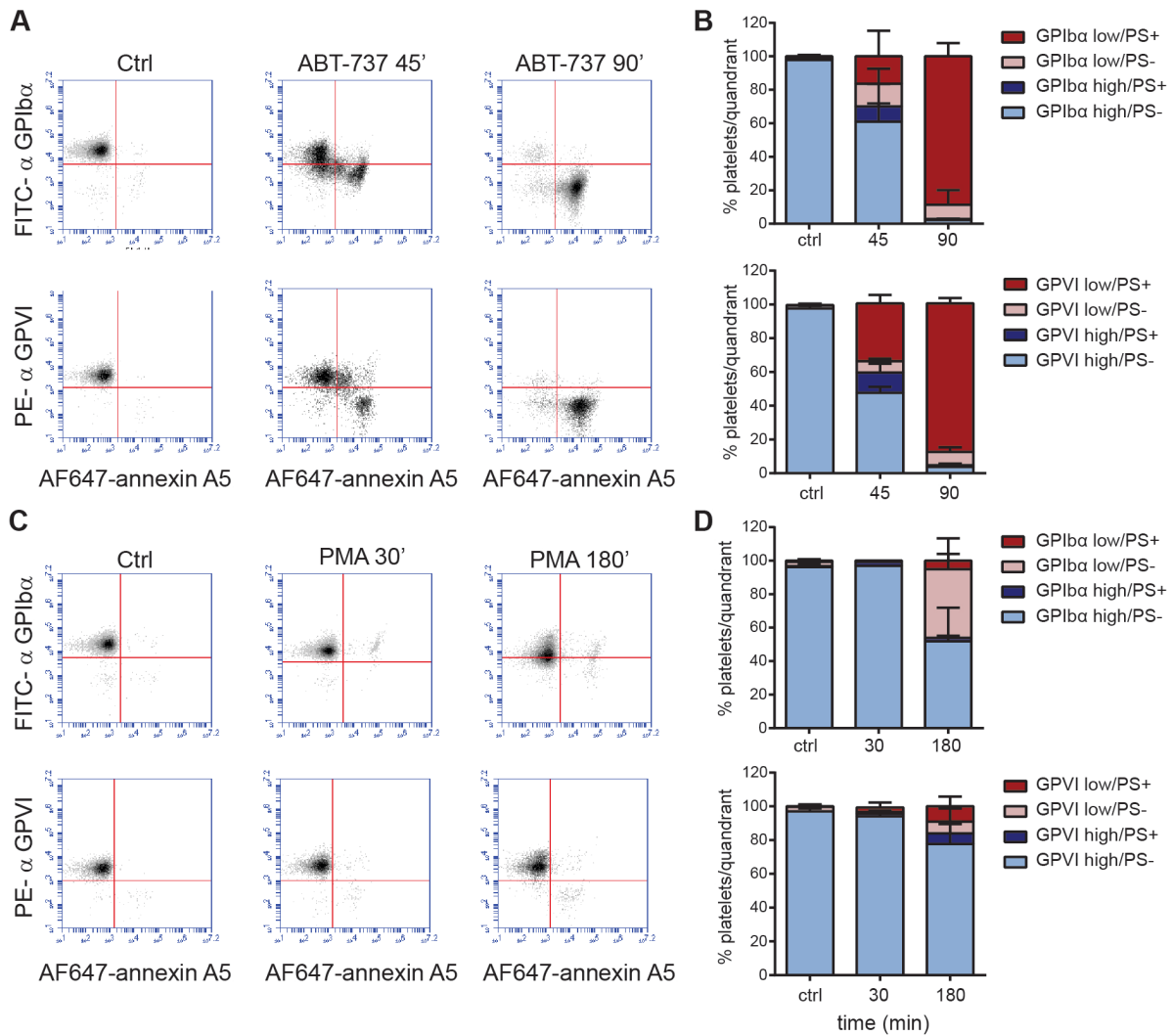
Inhibition or chelation of	Unstimulated	Ionomycin	CRP-XL +Thr	PMA	ABT-737	CCCP
<b>Control</b>	1.00	0.08 $\pm$ 0.02	0.17 $\pm$ 0.07	0.40 $\pm$ 0.12	0.09 $\pm$ 0.06	0.64 $\pm$ 0.14
<b>ADAM10/17</b>	0.99 $\pm$ 0.08	0.67 $\pm$ 0.04 *	0.79 $\pm$ 0.12*	0.79 $\pm$ 0.14*	0.85 $\pm$ 0.10*	1.01 $\pm$ 0.10*
<b>Calpain</b>	0.94 $\pm$ 0.09	0.24 $\pm$ 0.05 *	0.35 $\pm$ 0.11	0.38 $\pm$ 0.06	0.12 $\pm$ 0.01	0.45 $\pm$ 0.13
<b>Caspases</b>	0.99 $\pm$ 0.01	0.08 $\pm$ 0.02	0.22 $\pm$ 0.04	0.45 $\pm$ 0.10	0.87 $\pm$ 0.17*	0.73 $\pm$ 0.19
<b>PKC</b>	1.01 $\pm$ 0.08	0.04 $\pm$ 0.01	0.38 $\pm$ 0.11*	0.96 $\pm$ 0.10*	0.11 $\pm$ 0.03	0.50 $\pm$ 0.12
<b>Calcium</b>	0.85 $\pm$ 0.05	0.87 $\pm$ 0.02* <sup>§</sup>	0.70 $\pm$ 0.12*	0.52 $\pm$ 0.20	0.52 $\pm$ 0.17*	0.72 $\pm$ 0.07
<b>Calcium entry</b>	0.92 $\pm$ 0.13	0.87 $\pm$ 0.02* <sup>§</sup>	0.59 $\pm$ 0.05*	0.24 $\pm$ 0.07	0.13 $\pm$ 0.05	0.42 $\pm$ 0.14

<sup>§</sup>CaCl<sub>2</sub> omitted instead.

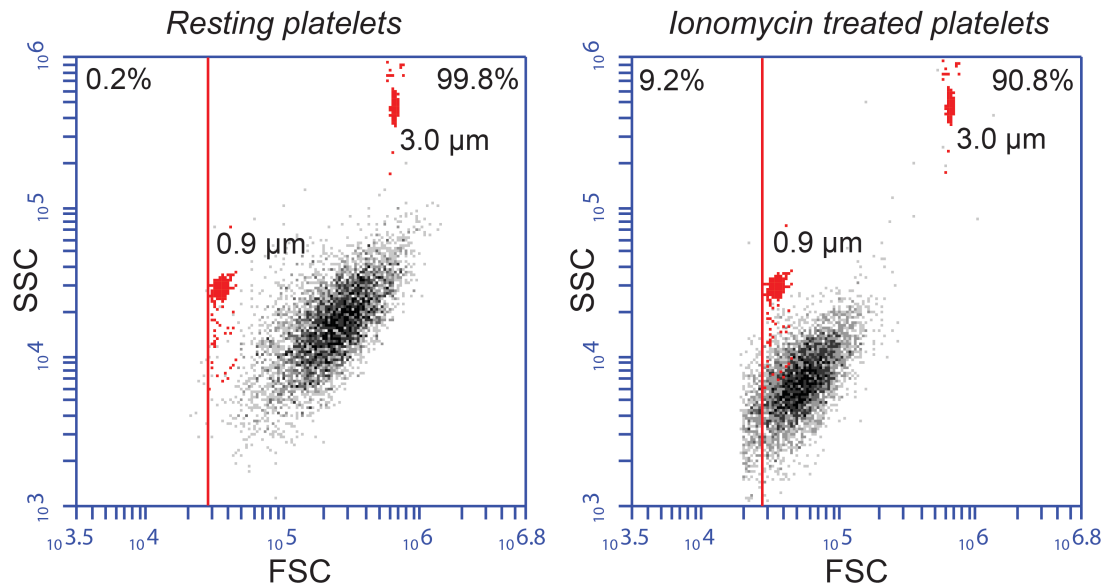
**Supplemental Figures:**



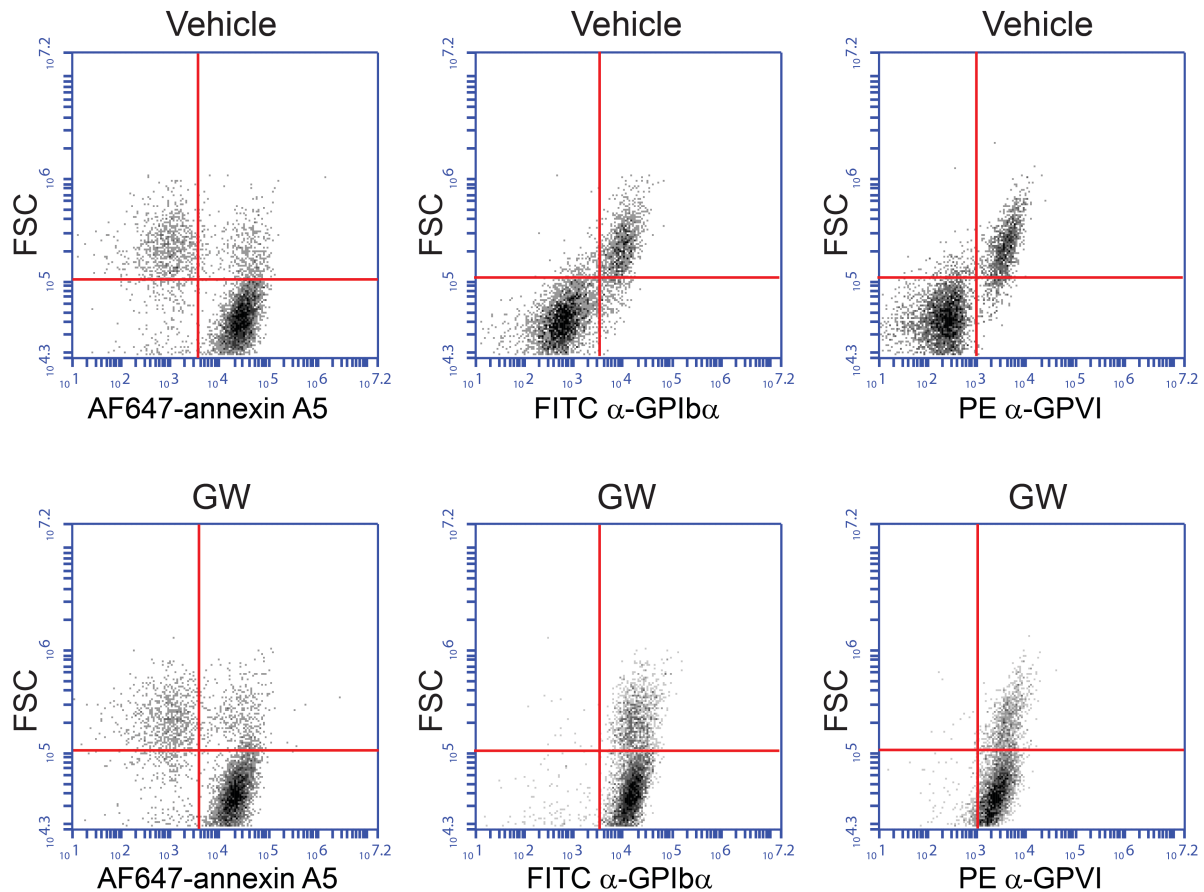
**Figure S1: Differential glycoprotein receptor shedding after platelet stimulation with ABT-737, PMA or CCCP.** Washed platelets, pre-incubated with DMSO (vehicle) or GW280264X (GW, 5  $\mu$ M), were stimulated with near-maximal concentrations of ABT-737 (**A-B**), PMA (**C-D**) or CCCP (**E-F**) for indicated times (30-300 min) at 37°C. Unstimulated platelets were used as control (Ctrl). Staining for GPIb $\alpha$  and GPVI was measured with fluorescent-labeled antibodies using flow cytometry (mean fluorescence intensities). Data were normalized to values of unstimulated platelets. Data are mean  $\pm$  SD, n = 3-7 (n $\geq$ 3 donors); \* $p$ <0.05 vs. unstimulated control, # $p$ <0.05 vs. vehicle.



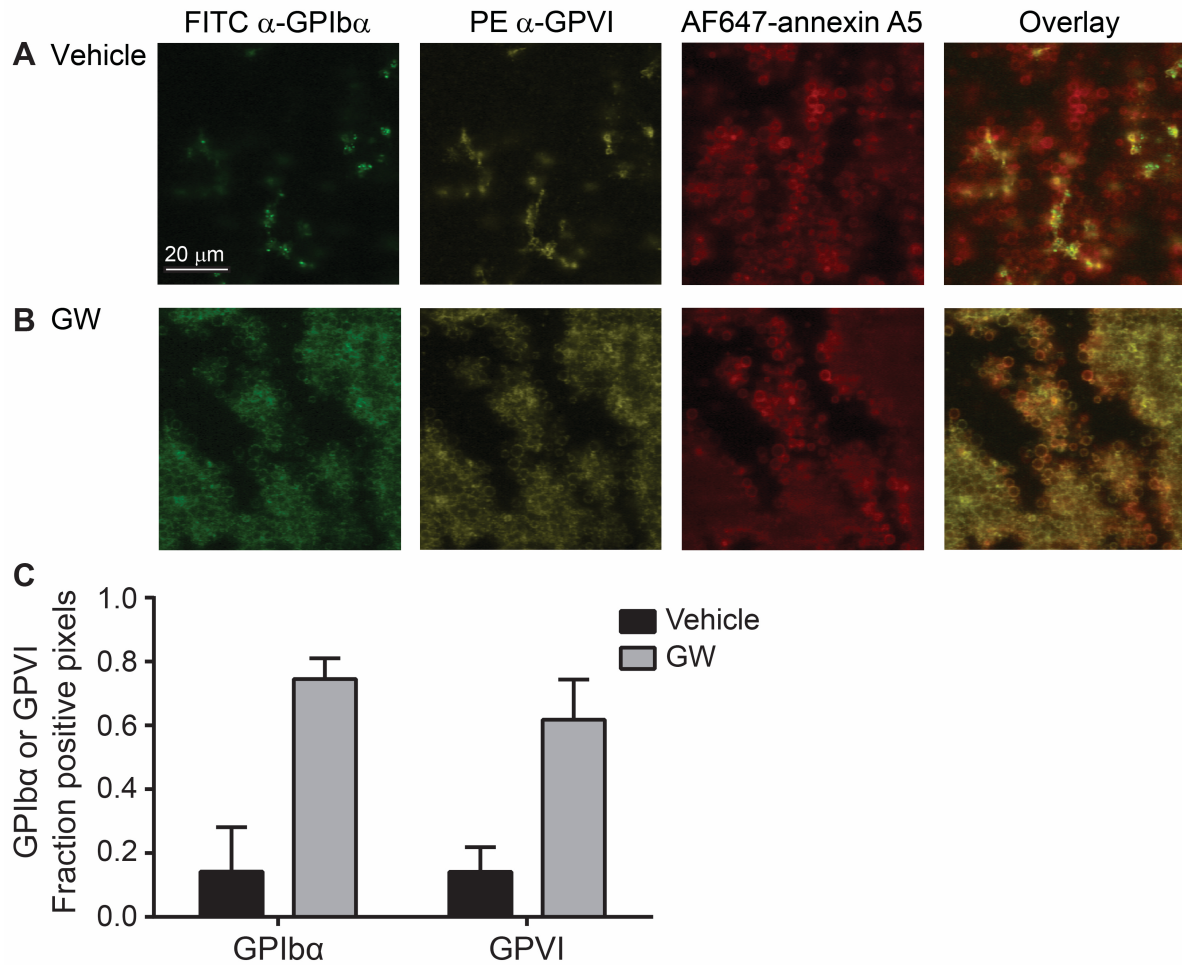
**Figure S2: Differential glycoprotein receptor shedding and PS exposure in platelets stimulated with ABT-737 or PMA.** Washed platelets were stimulated with ABT-737 (**A-B**) or PMA (**C-D**) for indicated times, and evaluated for glycoprotein expression, as for Figure S1. Unstimulated platelets were used as control (Ctrl). Platelets were dually stained for GPIba or GPVI expression and PS exposure (AF647-annexin A5) at indicated time points. **A**, Representative dot plots showing a gradual decrease in GPIb $\alpha$  and GPVI expression accompanying PS exposure. **B**, Quantification of four quadrants of platelet populations, as in Figure 2. **C**, Representative dot plots showing a decrease in GPIb $\alpha$  but not GPVI expression with limited PS exposure. **D**, Quantification of platelet populations. Data are means  $\pm$  SD, n = 3-5 (n $\geq$ 3 donors); \*p<0.05.



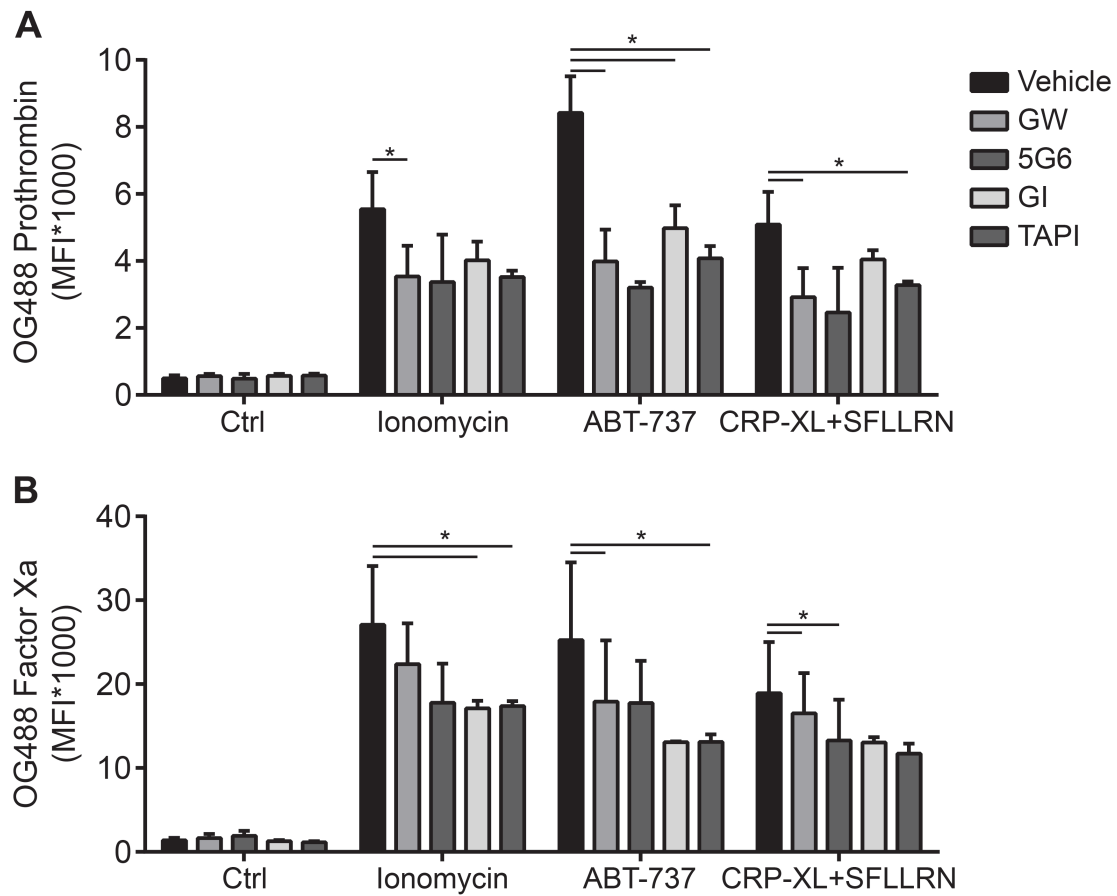
**Figure S3: Calibrated forward and side scatter profiles of resting and ionomycin-activated platelets.** Washed platelets ( $1 \times 10^8/\text{mL}$ ) were left untreated (resting) or were treated with  $10 \mu\text{M}$  ionomycin in the presence of  $2 \text{ mM CaCl}_2$  for 30 min at  $37^\circ\text{C}$ . Glycoprotein shedding was  $>95\%$  after activation. Forward scatter (FSC) and side scatter (SSC) profiles of the platelet events were measured, using standard flow cytometry settings. Scatter events from Megamix calibration beads are indicated in red. The majority of events (resting:  $99.8\%$ , ionomycin:  $90.8\%$ ) was comparable in size or was larger in FSC than the  $0.9 \mu\text{m}$  beads. Note that the ballooning morphology of the ionomycin-activated platelets makes them more translucent,<sup>20,21</sup> thus providing these cells with a lower FSC/SSC profile, in comparison to the non-transparent fluorescent beads.



**Figure S4: Unaltered scatter profiles of activated platelets undergoing glycoprotein shedding.** Washed platelets ( $1 \times 10^8/\text{mL}$ ) were pretreated with vehicle (DMSO) or ADAM10/17 inhibitor GW280264X ( $5 \mu\text{M}$ ) for 15 min. Subsequently, platelets were stimulated with  $5 \mu\text{g}/\text{mL}$  CRP-XL plus  $4 \text{ nM}$  thrombin for 180 min at  $37^\circ\text{C}$ , after which the platelets were labeled with AF647-annexin A5, FITC anti-GPIb $\alpha$  or PE anti-GPVI mAb. Shown are dot plots of forward scatter vs. indicated labels. Note that scattering of the platelet population with lower FSC values (corresponding to the ballooning, annexin A5-positive platelets) is not affected by ADAM10/17 inhibition.

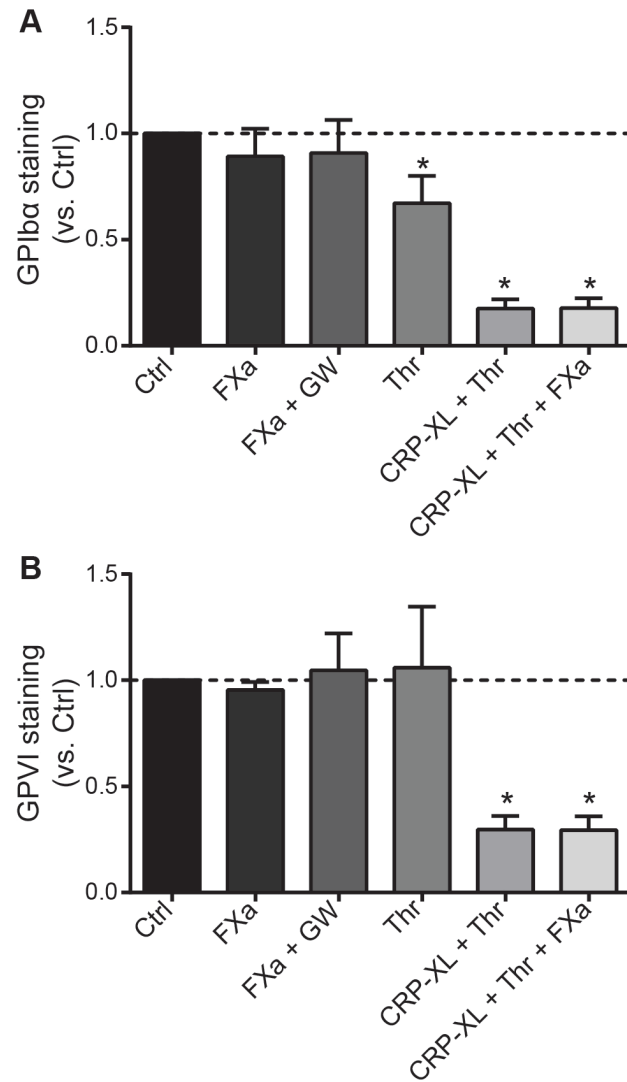


**Figure S5: Massive glycoprotein shedding of platelets in thrombi induced by ionomycin.** Thrombi were formed on collagen type I under arterial flow conditions, and subsequently post-perfused with 10  $\mu$ M ionomycin and 5 mM  $\text{CaCl}_2$  for 5 min in the presence or absence of GW280264X (GW). Platelets in thrombi were then stained for GPIb $\alpha$  and GPVI expression and PS exposure. Representative images of GPIb $\alpha$  and GPVI expression and PS exposure after ionomycin activation in the presence of vehicle (**A**) or GW (**B**). Bar = 20  $\mu$ m. Fractions of ballooned platelets staining for GPIb $\alpha$  or GPVI (**C**). Means  $\pm$  SD; n = 3, \* $p$ <0.05.

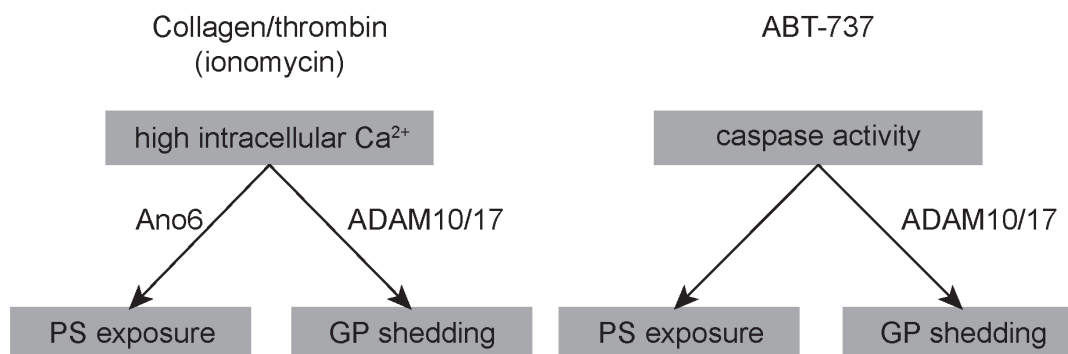


**Figure S6: Inhibitors of ADAM10/17 suppress coagulation factor binding to activated platelets.** Washed platelets were pre-incubated for 15 min with vehicle, GW280264X (GW, 5  $\mu$ M, specificity inhibition ADAM10  $\approx$  ADAM17), 5G6 Fab fragment, blocking ADAM17-mediated shedding of GPIIb/3a (10  $\mu$ g/mL), GI254023X (GI, 5  $\mu$ M, ADAM10 > ADAM17) or TAPI (5  $\mu$ M, ADAM17 > ADAM10). The platelets were then activated for 60 min (37°C) with ionomycin, ABT-737 or CRP-XL plus SFLLRN, as in Figure 7. Flow cytometry was used to assess binding of OG488-prothrombin (**A**) or OG488-factor Xa (**B**) to platelets. Coagulation factor binding is shown for the PS-exposing platelet population, identified with AF647-annexin A5. Means  $\pm$  SD, n = 3-6, \*p<0.05.





**Figure S7: Inability of factor Xa to induce direct GPIIb/IIIa and GPIIb/IIIa shedding.** Washed platelets, pre-incubated with DMSO (vehicle) or GW280264X (GW, 5  $\mu$ M) were stimulated with factor Xa (10  $\mu$ g/ml), thrombin (4 nM) or a combination of CRP-XL (5  $\mu$ g/ml), factor Xa and thrombin for 180 min. Platelet activation was evaluated for glycoprotein expression, as for Figure S1. Data were normalized to values of unstimulated platelets. Means  $\pm$  SD, n = 4, \* $p < 0.05$  (compared to control).



**Figure S8: Schematic representation of common and differential pathways involved in PS exposure and receptor shedding.** Abbreviations: Ano6, Anoctamin 6.

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