SUPPLEMENTARY APPENDIX

Functional plasminogen activator inhibitor 1 is retained on the activated platelet membrane following platelet activation

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Methods

Blood collection and preparation of platelet-rich plasma

Blood was drawn from healthy volunteers by a trained phlebotomist in to vacuettes (Greiner Bio-One Ltd) containing 3.2% sodium citrate for platelet-rich plasma (PRP) or acid-citrate-dextrose-A vacuettes for platelet isolation. Ethical approval was obtained from the University of Aberdeen College Ethics Review Board. PRP was isolated from whole blood by centrifugation at 170 *x g* for 10 min at ambient temperature.

Isolation of platelets, soluble and cellular fraction

Platelets were collected following centrifugation of whole blood at 260 *x g* for 15 min at ambient temperature to collect PRP. PRP containing 0.1 U/ml apyrase (Sigma-Aldrich) and acid citrate dextrose (ACD- 5.3 mM trisodium citrate, 3.5 mM citric acid and 12.2 mM glucose) was centrifuged at 870 *x g* for 15 min to obtain the platelet pellet. Platelet-poor plasma (PPP) was discarded and the platelet pellet washed in HEPES buffer (10 mM HEPES pH 6.6, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.1% glucose and 0.1% BSA containing 0.1 U/ml apyrase and ACD) at 870 x *g* for 15 min at ambient temperature. Pelleted platelets were resuspended in HEPES buffer (10 mM HEPES pH 7.45, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.1% glucose and 0.1% BSA containing 0.1 U/ml apyrase). Platelet counts were performed by the Haematology department at the Aberdeen Royal Infirmary using a Siemens Advia 2120i Hematology system.

In some cases, the soluble and cellular fraction were isolated. Platelets were activated for 30 min at 37°C with 1 µg/ml convulxin (CVX; Enzo Life Sciences) and 100 nM thrombin (Sigma-Aldrich). Activated platelets were centrifuged at 13,000 *x g* for 4 min and the soluble fraction, containing expelled granular contents collected. The pellet, containing the cellular components, was re-suspended in HEPES buffer to its original volume (10 mM HEPES pH 7.45, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.1 % glucose, 0.1 % BSA).

Flow cytometry analysis of platelets

Washed platelets (2 x 10^8 plt/ml) in HEPES buffer, pH 7.45, were stimulated for 45 min at ambient temperature with 1 µg/ml CVX ± 0.2 mM TRAP-6 (Sigma-Aldrich) or 100 nM thrombin in the presence of 2 mM $CaCl_2$. In some cases, platelets were pre-treated for 30 min with 5

mM Gly-Pro-Arg-Pro (GPRP) (Sigma-Aldrich) or 1 μg/ml tirofiban (Sigma-Aldrich) to inhibit fibrin polymerization or $\alpha_{IIb}\beta_3$, respectively. Rabbit polyclonal antibodies to either PAI-1 (5.8 μg/ml) labeled with DyLight 488 (DL488), fibrin(ogen) labeled with DL405 or Vn labeled with DL488 were added at the time of activation. After 40 min Annexin A5-alexa fluor 647 (AF647) (1/20) (BD Biosciences) was added in the presence of 2 mM $CaCl_2$ to label PS-exposing platelets. Exposure of PAI-1 and PS were analyzed using a BD LSRII cytometer with FACS DIVA 6.1.3 software. Inclusion of a rabbit IgG control showed 30% non-specific staining in both unstimulated and stimulated platelets. Platelets were gated using forward scatter (FSC) against side scatter (SSC) to exclude aggregates, and 10,000 events were collected per sample at excitation wavelengths 405, 488 and 633 nm. Data was analyzed using Flow Jo V.X.0.6 software (Tree Star Inc.) and expressed as median fluorescence intensity (MFI) or percentage of positive cells ± SEM.

Fluorescence imaging of platelets

Ibidi μ-slide $VI^{0.4}$ chambers were coated overnight at 4°C with collagen (20 μg/ml) (American Biochemical Pharmaceuticals). Excess collagen was removed and thrombin (100 nM) added for 1 h at ambient temperature. Slides were blocked with 5% BSA in PBS for 1 h before addition of washed platelets (0.5 x 10^8 plt/ml) at ambient temperature. In some cases, platelets were pre-treated with 5 mM GPRP or 1 μg/ml tirofiban prior to activation. An in-house rabbit polyclonal PAI-1 antibody-DL633 (5.8 μg/ml), rabbit polyclonal Vn antibody-DL488 (13 μg/ml) (Thermo Fisher Scientific), polyclonal antibody to fibrin(ogen)-DL488 (37 μg/ml) (Thermo Fisher Scientific), mouse monoclonal PE/Cy5-conjugated antibody to P-selectin (CD62P; 1/20; Biolegend) or a FITC-conjugated monoclonal antibody to the CD41 subunit (1/20; Stago) were included during stimulation. After 30 min of stimulation Annexin-A5 FITC or AF647 (1/20) was added in the presence of 2 mM $CaCl_2$ for an additional 15 min incubation. At 45 min platelets were visualized using a *x*63 1.40 oil immersion objective and Zeiss 710 laser scanning confocal microscope. Images were recorded on DIC and excitation wavelengths of 405, 488, 547 and 633 nm. The images were analyzed using Zen 2012 SP1 (Black edition).

Fluorescence imaging of PRP clots

Clots were formed from 30% PRP in the presence of 0.25 µM fibrinogen-Alex fluor 546 (AF546) (Thermo Fisher Scientific). In some cases, a neutralizing antibody to PAI-1 (400 µg/ml) was added for 30 min prior to activation. Clotting was initiated using 0.125 U/ml thrombin and 10 mM $CaCl_2$. Annexin A5-AF647 and a rabbit polyclonal antibody to PAI-1 DL633 or rabbit polyclonal antibody to Vn DL633 were incorporated into the clot. Clots were polymerized in Ibidi µ-slide $VI^{0.4}$ chambers at 37 °C for 2 h in a moist box. In some cases, 75 nM tPA (Genetech) was added to the edge of clot and lysis monitored by taking images every 10 s. For lysis experiments platelets were labelled with 0.5 µg/ml 39,39-dihexyloxacarbocyanine iodide (DiOC6) (AnaSpec). Clots were imaged using a *x* 63 1.4 oil immersion objective and Zeiss 710 laser scanning confocal microscope. Images were recorded on DIC and at excitation wavelengths of 405, 488, 547 and 633 nm and analysed using Zen 2012 SP1 (Black edition).

PAI-1 ELISA

An in-house enzyme-linked immunosorbent assay (ELISA) was performed as described previously[14] to quantify PAI-1 in platelet and plasma preparations. The ELISA is equally sensitivity to the active and latent form and can detect both free and complexed PAI-1[14]. Briefly, a rabbit polyclonal antibody to PAI-1 (10 µg/ml) was incubated overnight at 4°C before blocking the plate for 1 h with 1% BSA in PBS. Samples were incubated for 3 h at 37°C after which a biotinylated anti-PAI-1 antibody was added for 2 h at 37 °C. Biotin peroxidase was added for 1 h and the substrate, 3,3',5,5'-Tetramethylbenzidine (TMB), was applied. The reaction was stopped after 15 min with H_2S0_4. The plate was read at 450 nm with reference filter 620 nm using a Labsystems iEMS plate reader and Ascent software (version 2.6).

PAI-1 activity assay

PAI-1 activity was quantified using a commercial activity kit (Abcam). Briefly, samples were diluted and incubated with an excess of tPA to allow PAI-1-tPA complex formation. After 10 min incubation, 80 µl assay mix (containing plasminogen and a plasmin substrate) was added to the wells of a 96-well microplate before addition of 20 µl sample or standards. The plate was incubated at 37°C and read at 405 nm every 60 min for 8 h using a Labsystems iEMS plate reader and Ascent software (version 2.6). The absorbance at 405 nm is inversely proportional to PAI-1 enzymatic activity. A standard curve was generated, and the unknown

sample concentrations determined from the standard curve, PAI-1 activity was expressed as international units per ml (IU/ml).

Thrombodynamic analysis of clot formation and lysis

PRP clots were formed in the presence of 5 nM tPA according to manufacturer guidelines (HemaCore). In some cases, PRP was pre-treated with a neutralising polyclonal antibody to PAI-1 (400 µg/ml)[6, 46, 47] for 30 min prior to clot formation. Clot formation and lysis was monitored using time-lapse microscopy in a Hemacore Thrombodynamics T-2 analyser. Images were taken every 6 s for 60 min. Karmin v.0.54 software was used to analyse parameters of coagulation and fibrinolysis.

Turbidity clot lysis assay

PRP (30%), phospholipids (16 µM) and tPA (300 pM) ± neutralising polyclonal antibody to PAI-1 (400 µg/ml) in 10 mM TRIS pH 7.4, 0.01% Tween20 were added to 96-well plates. Clotting was initiated with 10.6 mM $CaCl_2$ and clot formation and lysis monitored using a Labsystems Multiskan Ascent 354 plate reader. Absorbance was recorded every 60 s for 4 h using Ascent software (version 2.6). Data was analysed by calculating time to 50% lysis using Shiny App software for clot lysis[48].

Chandler model thrombi

Thrombi were formed using the Chandler model[49]. Loops were formed using transparent vinyl tubing with 3 mm inner and 4.2 mm outer diameter. Pooled normal plasma (PNP) thrombi were formed containing 45 µg/ml FITC-labelled fibrinogen and 10.9 mM $CaCl_2$ ± a neutralizing antibody to PAI-1 (400 µg/ml). Loops were rotated at 30 rpm for 90 min to mimic arterial shear rates (400-600 s^{-1}). Thrombi were removed and washed in 0.9 % saline before adding to bathing buffer (10 mM Tris pH 7.5, 0.01 % tween 20) containing 1 µg/ml tPA at 37°C and samples taken every 30 min for 4 h. The plate was read at excitation 485 nm and emission 525 nm using a BioTek FLx800 fluorescence reader and Gen5 software. Fluorescence release is directly proportional to the rate of fibrinolysis in the sample.

Data Analysis

Results are expressed as mean ± SD or SEM. Statistical analysis was performed using Graph Pad Prism 5.04 using an unpaired Student's *t*-test or one-way ANOVA with Bonferroni post-

hoc test, $P < 0.05$ was considered significant. Lysis of model thrombi was fitted using centered 2nd ordered polynomial quadratic equation in Graph Pad Prism 5.04. Co-localization of proteins in confocal microscopy was calculated using Image J software and expressed as Pearson correlation co-efficient (*r*).

Supplemental Figure & Video Legends

Supplementary Figure 1 – PAI-1 co-localizes with the a-granule marker P-selectin on degranulated platelets

Platelets (0.5 x 10^8 plt/ml), were adhered to a slide coated with both collagen (20 µg/ml) and thrombin (100 nM) for 30 min at ambient temperature. Annexin V (red) was added to stain PS and left for a further 15 min before imaging by confocal microscopy. PAI-1 was detected using a rabbit polyclonal antibody labelled with DL550 (yellow) and P-selectin using a mouse monoclonal PE/Cy5-conjugated antibody (green). Images shown are representative of $n \geq 3$. Scale bars represent 2 µm.

Supplementary Figure 2 - Exposure of platelet-derived PAI-1 is enhanced on the activated.

Platelets (2 x 10^8 plt/ml), were unstimulated (US) or activated with CVX (1 µg/ml) ± TRAP-6 (200 nM) or thrombin (TH; 100 nM) and analyzed using flow cytometry. (A) Platelet-derived PAI-1 was detected with a rabbit polyclonal antibody labelled with DL488. (B) Vitronectin (Vn) was detected with DL488 labelled rabbit polyclonal antibody. (C) Fibrin(ogen) was analyzed using a rabbit polyclonal antibody labelled with DL405. Example histograms representative of $n \geq 4$.

Supplementary Figure 3 - Vitronectin co-localises with the fibrin fibres of platelet-rich clots.

PRP clots (30%) were formed in the presence of fibrinogen-AF546 (red) for 2 h at 37°C. Clotting was initiated with 0.125 U/ml thrombin and PS-exposing platelets were detected using AnnexinV-AF647 (green). Platelet-derived vitronectin (Vn) was detected using a rabbit polyclonal antibody (blue). Clots were imaged using confocal microscopy. Scale bar represents 10 µm. *Representative images of n=5.*

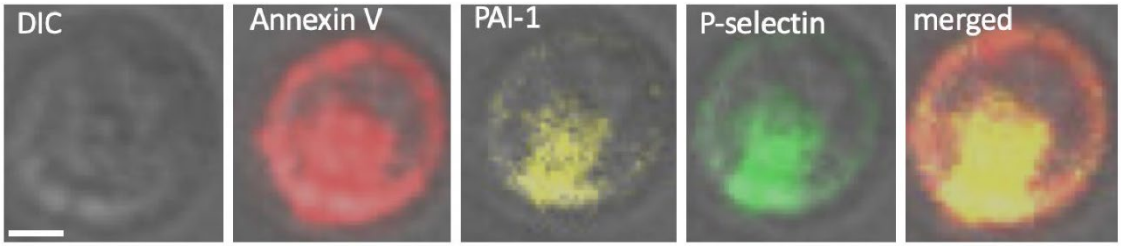
Supplementary video 1 – Lysis of platelet-rich clots is significantly faster following inhibition of PAI-1

PRP clots (30%) were formed with inclusion of fibrinogen-AF546 (red) for 2 h at 37°C in the absence (A) or presence (B) of a rabbit polyclonal neutralizing antibody to PAI-1 (400 µg/ml). Clotting was initiated with 0.125 U/ml thrombin. Platelets were labelled with DIOC-6 (0.5 µg/ml). Clots were lysed by addition of tPA (75 nM) and lysis monitored by confocal microscopy. Videos are representative of n=3.

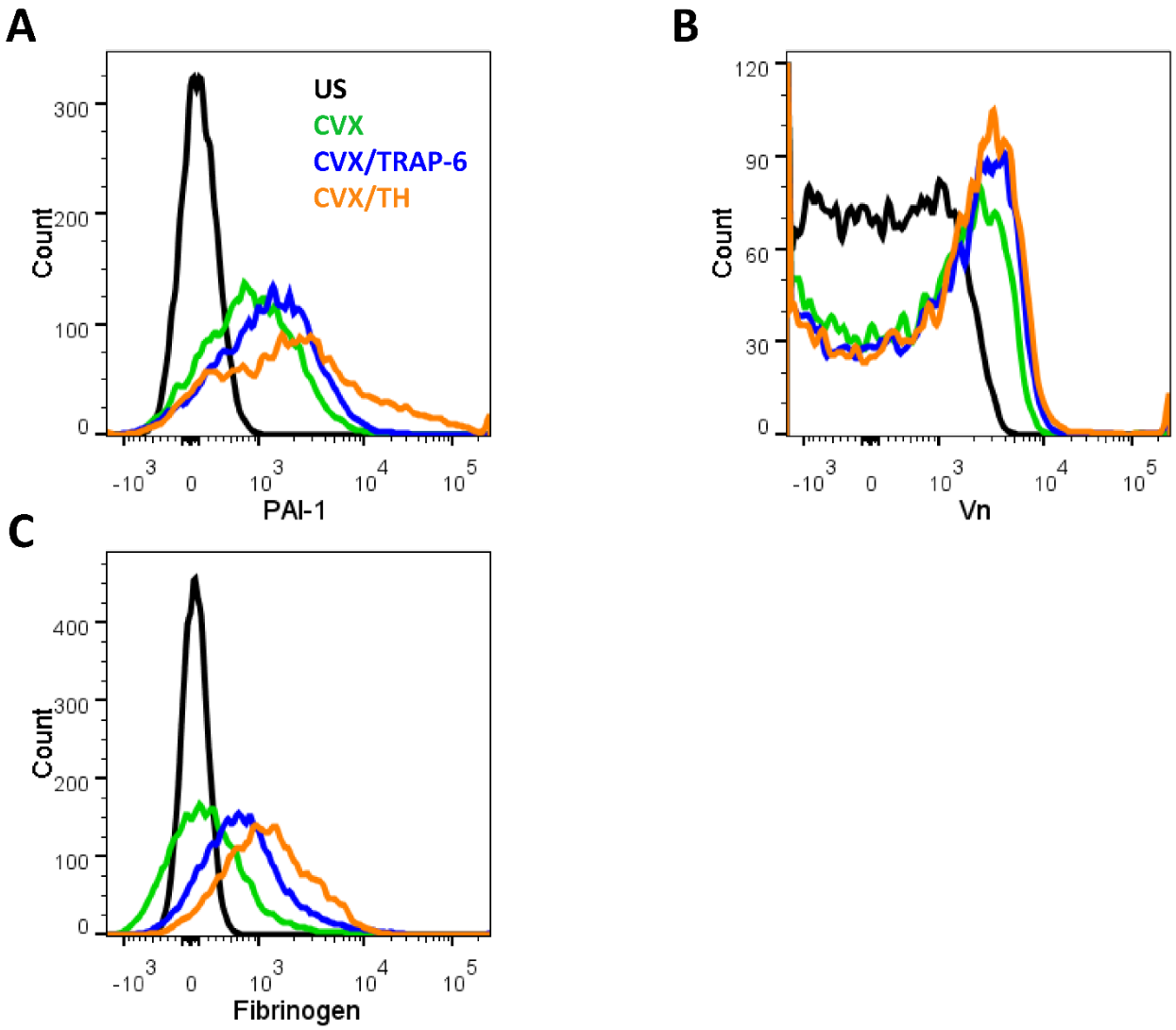
Supplementary video 2 – Thrombodynamic analysis of platelet-rich clot formation and lysis

Platelet-rich clots were formed in the presence of 5 nM tPA without (A) or with (B) pre-treatment with a neutralising polyclonal antibody to PAI-1 (400 μg/ml) for 30 min prior to initiation of clotting. Clot formation and lysis were monitored using a Hemacore Thrombodynamics T-2 analyser. Images were taken every 6 s over a 60 min time period. Videos are representative of *n*=4.

Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3