# Platelet interaction with von Willebrand factor is enhanced by shear-induced clustering of glycoprotein Ibα

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## Supplementary Appendix

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# Supplementary Methods

## *Materials and antibodies*

We used the following products (with sources): cytosolic phospholipase $A_2$ ($cPLA_2$) inhibitor $AACOCF_3$ (Santa Cruz Biotechnology, Santa Cruz, CA), arachidonic acid (AA; Bio/Data Corporation, Horsham, PA), bovine serum albumin (BSA) fraction V, methyl-β-cyclodextrin (mβCD), indomethacin (Sigma-Aldrich, St. Louis, MO), lipoxygenase-inhibitor 5, 8, 11-eicosatriynoic acid (ETI), prostacyclin ($PGI_2$) and Thromboxane $A_2$ receptor (TPα) agonist U46619 (Cayman Chemical, Ann Arbor, MI), human serum albumin fraction V (MP biomedicals, Santa Ana, CA), horm collagen (Nycomed, Linz, Austria), $PGI_2$ analog iloprost (Bayer AG, Leverkusen, Germany), Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE), ristocetin (Biopool Us Inc, Jamestown, NY), P38-mitogen-activated protein kinase (P38MAPK) inhibitor SB203580 (Alexis Biochemicals/Enzo Lifesciences BVBA, Zandhoven, Belgium), thrombin (Stago, Asnières sur Seine, France) and thrombin receptor activating peptide (TRAP-6, SFLLRN; Bachem, Bubdendorf, Switzerland). TPα antagonist SQ30741 was kindly provided by Bristol-Meyers-Squibb (Maarssen, The Netherlands). Cross-linked collagen related peptide (CRP) was a generous gift from Dr. Richard Farndale (Cambridge University, UK). Human VWF was purified as described.[1] The anti-VWF nanobody used to prevent binding to GPIbα was produced in house.[2] The synthetic peptide dRGDW and membrane-permeable myristoylated peptides MPαC (N-myristoyl-SIRYSGHpSL) and MαC (N-myristoyl-SIRYSGHSL) were produced by the NKI (Amsterdam, the Netherlands). The latter two mimic the cytoplasmic binding site for 14-3-3ζ on GPIbα surrounding the $Ser^{609}$ residue. Phosphorylation of $Ser^{609}$ is critical for 14-3-3ζ association.[3]

The antibody used for FRET analysis of GPIbα distribution was the recombinant 6B4-Fab fragment directed against aa 200-268 of GPIbα.[4] Other tools for FRET analysis were Alexa Fluor-488 and -594 protein labeling kits and Cholera toxin subunit B conjugated to Alexa Fluor-594 (CTB-594; Invitrogen, Carlsbad, CA), which binds to the raft marker monosialo-tetrahexosylganglioside (GM1). Antibodies or probes used for flow cytometry were directed against F-actin (Alexa Fluor-488 Phalloidin; Invitrogen), VWF (Abcam, Cambridge, UK), P-selectin and activated αIIbβ3 (PAC-1) (BD Pharmingen, San Diego, CA). Antibodies for immunoprecipitation were against GPIbα (clone HIP1; eBioscience, San Diego, CA) and for western blotting against GPIbα (clone SZ2; Beckman Coulter, Brea, CA), 14-3-3ζ (C-16, Santa Cruz Biotechnology), phospho-tyrosine (clone 4G10; Merck Millipore, Darmstadt, Germany), Phospho-P38MAPK (Thr180/Tyr182) and total P38MAPK (Cell Signaling Technology, Danvers, MA). Secondary antibodies were Alexa Fluor 680-conjugated goat-α-rabbit and goat-α-mouse (Invitrogen), and IRDye 800CW-conjugated goat-α-rabbit IgG (LI-COR Biosciences, Lincoln, NE).

### *Platelet preparation and incubations*

Human platelets were isolated with free-flow blood collection as described.[5] Procedures were approved by the local Medical Ethics Committee. Platelets were resuspended in Hepes-Tyrode's (HT) buffer ($2.5x10^{11}$ cells/L, pH 7.3) and allowed to recover for 30 minutes at room temperature prior to incubations (defined as control platelets). Reconstituted blood was prepared[6] by resuspending washed platelets in HT buffer ($3.3x10^{11}$ cells/L, pH 7.3) supplemented with 4% human albumin. Packed red blood cells were mixed with platelets in a 40:60 (v/v) ratio to obtain reconstituted blood with a platelet count of $2x10^{11}$/L and a hematocrit of 0.4 (L/L). In some experiments, VWF was added to a final concentration of 10 μg/mL.

Inhibitors used were against platelet activation (iloprost; 1 μM), integrin αIIbβ3 occupancy (dRGDW, 200 μM), TPα (SQ30741; 25 μM) and activation of P38MAPK (SB203580; 10 μM), $cPLA_2$ ($AACOCF_3$, 20 μM), cyclooxygenase-1 (indomethacin; 30 μM) and lipoxygenase (ETI; 25 μM), each preincubated for 10 minutes at 37°C. Membrane was depleted of cholesterol by incubating platelets with mβCD (10 mM) for 30 minutes at 37°C. In some experiments, platelets were incubated with control peptide MαC or MPαC (100 μM) for 5 minutes at 37°C. All incubations were without stirring unless indicated otherwise.

### *Platelet adhesion and rolling under flow conditions*

A parallel plate perfusion chamber[7] was used to investigate platelet adhesion and rolling. For adhesion experiments, coverslips were coated with VWF (10 μg/mL) or horm collagen (0.1 mg/mL) and blocked with 4% BSA. Whole blood or reconstituted blood was perfused at defined shear rates (37°C) and adhesion was visualized using an Axio Observer Z1 microscope equipped with an AxioCam MRm CCD camera with a Zeiss EC Plan-NeoFluar 40x/0.75 DICII lens (Carl Zeiss MicroImaging GmbH, Gottingen, Germany). Surface coverage was analyzed using ImageJ software (NIH, Bethesda, MD). For determination of rolling velocity, washed platelets were resuspended in HT buffer ($2.5x10^{11}$ cells/L, pH 7.3) supplemented with 4% human albumin and preincubated with iloprost and dRGDW. Platelets were perfused at 1,600 $s^{-1}$ over a VWF coated coverslip with half of the surface blocked with 4% BSA (upstream). Platelet rolling was analyzed in the middle of the perfusion chamber at the interface of surface immobilized BSA and VWF where flow is laminar. Rolling velocity was determined by tracking 40 platelets per experiment over a distance of 200 μm in time using Axiovision v4.8 software (Carl Zeiss MicroImaging GmbH).

### *Flow cytometric analysis*

Characterization of platelets by FACS was based on FSC and SSC-scatter (FACS-Calibur; BD Biosciences, San Jose, CA). Appropriate antibodies were added and incubated for 15 minutes at 37°C. A total of 10,000 platelets was analyzed for surface-expressed VWF, P-selectin, and activated αIIbβ3. For analysis of F-actin levels, platelets were fixed with 2% formaldehyde and permeabilized with 0.1% triton X-100 prior to phalloidin incubation.

### *Immunoprecipitations and western blots*

For immunoprecipitations, 900 µL washed platelets ($5x10^{11}$ platelets/L) were lysed with 100 µL of 10x RIPA lysis buffer (15 minutes, 0°C) and mixed with 110 µL (10% vol/vol) protein G beads (GE Healthcare, Little Chalfont, UK) together with antibody (2 µg/mL, o/n, 4ºC, rotating). For lysate analysis, platelet suspensions were lysed, centrifuged (10,000 g, 1 minute) and dissolved in sample buffer under reducing conditions. Proteins were separated by SDS-PAGE and western blotted. After blocking with Odyssey Blocking buffer, membranes were incubated with primary antibodies (1 µg/mL) and protein bands visualized with an Odyssey Imaging system (LI-COR Biosciences). Quantification was performed with ImageJ software. Variations in lane loading were adjusted by normalization to the immunoprecipitated protein.

## Supplementary Figures

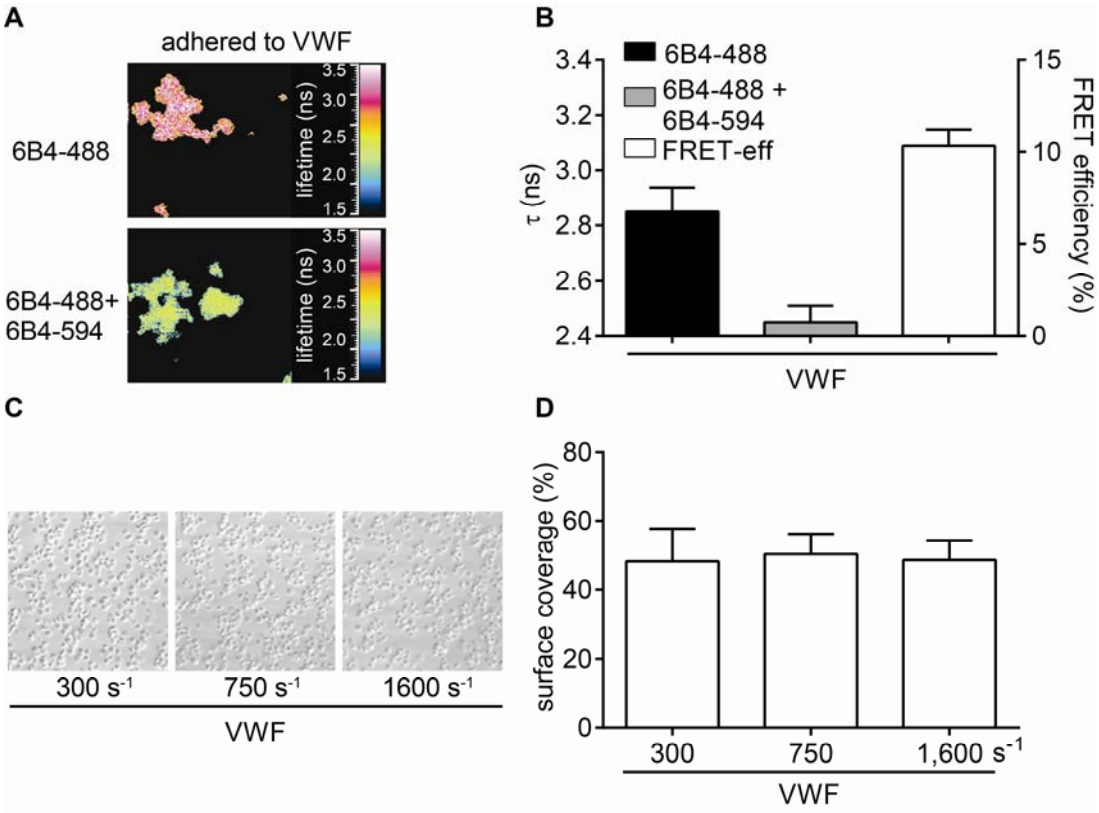


**Figure S1. Levels of GPIbα clustering in platelets adhered to VWF as small aggregates and platelet adhesion at different shear rates.** (A) Platelets adhered to VWF after whole blood perfusion (1 minute, 37°C) at a shear rate of 1600 $s^{-1}$ were analyzed for GPIbα distribution by FRET/FLIM. Platelets were fixed with 2% paraformaldehyde and stained with 1 µg/mL 6B4-488 (donor) in the absence (top panels) and presence of 1 µg/mL 6B4-594 (acceptor; bottom panels). The fluorescence lifetimes in nanoseconds (ns) are shown in false color images. (B) Quantification of fluorescence lifetime values of donor probe in the absence and presence of acceptor probe of platelets treated under the conditions of (A). Corresponding FRET efficiencies are calculated as described in Methods. (C) Platelet adhesion to VWF during whole blood perfusion at indicated shear rates. Snapshots were made after 5 minutes perfusion. (D) Quantification of platelet adhesion to VWF under the conditions of (C). Data are means ± SEM (n=3).

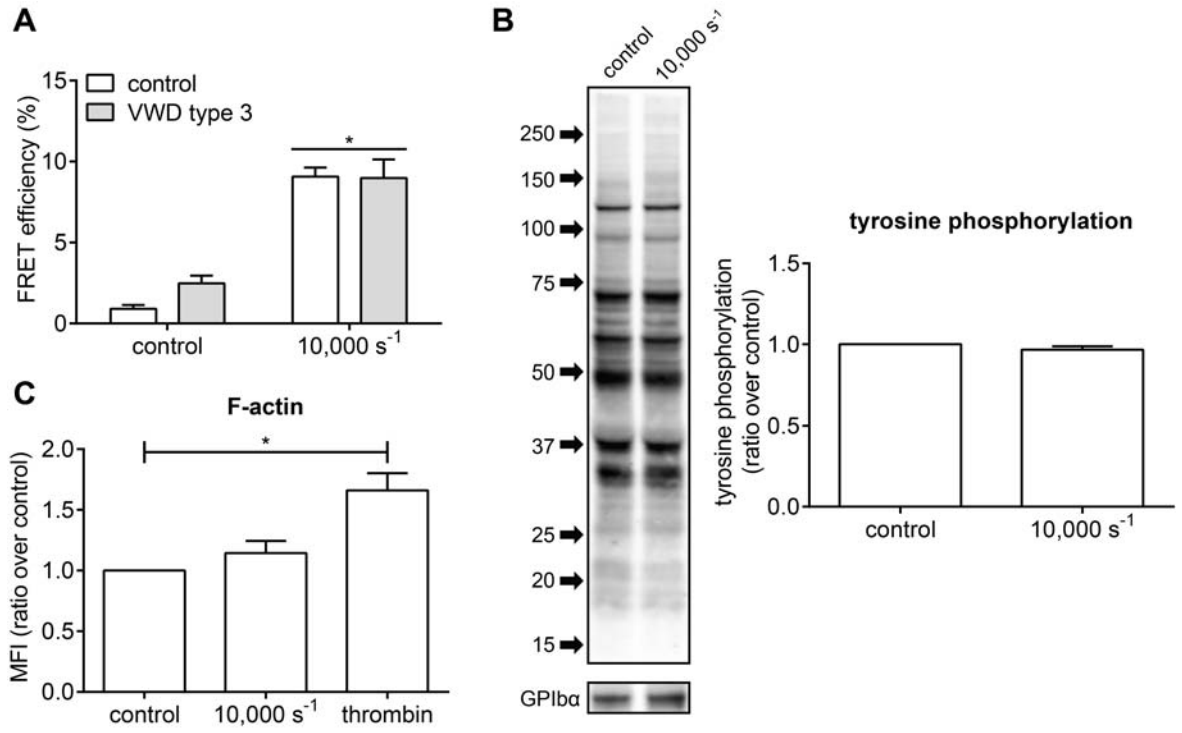


**Figure S2. Platelet exposure to high shear force induces GPIbα clustering in a patient with VWD type 3 and analysis of protein phosphorylation and cytoskeleton reorganization.** Platelets resuspended in HT buffer (pH 7.3) supplemented with 4% albumin were exposed to a shear rate of 10,000 $s^{-1}$ by perfusion (37$^{o}$C) through a microcapillary tube for 5 seconds. (A) Shear-induced GPIbα clustering occurs in the absence of VWF. Resting (control) or high shear-exposed platelets from healthy donors and from a VWD type 3 patient were and analyzed for GPIbα clustering by FRET/FLIM. (B) Analysis of tyrosine phosphorylation by probing whole platelet lysates with the anti-phospho-tyrosine antibody 4G10. Expression of GPIbα served as lane loading control. (C) FACS analysis of cytoskeleton reorganization using phalloidin as a probe to determine F-actin turnover in fixed, permeabilized platelets. Platelet stimulation with thrombin (1 U/mL; 5 minutes at 37 $^{o}$C) was used as a positive control. No significant difference between control and shear-exposed platelets was observed. Data are means ± SEM (n=4).

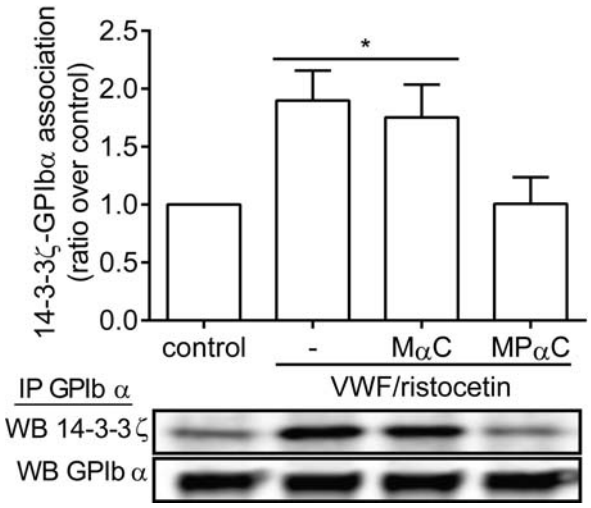


**Figure S3. Prevention of [14-3-3ζ-GPIbα] complex formation by MPαC.** Washed platelets were pre-incubated in the absence and presence of control peptide MαC and MPαC (100 μM) for 5 minutes at 37°C and subsequently stimulated with ristocetin (0.3 mg/mL) and VWF (10 μg/mL) at 37°C while stirring. After 2 minutes incubation, platelets were lysed and analyzed for [14-3-3ζ-GPIbα] complex formation. Data are means ± SEM (n=3).

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