SUPPLEMENTARY APPENDIX

Glycoprotein Ib clustering in platelets can be inhibited by α-linolenic acid as revealed by cryo-electron tomography

Simona Stivala,[1*] Simona Sorrentino,[2*] Sara Gobbato,[1] Nicole R. Bonetti,[1,3] Giovanni G. Camici,[1,4,5] Thomas F. Lüscher,[1] Ohad Medalia[2,6] and Jürg H. Beer[1,3]

[1]Laboratory for Platelet Research, Center for Molecular Cardiology, University of Zurich, Zurich, Switzerland; [2]Department of Biochemistry, University of Zurich, Zurich, Switzerland; [3]Internal Medicine, Cantonal Hospital of Baden, Baden, Switzerland; [4]University Heart Center, University Hospital Zurich, Zurich, Switzerland; [5]Department of Research and Education, University Hospital Zurich, Zurich, Switzerland and [6]Department of Life Sciences and the National Institute for Biotechnology in the Negev, Ben-Gurion University, Beer-Sheva, Israel

SSt and SSo contributed equally as co-first authors

 doi:10.3324/haematol.2019.220988

Received: March 5, 2019.

Accepted: August 14, 2019.

Pre-published: August 22, 2019.

Correspondence: *JÜRG H. BEER* - hansjuerg.beer@ksb.ch

Supplementary Data

Platelet isolation and cholesterol depletion

Shearing of platelets was performed on washed platelets isolated by centrifuging platelet rich plasma (PRP) at 340 RCF for 10 min in the presence of 1 μM PGE1, followed by washing with platelet wash buffer (26 mM Na_2HPO_4, 140 mM NaCl, pH=7.2) and resuspension in Tyrode's buffer (137 mM NaCl, 12 mM $NaHCO_3$, 5.5 mM glucose, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.3 mM Na_2HPO_4, pH=7.4). One hundred microliters of washed platelets were subjected to shear stress for 1 min at a rate of 10'000 s^{-1} (55 dynes/cm^2) in a cone-and-plate viscometer (CAP2000; Brookfield Inc., Middleboro, Massachusetts, USA). This shear rate has been shown to occur at clinically relevant 60–80% arterial stenoses[1]. The stainless-steel surfaces of the viscometer were precoated with 0.1% BSA in Tyrode's buffer for 1 min.
For platelet-rich plasma (PRP) isolation, blood was centrifuged at 200 RCF for 15 minutes, and PRP carefully removed and centrifuged again to remove contaminating leukocytes and erythrocytes. Cholesterol depletion of platelets was obtained by incubation of PRP with 10 mM methyl-beta cyclodextrin (Santa Cruz, US) at 37°C for 30 minutes.

Platelet adhesion to vWF under high-shear flow

Twenty-four-well plates were coated with human vWF (100 ug/ml, Hematologic Technologies Inc., US) for 1 hour, then washed with PBS +Ca^{2+} and Mg^{2+} and blocked with 0,1% BSA in PBS for 10 minutes. EDTA-anticoagulated blood was incubated with 30 μM ALA, EPA or stearic acid (from Cayman Chemical, US) or the same volume of vehicle (ethanol), at room temperature for 1 hour under gentle shaking, and platelets were concomitantly fluorescently labelled with calcein (4 μM final concentration, Enzo Life Science, US). Platelet adhesion was monitored on an inverted microscope (Leica Leitz DM IRB) with a 10x/0.22 NA objective and FITC filter by capturing images for 5 minutes after applying a flow of 100 dyn/cm^2 to the blood. Platelet-covered area (fluorescent area) was calculated in the region of view with the Bioflux software and expressed as μm^2.

Immunofluorescence staining for Ground State Depletion (GSD) microscopy

Samples were stained with a mouse anti-human GpIb-Alexa 647 antibody (MBL International, Woburn, US) in PBS for 2 hours at r.t., followed by 3 washes and post-fixation with 4% PFA. For integrin αIIbβ3 staining, platelets were incubated with a mouse anti-human primary

antibody (Abcam, Cambridge, UK) in PBS for 1 hour at room temperature, followed by 3 washes in PBS and a secondary antibody (donkey anti-mouse Alexa 647, Jackson Immunoresearch, West Grove, PA, USA) for 1 hour at room temperature[2]. Ground state depletion (GSD) and total internal reflection fluorescence (TIRF) microscopy was performed on a Leica SR GSD 3D microscope with a HCPL Apo 160x/1.43 NA objective. For imaging, samples were mounted in 200 mM phosphate buffer, pH=8.0, containing 15 mM cysteamine hydrochloride, 0.5 mg/ml glucose oxidase and 40 ug/ml catalase (all from Sigma-Aldrich, Buchs, Switzerland) as oxygen scavenging solution. We acquired 40'000 images exciting with a 633-nm laser and a 7.07 msec exposure; for TIRF, the penetration depth was 110 nm. Particle analysis in TIRF was performed with Fiji[3]. Alexa 647 switches reversibly between the on and off state, therefore, each Alexa 647 molecule might generate multiple detected events.

Cryo-electron tomography

Washed platelets obtained as described in "Platelet isolation" were seeded on gold grids coated with a Silicon mesh (R 1/4, 200 mesh, Quantifoli, Jena Germany). In case of shear activation, platelets were exposed to 10'000 s^{-1} shear rate for 1 min before being seeded. If indicated, platelets were treated with ALA as described above. Platelets were allowed to adhere on the grids for maximum 10 min and then they were fixed with 4% formaldhehyde for 5 min at room temperature. The time where platelets were allowed to spread on the grids was chosen based on a previous publication showing that GpIb clustering was stable for up to 10 minutes post-shear[4]. The EM grids were incubated in 0.05 M glycine/PBS for 15 min at room temperature to inactivate aldehyde groups present after fixation. Next, samples were blocked in blocking solution (5% BSA/0.1% cold water fish skin gelatin/PBS; Aurion) for 30 min at room temperature and subsequently washed 3 × 5 min with the incubation solution (0.2% BSA-c/PBS; Aurion). Immunogold analysis was performed using anti-human GpIb antibody (6D1, generous gift from B. Coller, Rockefeller University NY) at a dilution of 1:100 in the incubation solution (0.2% BSA-c/PBS; Aurion) for 1 hour at room temperature. Next, the EM grids were washed 6 × 5 min at room temperature with incubation solution (0.2% BSA-c/PBS; Aurion) before treatment with the gold conjugate (protein G coupled to 6 nm gold; Aurion) in incubation solution at a dilution of 1:40 for 2 hours at room temperature. After extensive washing (6 × 5 min at room temperature) with the incubation solution, fiducial markers of 10 nm were added (Aurion) and then the EM grids were plunge frozen in liquid ethane. Data acquisition was performed using an FEI Titan Krios transmission electron microscope equipped with a quantum energy filter and a K2-Summit direct electron detector (Gatan,

Pleasanton, USA). Tomograms were acquired with a magnification of 42,000× corresponding to a pixel size of 0.34 nm. The cumulative electron dose was ~80 electrons per ångström^2. To analyze the GpIb receptor density, the 3D coordinates of the 6 nm gold were selected using the tom_volxyz software from the TOM toolbox software package[5]. We used MATLAB to search how many 6 nm gold particles were present in a radius of 50 nm (using the selected coordinates) for each of the gold particle. The amount of gold particles found with this procedure is called: number of neighbors. In case of the negative control, no neighbors were found (data not shown). The plots of the distribution for number of neighbors for each receptor and the statistical analysis were performed using the OriginLab software. In particular, for the peak analysis we used the peak Fitting tool from OriginLab. For the analysis, we used 28 tomograms of platelets incubated with Calcium and Magnesium-containing buffer, 11 tomograms from shear activated platelets, 3 tomograms from the negative control. The analysis shown in the paper were performed on 6 tomograms for each condition.

Comparison between immunogold labeling and immunofluorescent data

The comparison between the GSD and the cryo-EM data was performed described previously[6]. Briefly, 25 tomograms were projected into 2-D images and assembled such to mimic the membrane of a platelet. Then the xy coordinates of GpIb (6 nm gold particles) were extracted and represented as 2D Gaussian functions with a full width at half maximum of 20 nm using MATLAB. Finally, these images were colored in red using Fiji[3].

References

1. Holme PA, Ørvim U, Hamers MJAG, et al. Shear-Induced Platelet Activation and Platelet Microparticle Formation at Blood Flow Conditions as in Arteries With a Severe Stenosis. Arterioscler Thromb Vasc Biol 1997;17(4):646 LP – 653.
2. Tamir A, Sorrentino S, Motahedeh S, et al. The macromolecular architecture of platelet-derived microparticles. J Struct Biol 2016;193(3):181–187.
3. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods 2012;9(7):676–682.
4. Gitz E, Koopman CD, Giannas A, et al. Platelet interaction with von Willebrand factor is enhanced by shear-induced clustering of glycoprotein Ibα. Haematologica 2013;98(11):1810–1818.
5. Nickell S, Förster F, Linaroudis A, et al. TOM software toolbox: acquisition and analysis for electron tomography. J Struct Biol 2005;149(3):227–234.
6. Turgay Y, Eibauer M, Goldman AE, et al. The molecular architecture of lamins in somatic cells. Nature 2017;543(7644):261–264.

Supplementary Figure 1 – Original cryo-ET images of the platelets displayed in Figure 2

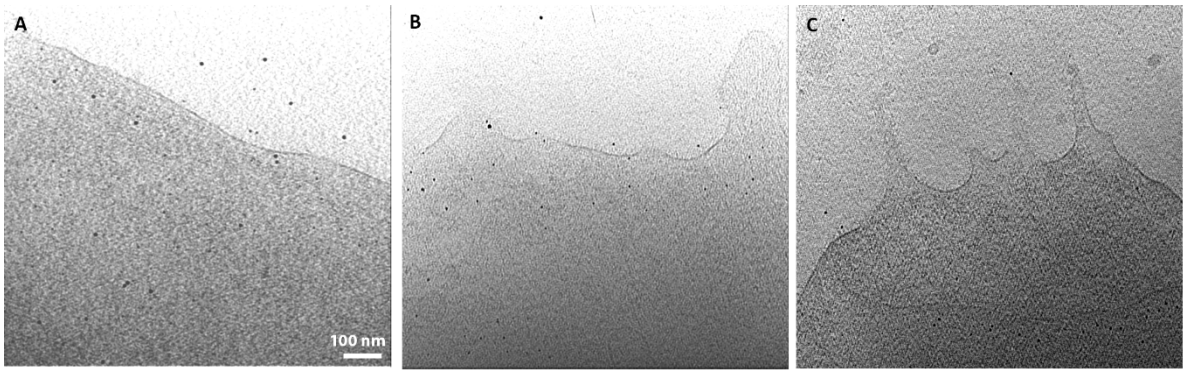

Figure S1. Original projection images from Fig. 2 where the 6 nm gold has not been labelled in red. The letter code has been kept the same.

Supplementary Figure 2 – Synthetic platelet, before and after filtering

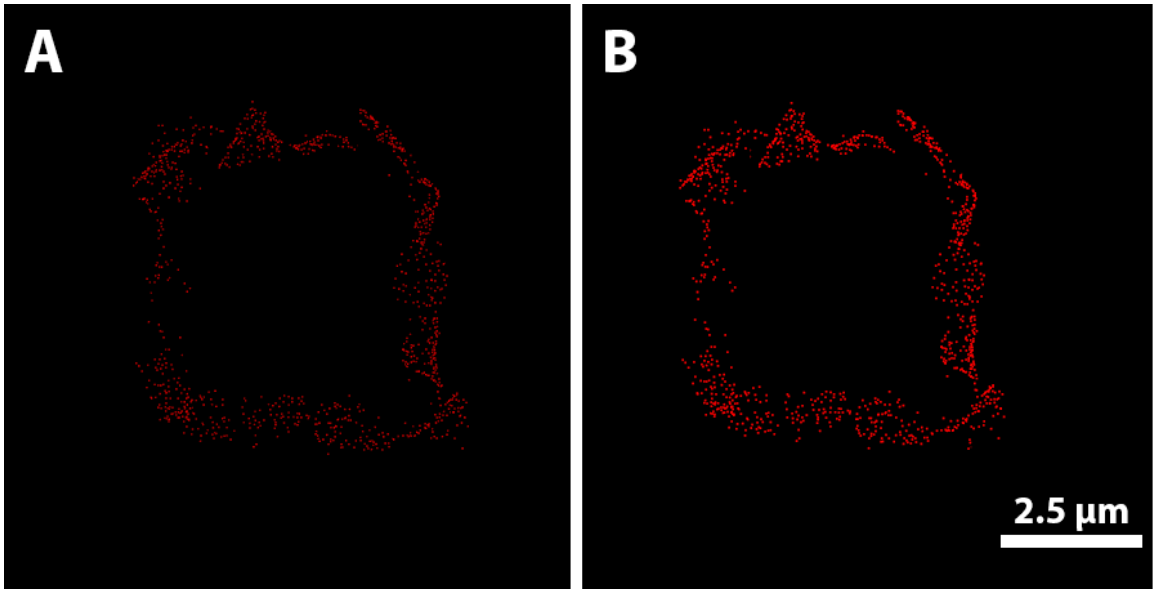

Figure S2. Synthetic platelet model. The coordinates of the antibody labelled GpIb (6 nm gold particles) from 17 tomograms represented as 2D Gaussian functions with a full width at half maximum of 10 nm (in A) and 20 nm (in B) using MATLAB. We choose to show the image from B in Figure 4 in order to match the resolution for GSD.

Supplementary Figure 3 – Ground state depletion (GSD) microscopy on integrin αIIbβ3

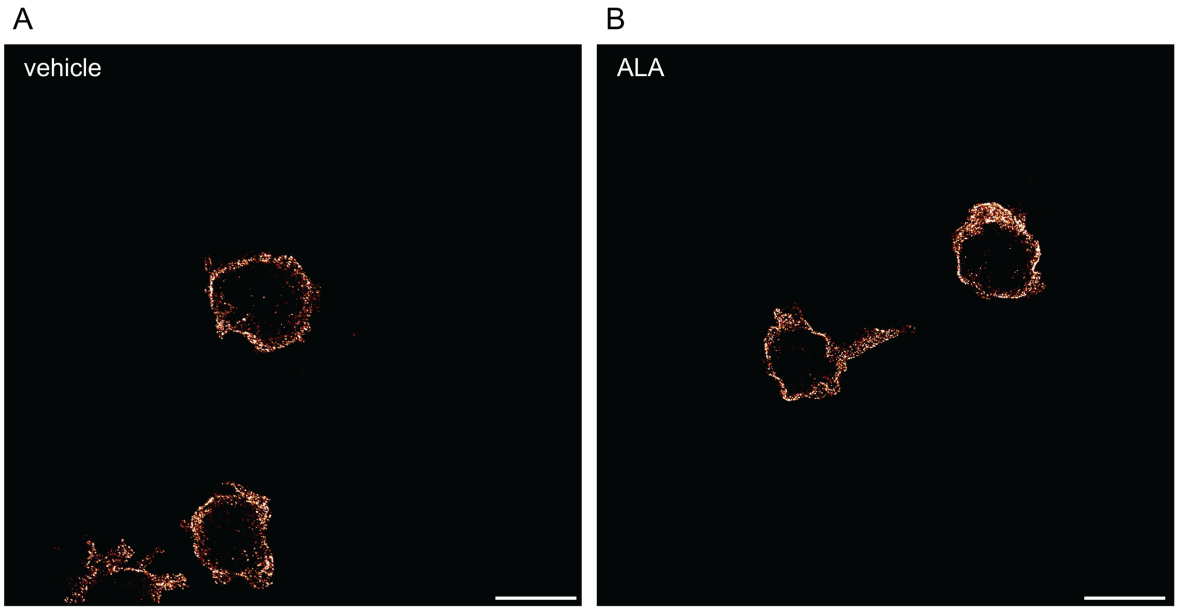

Figure S3. Superresolution microscopy by GSD on vehicle (A) and ALA-treated (B) platelets shows no difference in the distribution of the integrin αIIbβ3. Scale bar: 3 μm.

Supplementary Figure 4 – Inhibition of platelet adhesion to vWF by the long-chain n3-FA eicosapentaenoic acid (EPA)

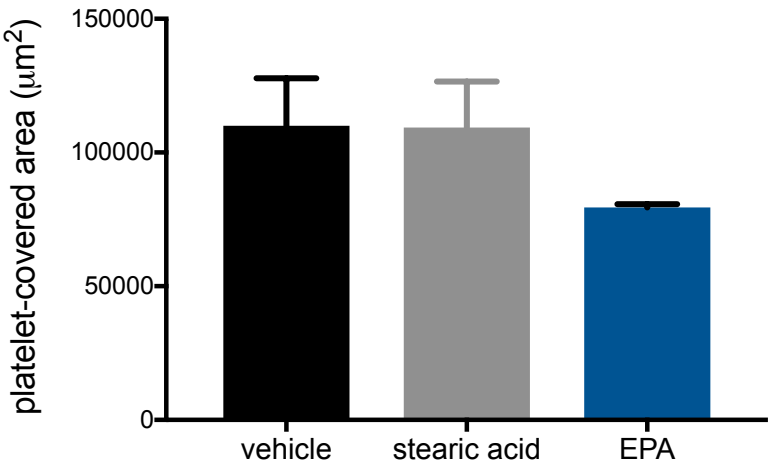

Figure S4. Adhesion experiments under high-shear flow show that the marine-derived n3-FA EPA has a similar inhibitory effect on the platelet-covered area on vWF, whereas the saturated fatty acid stearic acid has no effect on platelet adhesion (n=3).

Supplementary video 1. Platelet adhesion to vWF under high-shear flow after treatment with vehicle (0.1% ethanol).

Supplementary video 2. Platelet adhesion to vWF under high-shear flow after whole blood treatment with 30 μM alpha-linolenic acid.