SUPPLEMENTARY APPENDIX

Gradual increase in thrombogenicity of juvenile platelets formed upon offset of prasugrel medication

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Supplements

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Supplemental methods

Materials

2MeS-ADP and *D*-Phe-Pro-Arg chloromethyl ketone (PPACK) were from SantaCruz Biotechnology (Santa Cruz, CA, USA); fluorescein isothiocyanate (FITC)-conjugated PAC-1 antibody against active integrin $\alpha_{IIb}\beta_3$ and thiazole orange from Becton-Dickinson Bioscience (Franklin Lakes NJ, USA), and aspirin was from Sanofi (Paris, France). Ticagrelor and the clopidogrel active metabolite (CAM) were kindly provided by AstraZeneca R&D (Mölndal, Sweden). 5'-Cy5-conjugated oligo-dA_{20} and oligo-dT_{20} were obtained from Eurogentec (Maastricht, The Netherlands). Alexa Fluor (AF)-647 and Oregon Green (OG)-488 conjugated human fibrinogen were from Invitrogen (Bleiswijk, The Netherlands). Collagen type I came from Nycomed Pharma (Munich, Germany); 3,3'-dihexyloxa carbocyanine iodide ($DiOC_6$) from Anaspec (Fremont CA, USA); CellVue Maroon from eBioscience (San Diego CA, USA), and iloprost from Bayer Pharma (Berlin, Germany). Multiplate test kits were from Roche Diagnostics (Basel, Switzerland). Other compounds came from Sigma (St. Louis, MO, USA).

Patients and control subjects

This study was approved by the local medical ethics committee (MEC 12-3-075). All patients and healthy volunteers gave written informed consent for participation according to the Helsinki declaration. Sixteen patients were studied who were treated with prasugrel (10 mg/day) for one year and long-term aspirin (80-100 mg/day) due to a myocardial infarction with ST elevation. After one year of prasugrel treatment, blood was collected on the last day of prasugrel intake, and at 1, 2, 5 and 30 days after the last dose. From two patients, blood samples were also collected after 12 days to better understand the delayed regain of platelet function. Patients with a malignancy, active infection or a known platelet disorder were not included. Blood was obtained by venipuncture with a Vacutainer 21-gauge needle (Becton-Dickinson Bioscience). Blood collection was into Vacuette tubes, containing K_2-EDTA, for measurement of hemostatic variables and immature platelet fraction using a Sysmex XN-9000 analyzer, according to protocols of the supplier (Sysmex, Chuo-ku Kobe, Japan). Blood samples were also collected into 3.2% (w/v) trisodium citrate for platelet function measurements, and into hirudin for whole-blood platelet aggregation. Control experiments were performed with blood drawn from healthy volunteers. Collection was into trisodium citrate, or into acidic citrate dextrose (ACD, 80 mM trisodium citrate, 52 mM citric acid and 180 mM glucose) for the preparation of washed platelets.[1]

Preparation of platelet-rich plasma, platelets and red cells

Platelet-rich plasma (PRP) was prepared by centrifuging citrate-anticoagulated blood at 240 g for 15 minutes at room temperature. Platelet-free plasma was obtained by centrifuging citrate-anticoagulated blood twice at 2630 g for 10 minutes. Washed platelets were prepared from ACD-anticoagulated blood, as described [2], and were suspended in Hepes buffer pH 7.45 (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.1% glucose and 0.1% bovine serum albumin). Platelet counts were determined with a thrombocounter XP300 Sysmex analyzer (Sysmex, Chuo-ku Kobe, Japan).

Washed red blood cells were prepared by centrifuging citrate-anticoagulated blood at 240 g for 15 minutes at room temperature.[3] After removal of PRP, Hepes buffer pH 7.45 was added in a 1:2 volume ratio. The red blood cells were centrifuged twice at 2630 g for 10 minutes, with resuspension in Hepes buffer pH 7.45. This resulted in a preparation of >99.9% red cells.

Irreversible $P2Y_{12}$ inhibition in vitro

PRP from healthy donors was treated with 100 µM lysine aspirin for 30 minutes, and platelets were pelleted by centrifugation.[1] The resuspended cells were incubated for 15 minutes with 10 µM CAM or vehicle medium. Residual unbound CAM was removed by a second final centrifugation step, where 5 nM iloprost was added to prevent platelet activation. Mixtures of the washed CAM-treated and vehicle-treated platelets in Hepes buffer pH 7.45 were used for measurement of: platelet aggregation (light transmission aggregometry in the presence of 2 mM $CaCl_2$ and 0.1 mg/ml fibrinogen); integrin $\alpha_{IIb}\beta_3$ activation by flow cytometry in the presence of 75 µg/ml OG488-fibrinogen; and perfusion experiments with reconstituted whole blood.

Platelet aggregation

Aggregation of platelets in PRP was measured using a Chronolog aggregometer (Stago, Asnières sur Seine Cedex, France).[31] Preincubation with ticagrelor or vehicle medium was for 5 minutes at 37 °C. Aggregation of platelets in whole blood was measured by Multiplate impedance aggregometry (Roche Diagnostics, Basel, Switzerland) as described.[4] Aggregation was measured in response to ADP (6.4 µM), arachidonic acid (0.5 mM), collagen (3.2 µg/ml) or thrombin receptor-activating peptide SFLLRN (TRAP, 32 µM) at 37 °C during 6 minutes. Ticagrelor (1 µM) was added *in vitro* to block residual $P2Y_{12}$ activity, where indicated. Extent of platelet aggregation was assessed from the area under the impedance curve.

Flow cytometric analysis of platelet subpopulations

Flow cytometric measurements were performed on an Accuri C6 flow cytometer with CFlow Plus software (Becton-Dickinson Bioscience). To check for integrin $\alpha_{IIb}\beta_3$ activation, samples of citrated patient blood were diluted (1:20) into Hepes buffer pH 7.45, and incubated with 1 µM ticagrelor or vehicle control for 15 minutes at room temperature. Platelets were activated with 1 µM 2MeS-ADP in the presence of FITC-conjugated PAC-1 antibody (1.25 µg/ml) against the activated $\alpha_{IIb}\beta_3$ integrin for 10 minutes. Ticagrelor (1 µM) was added, where indicated. Activated platelets were identified as before.[5]

Juvenile platelets were identified using two different methods of mRNA staining, *i.e.* with thiazole orange [6] or by a novel method using Cy5-labelled oligo-dT, which binds to the poly-A tail of mRNA species. Thiazole orange (15% in filtered PBS: 136 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 6.46 mM Na_2HPO_4) was added to PRP for 30 minutes at room temperature, according to established procedures.[6] Platelets were then centrifuged at 5550 g for 3 minutes to remove excess and unbound thiazole orange, and resuspended in Hepes buffer pH 7.45. Samples were activated with 1 µM 2MeS-ADP in the presence of AF647-fibrinogen (75 µg/ml) for 15 minutes. For staining with 5'Cy5-oligo-dT, washed platelets in suspension (1 x 10^8/ml) were activated with 1 µM 2MeS-ADP in the presence of OG488-fibrinogen (75 µg/ml) for 15 minutes. Samples were fixed (15 minutes) with 0.2% formaldehyde and permeabilized (10 minutes) with 0.1% saponin. 5'-Cy5-oligo-dT (1 µM) was incubated for 15 minutes at 37 °C. For all samples, 5'Cy5-oligo-dA (1 µM) was used as a negative control probe to check for specificity of the staining with 5'Cy5-oligo-dT. Color compensation was not required as fluorescent spectra did not overlap.

The average percentage of juvenile platelets as analyzed by the thiazole orange staining and the oligo-dT staining was 6.7% (± 1.9%) and 21.5% (± 5.8%) respectively. The discrepancy in the percentage of detected juvenile platelets can be explained by the higher sensitivity of the oligo-dT staining to detect mRNA in comparison to thiazole orange. In order to use a uniform definition of juvenile platelets, the threshold for juvenile platelets was based on the IPF as determined by the Sysmex XN9000 analyzer, which is an internationally validated method in the clinic. An alternative analysis of juvenile platelets, based on the negative controls of both stainings, is presented in the supplements (Suppl. Fig. 3).

Thrombus formation in whole blood

Whole-blood thrombus formation on microspots in a parallel-plate flow chamber was measured, basically as described before.[7] Briefly, 0.5 µl microspots containing either collagen I (100 µg/ml) or fibrinogen (250 µg/ml) plus vWF (50 µg/ml) were perfused with citrate-anticoagulated whole blood, which was recalcified with 7.5 mM $CaCl_2$ and 3.75 mM $MgCl_2$ in the presence of 40 µM PPACK immediately before the experiment. Patient blood

samples were perfused through the chamber for 4 minutes at a wall-shear rate of 1600 s^{-1}, while 2MeS-ADP (0.1 µM, f.c.) was co-perfused with a second pump. Ticagrelor was added where indicated. Thrombi were stained with AF647-labelled fibrinogen and, when indicated, $DiOC_6$, as described elsewhere.[7] Brightfield and fluorescence microscopic images were captured with an EVOS fluorescence microscope, equipped with a 60x oil objective. Images were analysed using Metamorph (Molecular Devices, Sunnyvale CA, USA) and ImageJ (open access) software.[7]

For measurement of thrombus formation of reconstituted blood samples from healthy controls, mixtures of CAM- and vehicle-treated platelets (2.5 x 10^8/ml, final count) were added to washed red cells (45% hematocrit) and plasma (30-35% of total volume). In these experiments, the CAM-treated platelets were pre-labeled with the membrane probe CellVue Maroon (1.6 µM), whereas the vehicle-treated platelets were pre-labeled with the membrane probe PKH26 (0.8 µM). Both probes were not transferrable from cell to cell (data not shown). The reconstituted whole blood was again co-perfused with 2MeS-ADP over collagen. Microscopic DIC and confocal fluorescent images were taken using a Zeiss LSM7 microscope (Oberkochen, Germany).[7]

Statistical analysis

Statistical analysis was performed using the SPSS Statistics 22 package (Armonk, NY, USA). Statistical analysis was performed using a one-way-repeated-measures-ANOVA or with a Friedman test with a post hoc Wilcoxon signed rank test. Bonferroni correction was applied when comparing multiple groups.

References

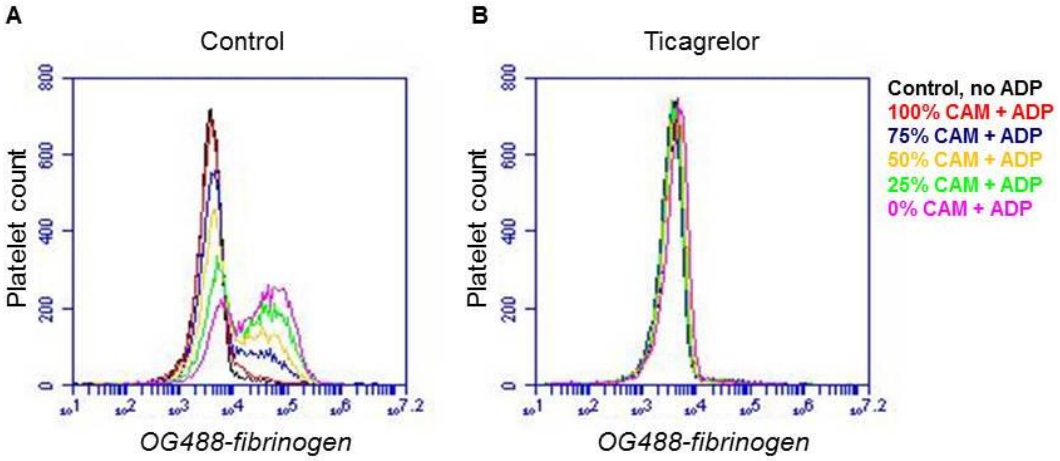
1. Van Gorp RM, Feijge MA, Vuist WM, Rook MB, Heemskerk JW. Irregular spiking in free calcium concentration in human platelets. Regulation by modulation of the inositol trisphosphate receptors. Eur J Biochem. 2002;269:1543-1552.
2. Schols SE, Feijge MA, Lance MD, et al. Effects of plasma dilution on tissue factor-induced thrombin generation and thromboelastography: partly compensating role of platelets. Transfusion. 2008;48:2384-2394.
3. Ninivaggi M, Feijge MA, Baaten CC, et al. Additive roles of platelets and fibrinogen in whole-blood fibrin clot formation upon dilution as assessed by thromboelastometry. Thromb Haemost. 2014;111:447-457.

4. Velik-Salchner C, Maier S, Innerhofer P, et al. Point-of-care whole blood impedance aggregometry versus classical light transmission aggregometry for detecting aspirin and clopidogrel: the results of a pilot study. Anesth Analg. 2008;107:1798-1806.
5. Van der Meijden PE, Bouman AC, Feijge MA, et al. Platelet dysfunction in thrombosis patients treated with vitamin K antagonists and recurrent bleeding. Plos One. 2013;8:e64112.
6. McCabe DJ, Harrison P, Sidhu PS, Brown MM, Machin SJ. Circulating reticulated platelets in the early and late phases after ischaemic stroke and transient ischaemic attack. Br J Haematol. 2004;126:861-869.
7. De Witt SM, Swieringa F, Cavill R, et al. Identification of platelet function defects by multiparameter assessment of thrombus formation. Nat Commun. 2014;16:e4257.

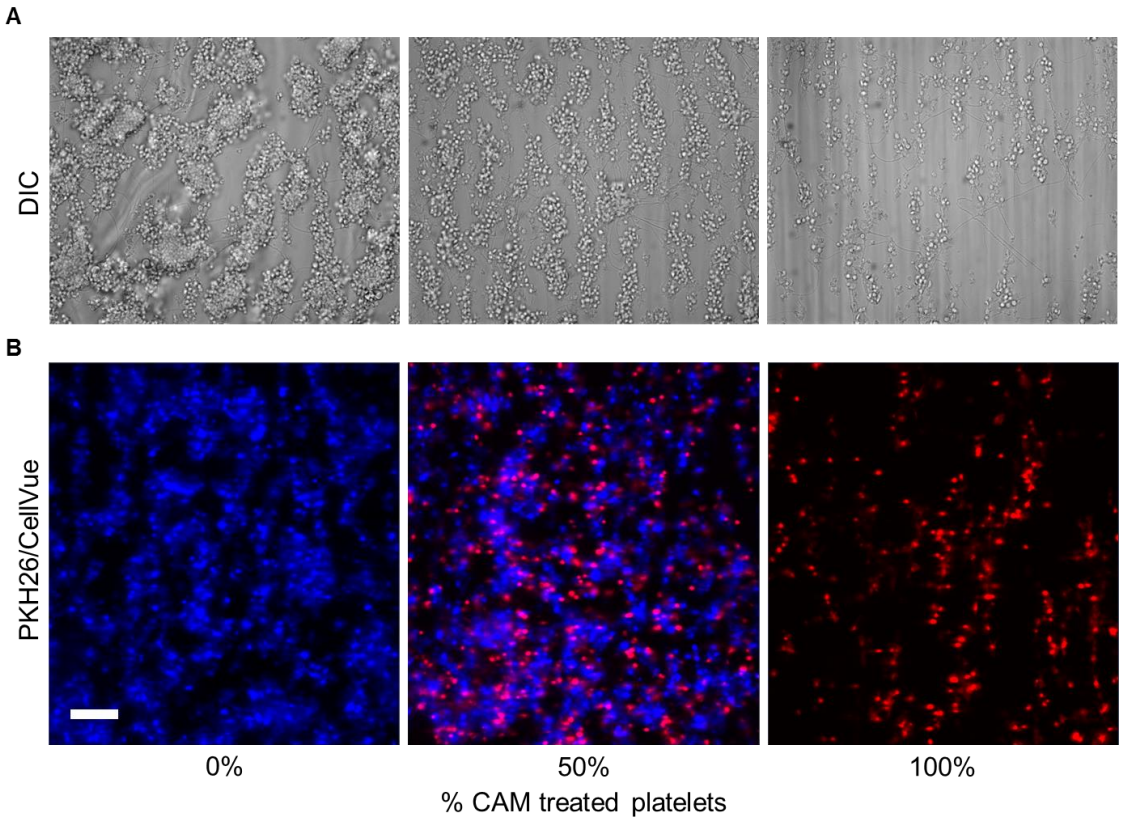
Supplemental Table 1. Study characteristics. Means ± SD.

Variables	
Patient characteristics	
Age *(years)*	59 ± 9
Female/male *(n)*	3/13
Diabetes mellitus *(n)*	3
Aspirin use *(n)*	16
Statins use *(n)*	13
Hematologic variables	
Hematocrit *(l/l)*	0.435 ± 0.035
Platelet count *(10^9/l)*	239 ± 81
Mean platelet volume *(fl)*	10.8 ± 0.7
Immature platelet fraction (IPF) Sysmex *(%)*	3.6 ± 1.9

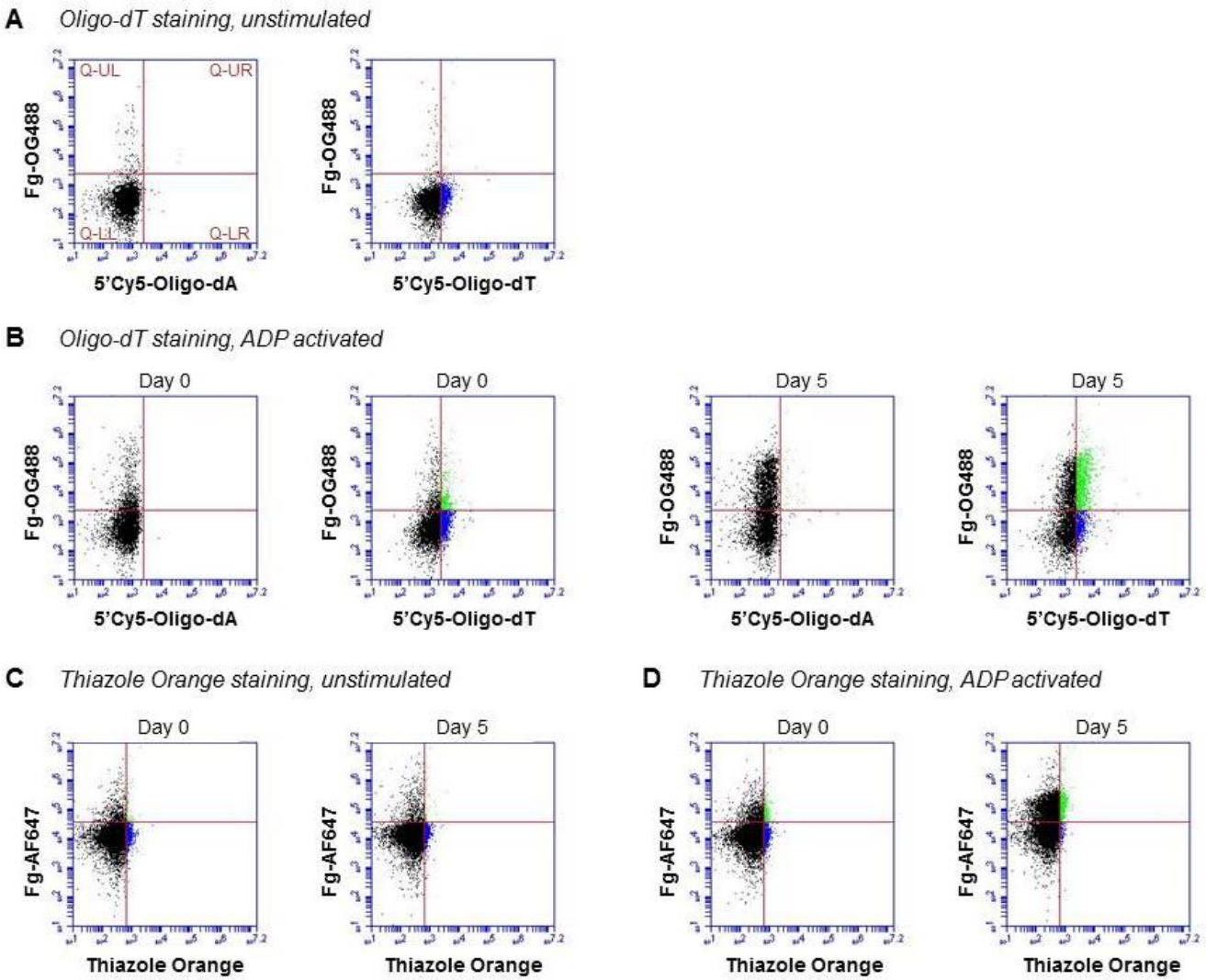
Supplemental figures



Suppl. Figure 1. Impaired fibrinogen binding of populations of $P2Y_{12}$-inhibited platelets. Mixtures of control platelets and CAM-treated platelets were preincubated with 1 µM ticagrelor or vehicle, and stimulated for 15 minutes with 20 µM 2MeS-ADP in the presence of OG488-fibrinogen. Fibrinogen binding was assessed by flow cytometry. Shown are representative histograms of fibrinogen binding after stimulation in the presence of vehicle (**A**) or ticagrelor (**B**).



Suppl. Figure 2. Impaired contribution to thrombus formation of $P2Y_{12}$-inhibited platelets. Reconstituted blood with different fractions of CAM-treated platelets was perfused 4 minutes over collagen at 1600 s^{-1} in the presence of 2MeS-ADP. Uninhibited platelets were prelabeled with PKH26 (blue) and CAM-treated platelets with CellVue Maroon (red). Shown are representative DIC (**A**) and dual-color fluorescence (**B**) images. Bars = 25 µm. See further, Figure 1.



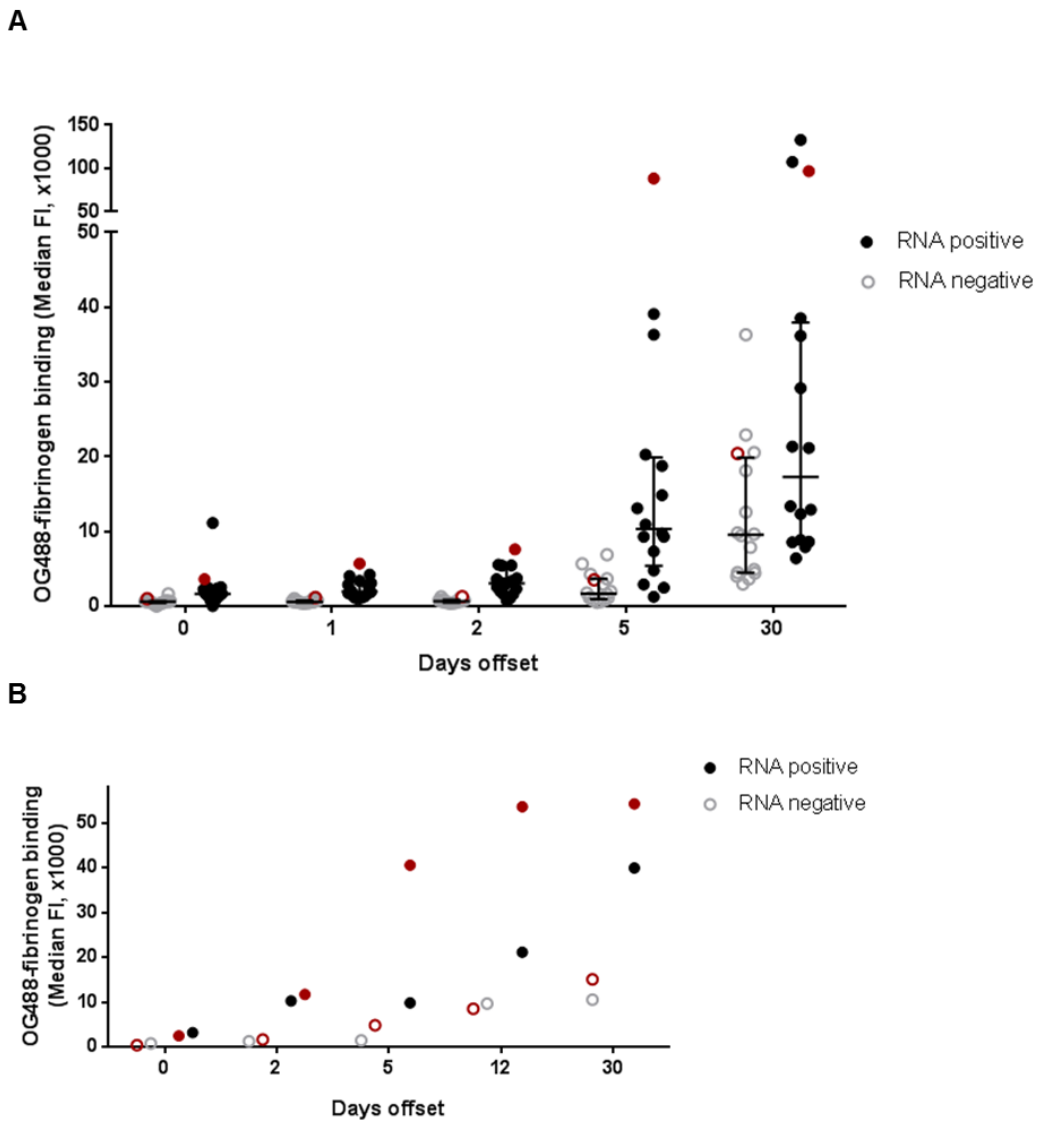
Activation ratio		*Day 0*	*Day 1*	*Day 2*	*Day 5*	*Day 30*
Oligo-dT staining	*Mature*	0.17 (±0.03)	0.24 (±0.03)	0.33 (±0.03)	0.82 (±0.10)	2.70 (±0.26)
	Juvenile	0.44 (±0.08)*	0.66 (±0.11)*	0.84 (±0.09)***	1.81 (±0.22)**	6.30 (±0.99)*
Thiazole Orange	*Mature*	0.18 (±0.04)	0.24 (±0.04)	0.41 (±0.06)	1.02 (±0.12)	3.35 (±0.59)
	Juvenile	0.38 (±0.08)*	0.50 (±0.08)***	0.92 (±0.13)***	2.42 (±0.27)***	8.18 (±1.32)**

Suppl. Figure 3. Alternative analysis confirms higher reactivity of juvenile platelets and increased activation in time upon prasugrel offset

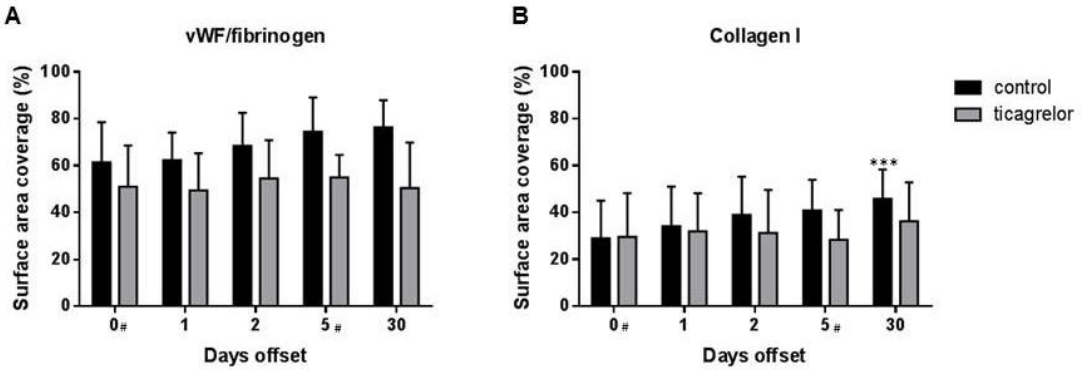
Juvenile platelets were detected using the oligo-dT staining or the thiazole orange staining, as described for Figure 5. Shown are representative dot plots of oligo-dA/dT or thiazole orange vs. Fg-OG488/AF647. Thresholds for juvenile and mature platelets were based on the negative controls oligo-dA and PBS for the oligo-dT and thiazole orange staining respectively. Thresholds for activated platelets were based on unstimulated samples. Unstimulated juvenile platelets (blue) and activated juvenile platelets (green) are depicted. Representative dot plots of unstimulated **(A)** or ADP-activated

(B) mature and juvenile platelets as identified by the oligo-dT staining. Representative dot plots of unstimulated **(C)** or ADP-activated **(D)** mature and juvenile platelets using the thiazole orange staining.

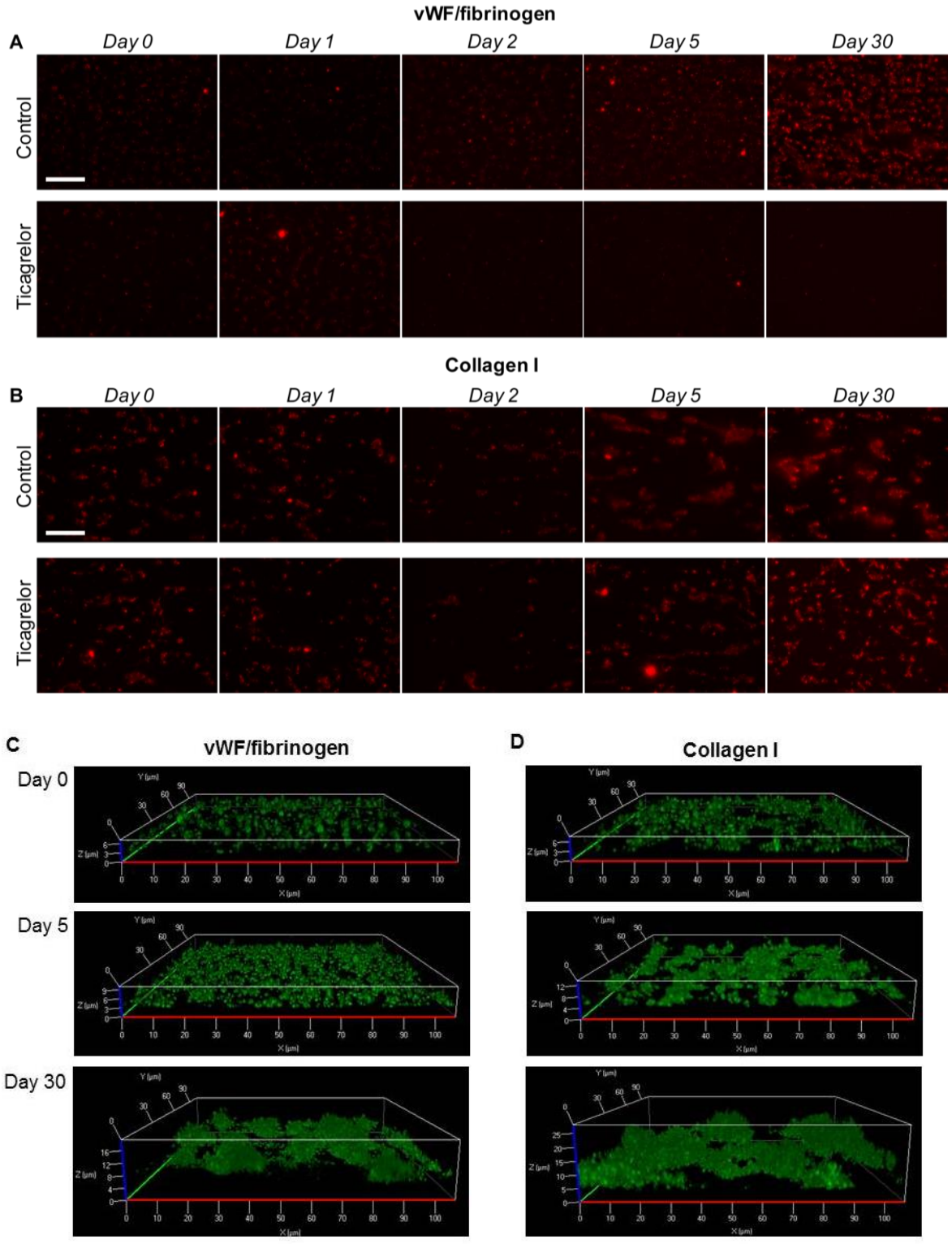
Table. Using the percentage of platelets in each quadrant of the dot plots, the activation ratio was calculated for both mature (Q-UL/Q-LL) and juvenile platelets (Q-UR/Q-LR) using either staining. Mean ± SEM (n =16), *p < 0.05, **p < 0.01, ***p < 0.001 *vs.* corresponding mature ratio (ANOVA with Bonferroni correction).



Suppl. Figure 4. Increased ADP-induced $\alpha_{IIb}\beta_3$ activation of juvenile platelets formed upon prasugrel offset. Platelets from patients during offset from prasugrel were activated and analyzed by flow cytometry. Juvenile platelets were identified by staining with the mRNA probe, 5'Cy5-oligo-dT. See further Figure 5. Washed platelets were activated with 1 µM 2MeS-ADP in the presence of OG488-fibrinogen. The cells were fixed and permeabilized with saponin to allow staining of mRNA by incubation with 5'Cy5-oligo-dT. Shown is extent of $\alpha_{IIb}\beta_3$ activation of mRNA-positive and -negative platelets, per subject. **(A)** Data from 16 patients, medians ± IQR. **(B)** Data from additional patients including day 12 time points. Red dots are from patients with a high immature platelet fraction (IPF ≥ 7.0%).



Suppl. Figure 5. Unchanged platelet adhesion in thrombus formation under flow upon prasugrel offset. Whole blood samples from patients (at indicated days after stopping prasugrel intake) were co-perfused with 2MeS-ADP over microspots containing vWF/fibrinogen (**A**) or collagen type I (**B**), as in Figure 6. Brightfield images were analyzed for surface area coverage of all platelets (single platelets and aggregates) at different offset days. Means ± SD (n = 16), ***p < 0.001 *vs.* day 0 (ANOVA with Bonferroni correction); $^{\#}$n = 15.



Suppl. Figure 6. Restored thrombus formation under flow upon prasugrel offset. Whole blood samples from patients (at indicated days after stopping prasugrel intake) were co-perfused with 2MeS-ADP over microspots containing vWF/fibrinogen or collagen type I, at a shear rate of 1600 s^{-1} for 4 minutes. See also Figure 6. Staining of thrombi was with AF647-fibrinogen and the platelet membrane label $DiOC_6$. Vehicle (control) or 10 µM ticagrelor was added to the blood. (**A**, **B**) Shown are (for a representative patient) fluorescence images of fibrinogen binding (red) of the thrombi

formed on vWF/fibrinogen or collagen I. Bars = 25 µm. (**C**, **D**) Representative confocal z-stacks of thrombi on vWF/fibrinogen or collagen I stained with $DiOC_6$ (green).