SUPPLEMENTARY METHODS

Blood reconstitution

An ABO-matched blood sample from healthy non-medicated volunteers was collected in heparin vacutainers® (REF368480, BD Diagnostics, Franklin Lakes, NJ) with measures for preserving platelet quiescence [1]. The blood was centrifuged at 250g to prepare platelet rich plasma (PRP) and red blood cells (RBC). The PRP and buffy coat were transferred to a new tube and centrifuged at 4500g to yield platelet-poor plasma. The cell pellet was discarded. To obtain reconstituted blood, packed RBC and plasma from this fresh blood were mixed with platelets from the respective research conditions aiming at 40 percent hematocrit and 250,000 platelets/µL. In the reconstituted blood a mean (±SD) platelet concentration of 236±21 $x10^3$platelets/µL and a hematocrit of 40.4±1.1% was become for RF-PRT and 248±16 $x10^3$platelets/µL and a hematocrit of 40.5±1.3% was become for AS-PCT (both n=6).

Platelet function during blood perfusion in microfluidic conditions

Perfusion experiments were performed using Vena8 Fluoro+^{TM} biochips (Cellix Ltd, Dublin, Ireland). The microfluidic channels were incubated with 50µg/mL type I collagen (Takeda, Osaka, Japan) or 5IU/mL VWF (Wilfactin®, CAF-DCF, Brussels, Belgium) at 4°C overnight and next blocked with 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 7.4 with 0.9% NaCl (HBS) containing 1.0% (w/v) bovine albumin and 0.1% (w/v) D-glucose at room temperature for 30 minutes. Blocking buffer and remainder collagen solution were rinsed with 1mL HBS buffer, pH 7.4 at 200dynes/cm^2. The reconstituted blood in the RF-PRT study was labeled with 1µM 3,3'-dihexyloxacarbocyanine iodide (DiOC6) (Sigma-Aldrich, Saint Louis, MO) for 10 minutes at 37°C. The reconstituted blood samples in the AS-PCT were labeled with calcein-AM (5µM final) (Life technologies, Carlsbad, CA) or where indicated with DiOC6 as mentioned above.

Paired samples (control vs treated) were perfused simultaneously and in duplicate using an automated microscope stage and a channel splitting manifold. Perfusion was performed at

50dynes/cm^2 (1,100s^{-1} flow rate) over collagen and at 135dynes/cm^2 (3000s^{-1} flow rate) over VWF using a Mirus Evo microfluidic pump (Cellix). Where indicated 50ng/mL of tirofiban (aggrastat®, Sigma-Aldrich) was added to block integrin $\alpha_{IIb}\beta_3$ binding. was Every 20 seconds, three side-by-side images were snapped in real-time in each lane during five minutes using an inverted fluorescent microscope at 100X magnification equipped with a Colibri-LED fluorescent light source (488nm) and high resolution CCD camera (all Carl Zeiss, Oberkochen, Germany). One single image per time point was generated by digitally stitching the three side-by-side images (ZEN2012 software (Carl Zeiss)). Surface coverage increased linearly in function of time for at least five minutes (Figure S1). Pixels were positive for platelet deposition using a fixed threshold of 400-4096 arbitrary fluorescence units. Slopes were determined by linear regression (Prism®, GraphPad Software Inc, La Jolla, CA) and are a measure for the rate at which thrombi deposit on collagen. Blood reconstituted with RF-PRT platelets maximally contained 12.5µM RF which did not interfere with the readout (data not shown).

Blood gases and platelet count

Glucose, lactic acid and pH were measured immediately after sampling by a point-of-care instrument (Siemens Rapidpoint® 500, Munich, Germany). Whole blood counts and mean platelet volume (MPV) were determined using an automated hematology analyzer (pocH-100i™, Sysmex, Kobe, Japan).

Flow cytometry

Expression of glycoprotein Ibα (anti-CD42b~fluorescein, Life technologies, Carlsbad, CA), activated integrin $\alpha_{IIb}\beta_3$ (PAC-1~fluorescein, BD Biosciences, Erembodegem, Belgium), P-selectine (anti-CD62P~phycoerythrin, BD Biosciences) and Annexin V (Annexin V~Peridinin chlorophyll-Cy5.5; BD Biosciences) was determined using an acoustic focusing Attune® flow cytometer (Life Technologies). Platelets were incubated with labeled antibodies or ligand for 10 minutes at room temperature in HBS, supplemented with 1mM $MgSO_4$, pH 7.4 and diluted

thousand fold immediately before readout as described in established methods[2]. For Annexin V measurements, buffers were supplemented with 2mM $CaCl_2$. For measurements of integrin $\alpha_{IIb}\beta_3$ activation on stimulated platelets, the PAR1 agonist thrombin related activating peptide SFLLRN (PAR1AP, Sigma-Aldrich) was added at 30µM. The signals of the isotype antibody controls were used to set threshold gates including 0.5% of 10,000 negative events. Mean or median fluorescence intensities and percentage positive events were determined of 10,000 cells staining positive for the platelet marker CD61 (anti-CD61~allophycocyanin, Life Technologies).

Aggregation

Platelet aggregation was determined by light transmission in a dual-channel lumiaggregometer (Chrono-log, Helena Laboratories, Haverton, PA) using three different platelet agonists, each at two concentrations. A saturating concentration to boost aggregation and a threshold (i.e. halfmaximal or EC50) concentration to investigate platelet sensitivity. Both concentrations were determined in separate serial dilution experiments (data not shown) using the same assay conditions as below. For collagen (American Biochemical & Pharmaceuticals, Epsom, United Kingdom) 25µg/mL and 10µg/mL was used, PAR1AP at 10µM and 6µM and ristocetin (American Biochemical & Pharmaceuticals) at 1.5mg/mL and 0.6mg/mL. Aggregation cuvettes contained platelets diluted to 250,000 platelets/µL with the corresponding autologous platelet free plasma with additive solution. The latter was prepared by centrifugation at 4500g for 20 minutes to pellet the platelet fraction. The readout comprises maximal aggregation (amplitude (%)), rate (slope), and total aggregation potential (area under curve). In all cases maximal aggregation is shown, but the corresponding auxiliary parameters were comparable.

VWF binding to glycocalicin

Binding of VWF to GPIbα was determined with the VWF:RCo enzyme-linked immunosorbant assay (ELISA) as previously published[3]. In brief, the anti-GPIbα monoclonal antibody 24B3 is

coated and following a blocking step, the binding sites are saturated with glycocalicin derived from a normal human plasma pool (n=38). Next, the suspending media of the platelet concentrates are incubated in the presence of 1mg/mL ristocetin to allow binding of VWF to the immobilized glycocalicin. After washing excess medium, bound VWF is detected by horse radish peroxidase labeled anti-VWF polyclonal antibody (Dako, Glostrup, Denmark).

Statistical analysis

Comparison of mean values of the study arms in function of time was with repeated measures two-way ANOVA with Tukey's or Sidak's correction for multiple comparisons testing and a 0.05 significance level. Analyses were performed with Prism® software version 6.01 (GraphPad Software Inc).

REFERENCES

1. Cattaneo M, Cerletti C, Harrison P, Hayward CP, Kenny D, Nugent D, et al. Recommendations for the Standardization of Light Transmission Aggregometry: A Consensus of the Working Party from the Platelet Physiology Subcommittee of SSC/ISTH. J Thromb Haemost 2013;11(6):1183-9.

2. Goodall AH, Appleby J. Flow-cytometric analysis of platelet-membrane glycoprotein expression and platelet activation. Methods Mol Biol 2004;272:225-53.

3. Vanhoorelbeke K, Pareyn I, Schlammadinger A, Vauterin S, Hoylaerts MF, Arnout J, et al. Plasma glycocalicin as a source of GPIbα in the von Willebrand factor ristocetin cofactor ELISA. Thromb Haemost 2004;93(1):165-71.

SUPPLEMENTARY DATA

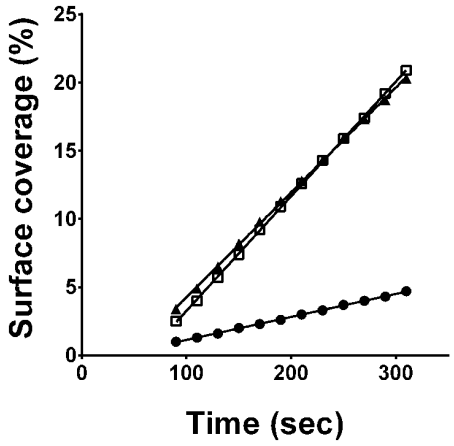

Figure S1. Platelet deposition in function of time as measured by surface coverage (%) in a single representative microfluidic flow experiment. Platelet deposition in microfluidic flow chamber experiments is determined by real-time video microscopy and subsequent image analysis of the platelet surface coverage (%) in function of time. This relationship is linear for at least five minutes and regression analysis allows for determination of the slope to deduce kinetics of hemostasis. Conditions in this case were untreated control (☐), gamma treated (▲) and photochemically RF-PRT treated platelets (●).

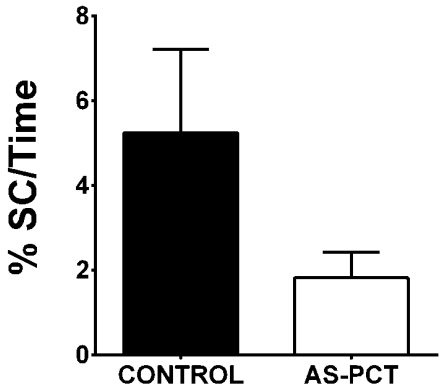

Figure S2 Thrombus formation differences on immobilized collagen are independent of the fluorescent label used. Analogous to the data in Figure 1 biologically paired reconstituted blood samples, containing either control (*closed bar*) or AS-PCT (*open bar*) platelets were perfused at 50dynes/cm² over immobilized collagen on day two. Thrombus formation kinetics were determined from the linear relationship of surface coverage (%SC) in function of perfusion time by regression analysis (Figure S1). In this case however, the cell suspension was labeled with DiOC6 instead of calcein that was used to generate the data for AS-PCT in Figure 1. Data are depicted as mean with standard deviation as whiskers (n=3).

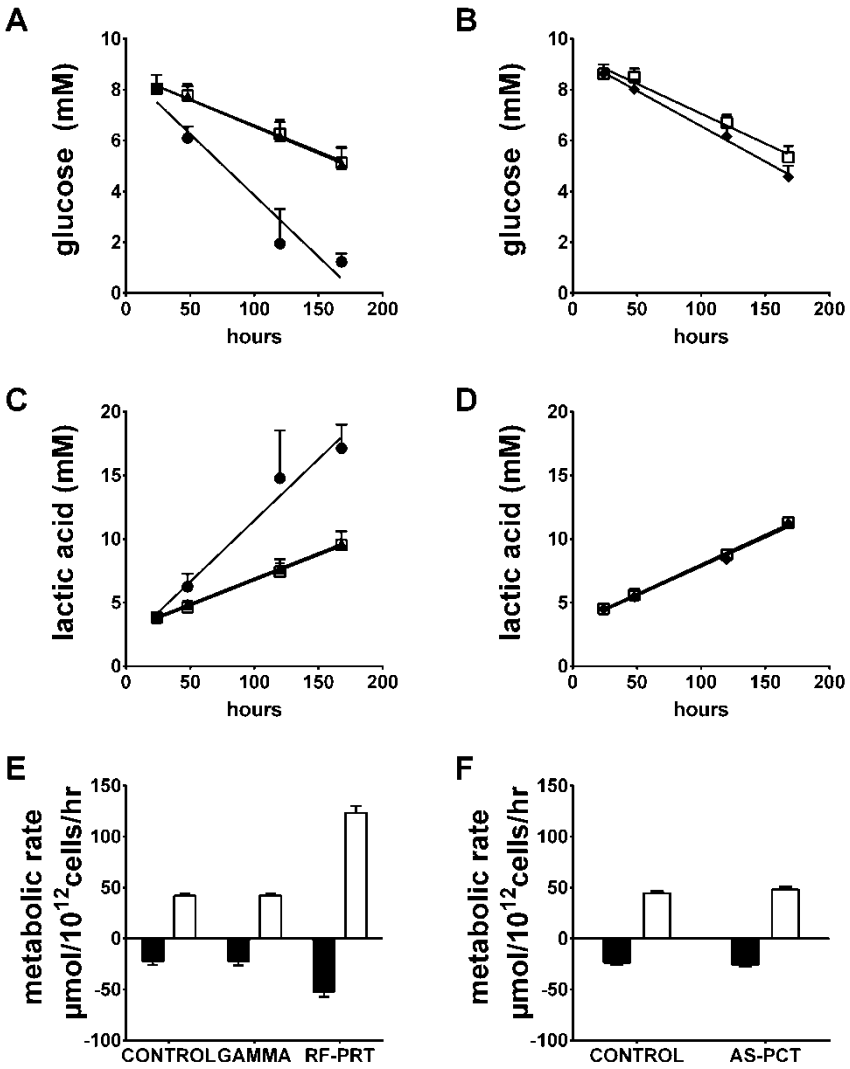

Figure S3 Glucose consumption and lactic acid production rates are significantly increased by RF-PRT but not AS-PCT. (A and B) Glucose consumption and (C and D) lactic acid production in function of storage time (in hours) is depicted for untreated control (☐), gamma treated (▲), RF-PRT (●) and AS-PCT (◆) treated concentrates. (E and F) The glucose consumption (*filled bars*) and lactic acid production rates (*open bars*) are derived from the data in A and B by linear regression analysis. Data are shown as means with standard deviation (n=6).

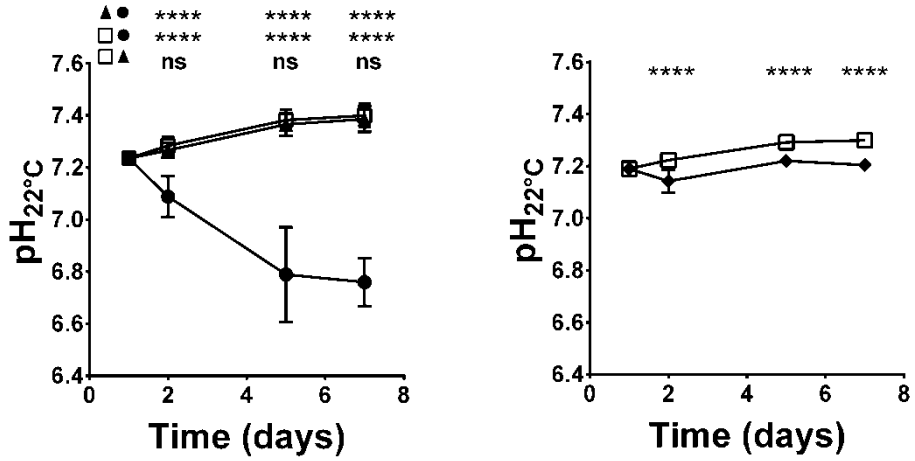

Figure S4 The pH of RF-PRT platelet concentrates gradually declines. The pH at ambient temperature of control (☐), gamma treated (▲), RF-PRT treated (●) and AS-PCT treated (◆) platelet concentrates (n=6) was determined in function of storage time. The means were statistically analyzed by two-way ANOVA and Tukey's or Sidak's multiple comparisons algorithm and the results are shown on top of each panel (ns = not significant; ****P<.0001).

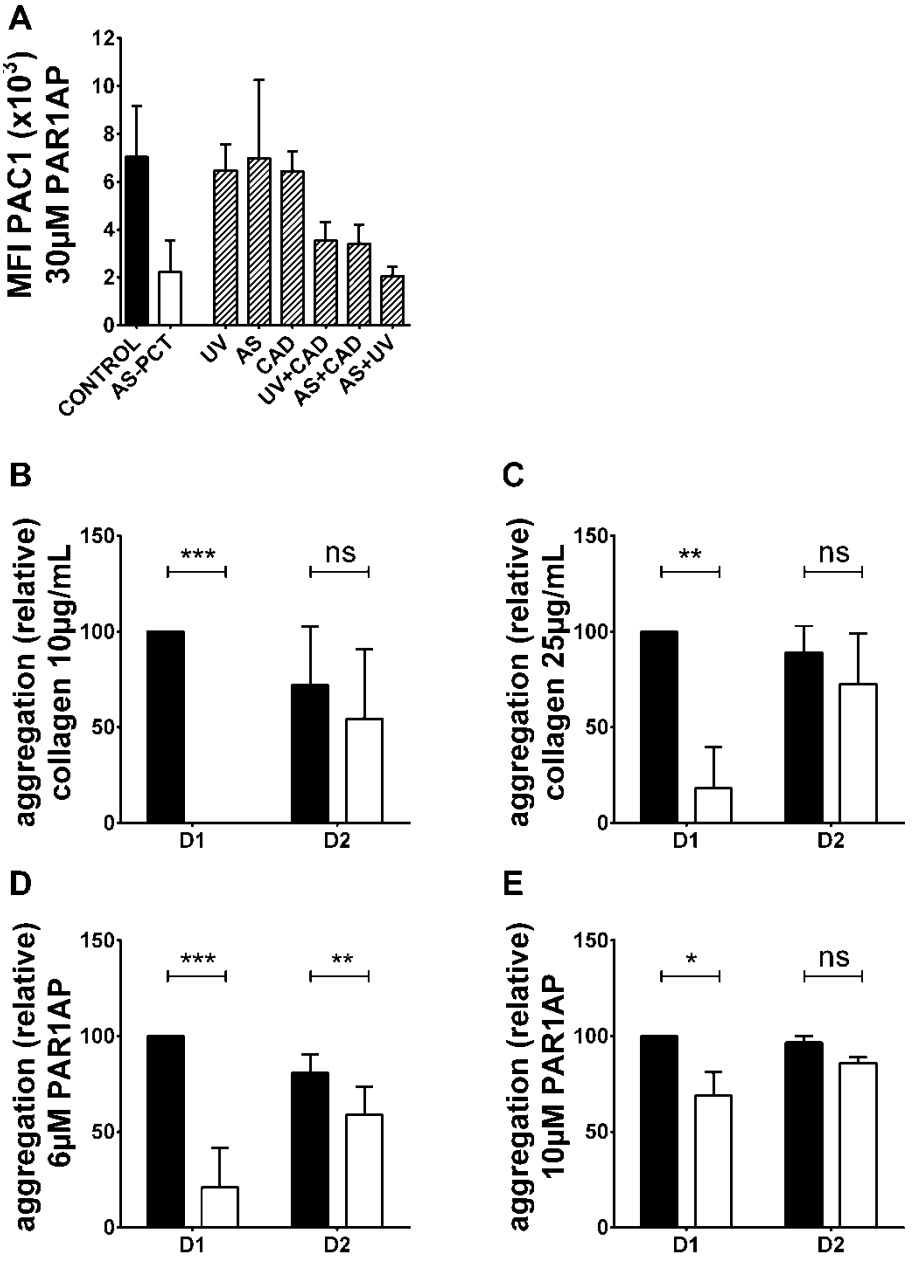

Figure S5 Photochemistry on itself has the largest impact on platelet activation. (A) Integrin $\alpha_{IIb}\beta_3$ activation to 30µM PAR1AP was measured by labeled PAC1 binding in flow cytometry. Platelet concentrates were treated with all possible combinations of the three principal AS-PCT steps (addition of photosensitizer (AS), illumination (UV) and adsorption (CAD)). Negative controls (*closed bar*) were not treated and positive controls (*open bars*) were treated according to the standard AS-PCT protocol. The other conditions (*shaded bars*) were either treated by UV, AS and CAD alone or by a combination of UV+CAD, AS+CAD and AS+UV. The median fluorescent PAC1 signal (MFI) of a minimum of two (AS, UV,

AS+CAD, UV+CAD) or three (both control conditions, CAD and AS+UV) repeat experiments is shown. (B-E) Aggregation by light transmission was performed with collagen (B and C) and PAR1AP (D and E) each in a threshold (B and D) and high (C and E) concentration (n=4). The maximal amplitude is depicted relative to the result of untreated control platelets on day one. Data of paired samples were collected immediately following photosensitization without incubation on CAD on day 1 (D1) and following overnight (16h) incubation on CAD (D2). Two-way ANOVA with SIDAK's multiple comparisons test was used to detect significant differences (*P<.05; **P<.01; ***P<.001). All data are shown as mean values with standard deviations.