Removing blood platelets from human plasma to reveal extracellular vesicle-associated miRNA profiles

Supplementary File 2. MIFlowCyt checklist

Requirement	Please Include Requested Information
1.1. Purpose	The purpose of this experiment was to determine the concentration of platelets (CD61+/CD41+), activated platelets (CD61+/CD62p+) and erythrocyte ghosts (ery-ghosts; CD235a+) in plasma- and extracellular vesicle (EV) samples, and to investigate how these remaining cells affect downstream analyses of miRNA that is thought to be associated with EVs. Flow cytometry was used to measure (activated) platelets and ery-ghosts before and after filtration of the samples using 0.8-µm pore-sized polycarbonate membrane filters. We hypothesized that filtration would remove platelets and ery-ghosts, and that this removal would lower the concentration of miRNAs that could be detected.
1.2. Keywords	Platelets, erythrocytes, plasma, extracellular vesicles, filtration, miRNA
1.3. Experiment variables	EDTA blood samples were centrifuged twice, according to two different blood processing protocols. The obtained plasma was used as input for size-exclusion chromatography CL-2B (SEC2B) to enrich for EVs. Plasma and EV samples were filtered using a 0.8-µm pore-sized polycarbonate membrane filter, to remove platelets and ery-ghosts.
1.4. Organization name and address	Amsterdam University Medical Centers Location University of Amsterdam Meibergdreef 9 1105 AZ Amsterdam The Netherlands
1.5. Primary contact name and email address	Jillian W.P. Bracht; j.w.p.bracht@amsterdamumc.nl
1.6. Date or time period of experiment	29th September 2021 - 14th October 2021
1.7. Conclusions	Platelets and ery-ghosts are present in plasma- and EV samples. Platelet concentrations correlated with miRNA levels of let7a-5p, miR-21-5p and miR-223-5p, indicating that the presence of platelets may lead to an overestimation of the concentration of miRNAs that are associated with EVs. A 0.8-µm polycarbonate filter removes pre-activated platelets and ery-ghosts from plasma- and EV samples. To avoid interference of platelets on downstream EV-miRNA analysis, it is

	recommended to remove these platelets, using filtration. Activated platelets were not removed using filtration. Plasma should be filtered before freezing samples, because upon freezing residual platelets may fragment into particles detected as CD61+ EVs.
1.8. Quality control measures	All samples were measured manually in Falcon® tubes (BDAA352052 VWR, Radnor, PA). The flow rate of the FACSCanto™ II (BD Biosciences, San Jose, CA) was determined using TruCount beads (BD Biosciences). The side scattering and fluorescence detectors of the FACSCanto II were calibrated.
1.9 Other relevant experiment information	The experiment was performed in triplicate and includes samples of three healthy volunteers, processed at different days.
2.1.1.1. Sample description	Freshly prepared double-centrifuged plasma (section 2.1.1.2) from three healthy volunteers. Plasma samples were prepared using two different centrifugation protocols. This plasma was used as input for SEC2B to enrich for EVs.
2.1.1.2. Biological sample source description	Venous blood was collected from three healthy volunteers using a 21-Gauge needle. The first 2 mL of blood was discarded, then 4 tubes of EDTA blood (6 mL; BD Biosciences) were collected from each donor. Plasma was prepared by two centrifugation protocols, using a Rotina 380 R centrifuge (Hettich, Tuttlingen, Germany): • International Society on Thrombosis and Haemostasis (ISTH) protocol: plasma was prepared by double centrifugation, according to the ISTH protocol[1]. Whole blood was centrifuged for 15 minutes at 2,500 x g and 20 °C, with the centrifuge acceleration set at 9, and deceleration set at 1. Single centrifuged plasma was collected to exactly 10 mm above the buffy coat (with a lego brick), and transferred to a 15 mL polypropylene tube (Greiner Bio-One B.V., Alphen aan den Rijn, The Netherlands). The obtained plasma was then centrifuged for 15 minutes at 2,500 x g and 20 °C, with the centrifuge acceleration set at 9, and deceleration set at 1. Double centrifuged plasma was collected to exactly 10 mm above the cell pellet (with a lego brick). • Non-ISTH protocol[2]: whole blood was centrifuged for 7 minutes at 900 x g and 20 °C, with the centrifuge acceleration and deceleration set at 9. Single centrifuged plasma was collected to approximately 10 mm (estimated by eye) above the buffy coat, and transferred to a 15 mL polypropylene tube (Greiner Bio-One B.V.). The obtained plasma was then centrifuged for 10 minutes at 2,500 x g and 20 °C, with the centrifuge acceleration and deceleration set at 9. Double centrifuged plasma was

	collected to approximately 10 mm (estimated by eye) above the cell pellet. Double centrifuged plasma was (1) filtered with a 0.8-µm pore-size polycarbonate membrane filter (Isopore^{TM}, Merck Millipore, Darmstadt), or (2) used as starting material to isolate extracellular vesicles with SEC2B. SEC2B columns were prepared by addition of 10 mL washed Seapharose CL-2B (GE Healthcare, Uppsale, Sweden) to 15 mL Kinesis TELOS Chromatography Filtration Columns with double 20 µm Polyethylene Frits (Cole-Parmer, St Neots, UK). A Kinesis TELOS Polyethylene Frit 20 µm (Cole-Parmer) was placed on top of the stacked Sepharose. One mL of double centrifuged plasma was loaded onto a SEC2B column and 0.5 mL fractions were collected directly after sample loading. Dulbecco's Phosphate-Buffered Saline (DPBS; Gibco, Waltham, MA) was added to the column as running buffer, after the plasma sample had almost completely entered the Sepharose. EV-enriched fractions (F8-10) were pooled and used for further analyses. One mL of the pooled EV fraction was filtered using the polycarbonate membrane filters.
2.1.1.3. Biological sample source organism description	Healthy human volunteers.
2.2 Sample characteristics	Plasma is expected to contain EVs, (activated) platelets, ery-ghosts, lipoproteins and proteins. EV-enriched SEC fractions are expected to be enriched for EVs, but samples will also contain lipoproteins and proteins and are expected to contain (activated) platelets and ery-ghosts. Samples were stained for platelet- (CD61+/CD41+), activated platelet- (CD61+/CD62p+) and erythrocyte- (CD235a+) markers.
2.3. Sample treatment description	Plasma was prepared according to two different protocols (section 2.1.1.2). Plasma sample were used as sample input for SEC2B, and EV enriched SEC fractions (F8-10) were pooled. Plasma samples and EV enriched SEC fractions were then (1) analyzed directly, and (2) analyzed after filtration with a 0.8-µm polycarbonate filter.
2.4. Fluorescence reagent(s) description	**Table S1.1** contains an overview of the staining reagents. Anti-CD41-PE (Biocytex, Marseille, France), CD61-APC (Invitrogen, Waltham, MA), CD62p-PE (Beckman Coulter, Brea, CA) and anti-CD235a-FITC (Dako, Amstelveen, The Netherlands) were pre-diluted in DPBS (Corning, Amsterdam, The Netherlands) and centrifuged at 18.890 x g to remove antibody aggregates. Twenty µl of each sample was added to 30 µl HEPES buffer (137 mmol/L NaCl (6404, Merck Millipore), 20 mmol/L Hepes (10110, Merck Millipore), 5.6 mmol/L D-glucose (8337, Merck

	Millipore), 0.1%, BSA (A9647, 0.1%, Sigma-Aldrich, St. Louis, MO, 3.3 mmol/L fc $NaH_2PO_4.H_2O$ (6345, Merck Millipore), 2.7 mmol/L fc, KCl (24936, Merck Millipore), and 1 mmol/L fc $MgCl_2.6H_2O$ (5833, Merck Millipore) in Milli-Q (Baxter, TKF7114, Deerfield, IL)). Subsequently, 5 µl pre-diluted anti-CD235a-FITC, 10 µl pre-diluted mix of anti-CD61-APC and anti-CD41-PE or 10 µl pre-diluted mix of anti-CD61-APC and anti-CD62p-PE was added. After a 30 minute incubation in the dark at room temperature (RT), samples were fixated with 200 µl HEPES buffer/0.3% paraformaldehyde (PFA; 104005, Merck Millipore) for 1 hour in the dark at RT. In case samples exceeded an event rate of > 10,000 evts/sec during measurements, samples were further diluted in HEPES buffer/0.3% PFA.
3.1. Instrument manufacturer	BD Biosciences
3.2. Instrument model	FACSCanto™ II
3.3. Instrument configuration and settings	Detector voltages of the FACSCanto II were set using CS&T beads (BD, custom made, lot. 32272). Stained samples were measured for 120 seconds at a flow rate of 60 µL/min on a FACSCanto II, equipped with a 405-nm laser, 488-nm laser, and 633-nm laser. The trigger threshold was set at FSC 200 arbitrary units or SSC 200 arbitrary units or fluorescence APC, FITC or PE 200 arbitrary units. For FSC and SSC, the PMT voltages were 233 V and 373 V, respectively. APC signals were collected with the 633 Red detector (660/20 nm filter, PMT voltage 532 V). FITC signals were collected with the 488 Blue detector (530/30 nm filter, PMT voltage 478) and PE signals were collected with the 488 Blue detector (585/42 nm filter, PMT voltage 462). TruCount beads (BD Biosciences) were dissolved in 500 µL Milli-Q water and were used to determine the flow rate, according to manufacturer's instructions.
4.1. List-mode data files	A summary of all flow cytometry scatter plots and gates applied are available via: https://doi.org/10.6084/m9.figshare.c.6126783.v1
4.2. Compensation description	No compensation was required, because no fluorophore combinations were used that have overlapping emission spectra.
4.3. Data transformation details	Light scattering calibration and fluorescence calibration were applied, as indicated below. Concentrations reported in the manuscript describe the number of particles that fulfil the gating criteria per mL. **Light scatter calibration** We used Rosetta Calibration (v1.28, Exometry BV, Amsterdam, The Netherlands) to relate side scattering (SSC) to the effective scattering cross section and diameter of platelets and ery-ghosts. **Figure S1.1** shows a print screen of the scatter calibration settings. Platelets were modelled as core-shell particles with a core refractive index of 1.38, a shell refractive index of 1.48, and

	a shell thickness of 6 nm. Ery-ghosts were modelled as core-shell particles with a core refractive index of 1.35, a shell refractive index of 1.48, and a shell thickness of 6 nm. For each measurement, we added the SSC cross sections and particle diameters to the flow cytometry datafiles. The SSC trigger threshold corresponds to a side scattering cross section of 27 nm^2. **Fluorescence calibration** Calibration of the fluorescence detectors from arbitrary units (a.u.) to molecules of equivalent soluble fluorochrome (MESF) was accomplished using 2-µm APC Quantitation Beads (2321-175, BD), Quantum™ FITC-5 MESF Beads (13734, Bangs Laboratories, Inc Fishers, IN) and SPHERO™ PE Calibration Particle Kit, 3.0-3.4 µm (ECFP-F2-5K, AK01, Spherotech Inc., Irma Lee Circle, IL). Calibrations of the APC, FITC and PE detectors were performed on 2022-01-24. For each measurement, we added fluorescent intensities in MESF to the flow cytometry data files by custom-build software (MATLAB R2018a) using the following equation: $I(MESF) = 10^{a \cdot \log_{10} I(a.u.) + b}$ Equation S1 where I, is the fluorescence intensity, and *a* and *b* are the slope and the intercept of the linear fits respectively, see **Table S1.2**.
4.4.1. Gate description	All gates were manually set using FlowJo (v 10.8.1, Flowjo, Ashland, OR). Gates for platelets, activated platelets and ery-ghosts were set based on fluorescence intensities (CD61+/CD41+, CD61+/CD62p+ and CD235a+, respectively) and light scattering intensities. An example of the gating strategy is depicted in **Figure S1.2.1, S1.2.2. and S1.2.3.**
4.4.2. Gate statistics	The concentration of positive events was calculated by taking into account the flow rate, measurement time and dilutions performed during sample preparation.
4.4.3. Gate boundaries	An overview of all gates can be found in the data summary file via: https://doi.org/10.6084/m9.figshare.c.6126783.v1

APC: Allophycocyanin; CD: cluster of differentiation; EDTA: Ethylenediamine tetraacetic acid; Ery-ghost: erythrocyte ghost; FITC: Fluorescein Isothiocyanate; FSC: forward scattering; PE: Phycoerythrin; PMT: photomultiplier tube; SSC: side scattering.

References

1. Lacroix R, Judicone C, Poncelet P, et al. Impact of pre-analytical parameters on the measurement of circulating microparticles: towards standardization of protocol. *J Thromb Haemost.* 2012;10(3):437-446.
2. van Eijndhoven MA, Zijlstra JM, Groenewegen NJ, et al. Plasma vesicle miRNAs for therapy response monitoring in Hodgkin lymphoma patients. *JCI Insight.* 2016;1(19):e89631.

Table S1.1: Overview of staining reagents. Characteristics being measured, analyte, analyte detector, reporter, isotype, clone, concentration, manufacturer, catalog number and lot number of used staining reagents.

Characteristic measured	**Analyte**	**Analyte detector**	**Reporter**	**Isotype**	**Clone**	**Concentration during staining (µg mL^{-1})**	**Manufacturer**	**Catalog number**	**Lot number**
Integrin	Human CD61	Anti-human CD61 antibody	APC	IgG1	VI-PL2	0.69	Invitrogen	17-0619-42	2284236
Integrin	Human CD41	Anti-human CD41 antibody	PE	IgG1	PL2-49	0.63	Biocytex	5112-PE100T	091251
Glycoprotein	Human CD235a	Anti-human CD235a antibody	FITC	IgG1	JC159	4.55	Dako	F0870	20064863
Selectin	Human CD62p	Anti-human CD62p antibody	PE	IgG1	CLB-Thromb/6	1.04	Beckman Coulter	IM1759U	200053

APC: Allophycocyanin; FITC: Fluorescein Isothiocyanate; IgG: Immunoglobulin G; PE: Phycoerythrin

Table S1.2: Overview of fluorescence calibrations

	Calibration date	Slope	Intercept	R^2
APC	2022-01-24	1.3014	-1.539	0.9902
PE	2022-01-24	0.9966	0.4940	1
FITC	2022-01-24	1.0737	1.2195	0.9994

Figure S1.1.

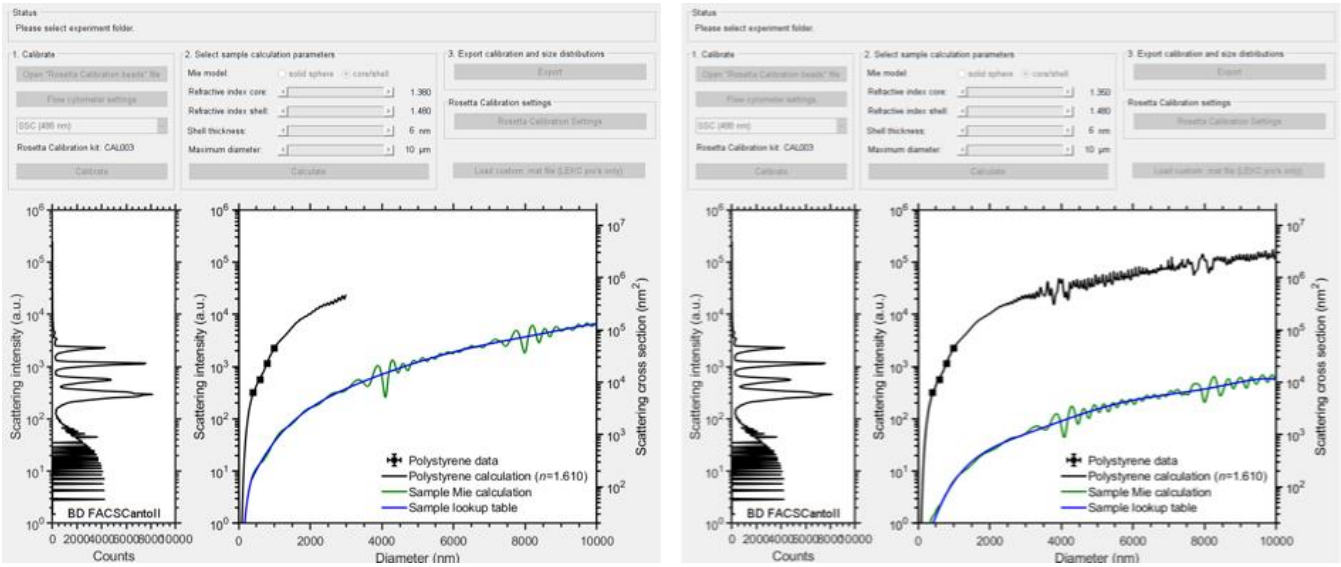
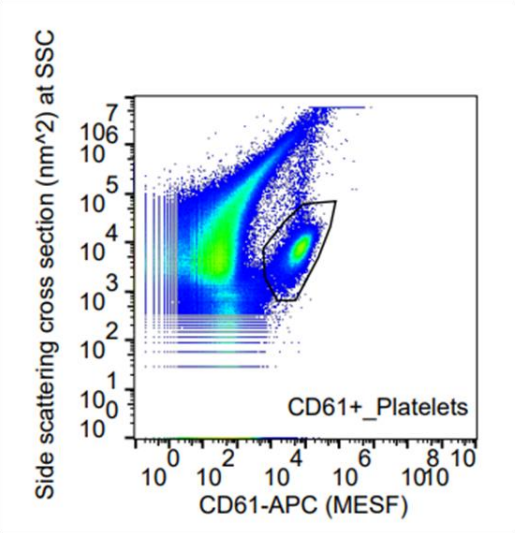


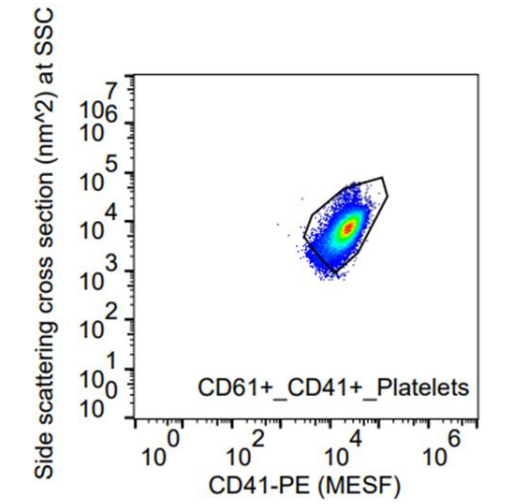
Figure S1.1. *Side scatter calibration of the FACSCanto II. To relate scatter to the approximate diameter of (activated) platelets (left), we assumed platelets to be core-shell particles with a core refractive index of 1.38, a shell refractive index of 1.48, and a shell thickness of 6 nm. To relate scatter to the approximate diameter of ery-ghosts (right), we assumed ery-ghosts to be core-shell particles with a core refractive index of 1.35, a shell refractive index of 1.48, and a shell thickness of 6 nm.*

Figure S1.2.1.

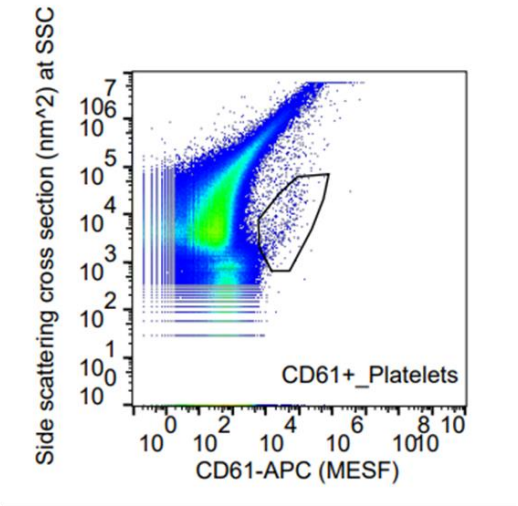
1) CD61+ population (unfiltered)



2) CD61+_CD41+ population (unfiltered)



3) CD61+ population (filtered)



4) CD61+_CD41+ population (filtered)

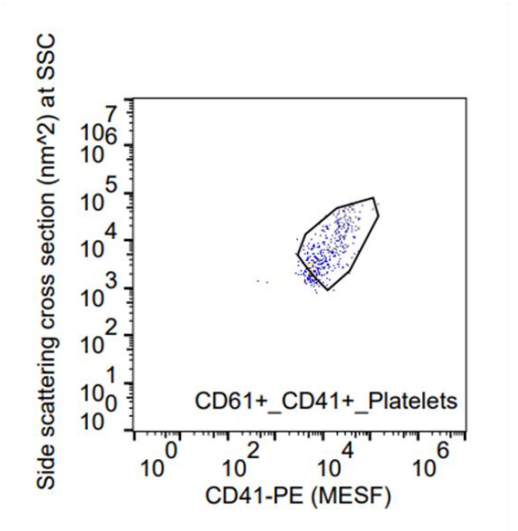
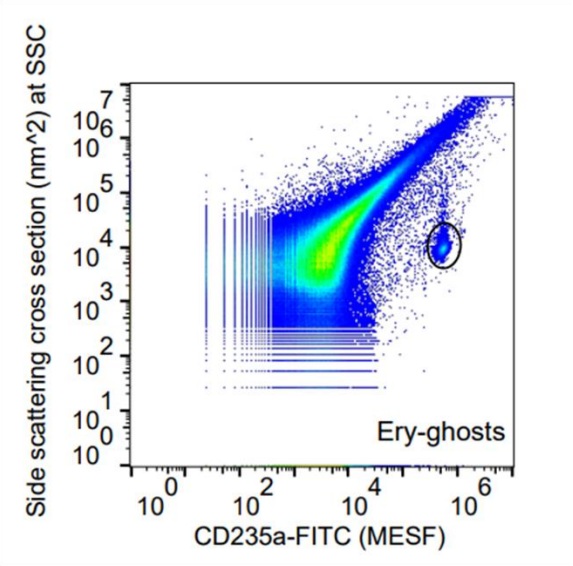


Figure S1.2.1. *Gating strategy of platelets measured by the FACSCanto II. The plots show side scatter cross sections vs. fluorescence. (Panel 1, 2) Platelet gate on the unfiltered plasma sample. The CD61-APC+/CD41-PE+ population is defined as platelets. (Panel 3, 4) Platelet gate on the filtered plasma sample. The CD61-APC+/CD41-PE+ population is defined as platelets.*

Figure S1.2.2.

1) CD235a+ population (unfiltered)



2) CD235a+ population (filtered)

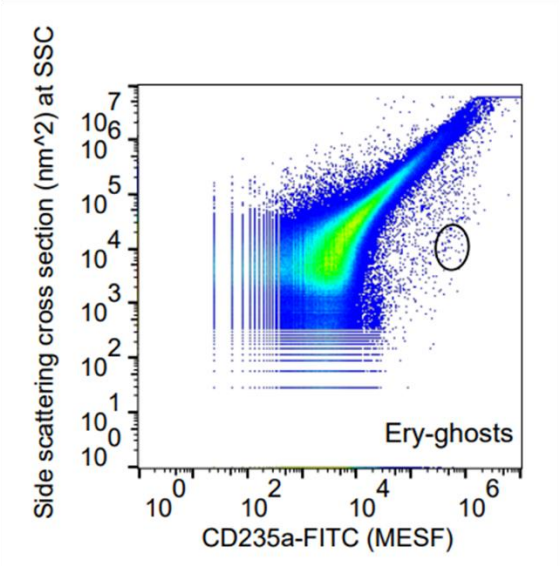
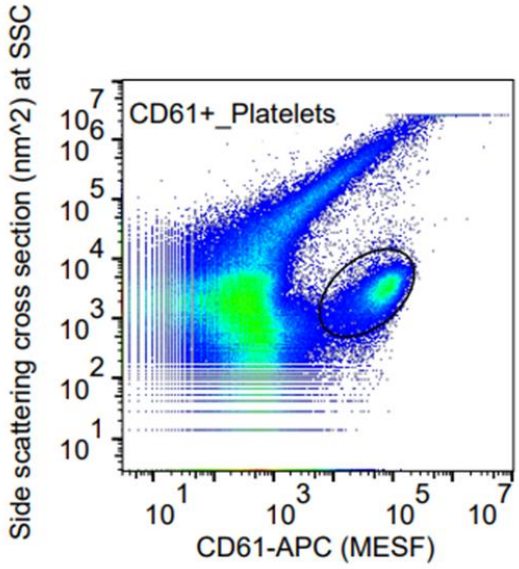


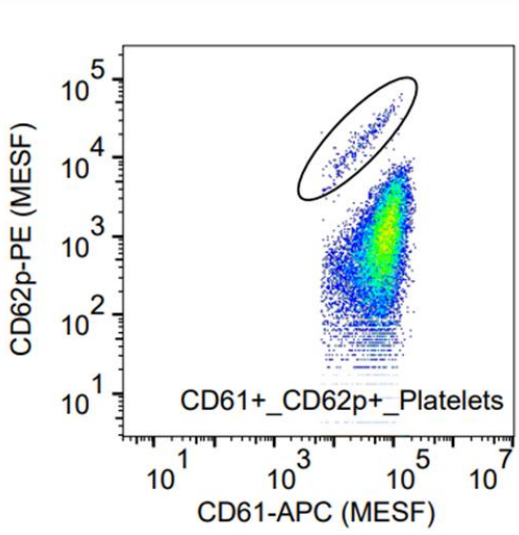
Figure S1.2.2. *Gating strategy for erythrocyte ghosts (ery-ghosts) measured by the FACSCanto II. The plots show side scatter cross sections vs. fluorescence for unfiltered (panel 1) and filtered (panel 2) plasma samples. The CD235a-FITC+ population is defined as ery-ghosts.*

Figure S1.2.3.

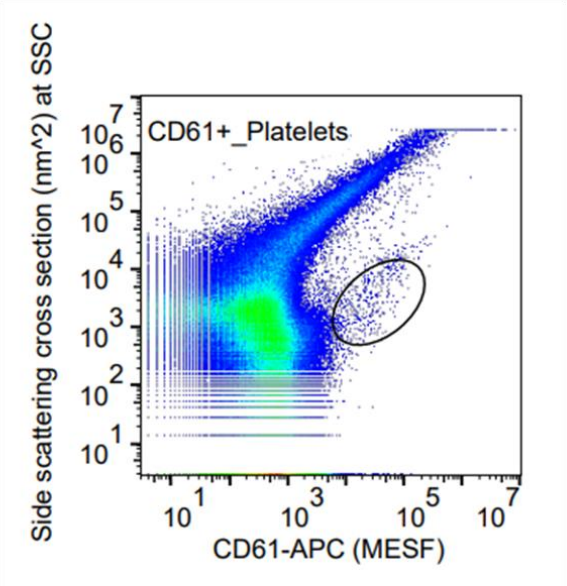
1) CD61+ population (unfiltered)



2) CD61+_CD62p+ population (unfiltered)



3) CD61+ population (filtered)



4) CD61+_CD62p+ population (filtered)

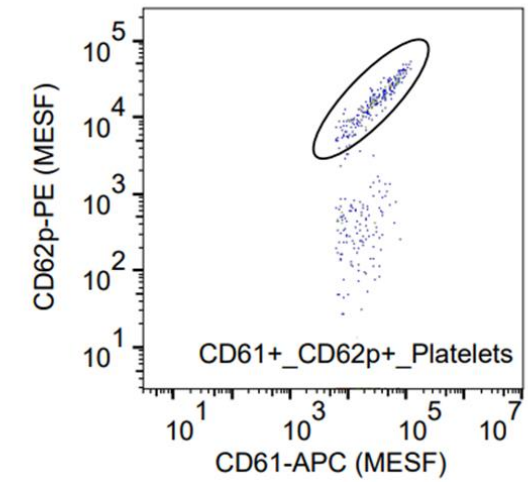


Figure S1.2.3. *Gating strategy of activated platelets measured by the FACSCanto II. Panel 1 and 3 show plots of side scatter cross sections vs. fluorescence in filtered and unfiltered plasma samples, respectively. The CD61-APC+ population is defined as platelets. Panel 2 and 4 show fluorescence (CD61-APC, MESF) vs. fluorescence (CD62p-PE, MESF) in filtered and unfiltered samples, respectively. The CD61-APC+/CD62p-PE+ population is defined as activated platelets.*