Supporting information 3 MIFlowCyt checklist of Filtration experiments, "Removal of platelets from blood plasma to improve the quality of extracellular vesicle research"

Requirement	Please Include Requested Information
1.1. Purpose	The aim of the flow cytometry experiment was to compare the concentrations of platelets stained with CD61-APC in double centrifuged plasma before and after filtration with a polycarbonate membrane filter having a 0.8-µm pore diameter. We hypothesized that the use of a polycarbonate filter would remove platelets effectively.
1.2. Keywords	Platelets, plasma, filtration
1.3. Experiment variables	Filtration of the samples using a polycarbonate membrane filter having a 0.8-µm pore diameter.
1.4. Organization name and address	Amsterdam University Medical Centers Location Academic Medical Centre Meibergdreef 9 1105 AZ Amsterdam The Netherlands
1.5. Primary contact name and email address	Britta A. Bettin, b.a.bettin@amsterdamumc.nl
1.6. Date or time period of experiment	7th October 2020
1.7. Conclusions	Double centrifugation followed by filtration using a polycarbonate membrane filter having a 0.8-µm pore diameter resulted in a 146-fold lower platelet concentration compared to double centrifugation without filtration.
1.8. Quality control measures	All samples were measured manually in Falcon® tubes (BDAA352052 VWR, Radnor, PA). The flow rate was determined using TruCount beads (BD Biosciences, San Jose, CA).
1.9 Other relevant experiment information	The experiment was conducted within one day.
2.1.1.1. Sample description	Freshly prepared double-centrifuged plasma (section 2.1.1.2) from a healthy volunteer (section 2.1.1.3).
2.1.1.2. Biological sample source description	Blood was collected from one healthy individual who denied having a disease or using drugs and/or medication. Venous blood was collected using a 21-Gauge needle, and the first 3.5 mL of blood was discarded. One tube with 6 mL of EDTA blood (BD Biosciences) was collected, mixed gently, and processed within 15 minutes. To prepare plasma, the blood collection tube was double centrifuged using a Rotina 380 R equipped with a swing-out rotor and radius of 155 mm (Hettich Zentrifugen, Tuttlingen, Germany). Whole blood was double centrifuged at 2,500 g, 15

	minutes, 20°C, acceleration speed 9, deceleration speed 1. Plasma was collected 10 mm above the buffy coat with a plastic Pasteur pipette (VWR, Radnor, PA) and transferred into 15-mL polypropylene centrifuge tubes (Greiner Bio-One B.V., Alphen aan den Rijn, The Netherlands). Subsequently, plasma was centrifuged at 2,500 g, 15 minutes, 20°C, acceleration speed 9, deceleration speed 1. Plasma was collected to 10 mm above the pellet, transferred into a new 15-mL polypropylene centrifuge tube (Greiner Bio-One B.V.), mixed by pipetting, and further transferred to 1.5-mL low protein binding Eppendorf tubes (Thermo Fisher Scientific, Waltham, MA). To further reduce the concentration of platelet contaminants in plasma, polycarbonate membrane filters having a 0.8-µm pore diameter and a 2.5-cm filter diameter (IsoporeTM, Merck Millipore, Darmstadt, Germany) were used. The concentration of platelets were measured in fresh, unfiltered and filtered plasma.
2.1.1.3. Biological sample source organism description	Healthy human volunteer.
2.2 Sample characteristics	Plasma is expected to contain detectable extracellular vesicles, lipoproteins proteins, and platelets.
2.3. Sample treatment description	Plasma was either double centrifuged, or double centrifuged followed by filtration (section 2.1.1.2).
2.4. Fluorescence reagent(s) description	Table S1 contains an overview of the staining reagents. Prior to staining, samples were diluted 2.75-fold in HEPES buffer. HEPES buffer was 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 MiliQ (Baxter, TKF7114, Deerfield, IL). To remove antibody aggregates, anti-human CD61-APC antibody (17-0619-42, eBioscience; clone VI-PL2; final concentration 8.33 µg/mL) was diluted in Dulbecco's Phosphate-buffered saline (DPBS, 21-031-CV, Corning, NY) and centrifuged at 18,890 · g for 5 minutes. To measure the concentration of platelets, 20 µL diluted plasma was incubated with 30 µL HEPES buffer and 5 µL antibody and kept in the dark for 30 minutes at room temperature. After 30 minutes, samples were 9-fold post-diluted and fixated with 200 µL HEPES/0.3 % paraformaldehyde (PFA; 104005, Merck Millipore) in the case of plasma and 41-fold HEPES/0.3 % PFA in the case of filtered plasma. Half the volume of plasma was filtered using a polycarbonate membrane with a 0.8-µm pore diameter and the other half was used as a control. To allow fixation, the HEPES/0.3 % PFA diluted samples were incubated for 1 hour in the dark at room temperature. Afterward, 450 µL of plasma or 500 µL of filtered plasma were transferred into TruCount beads vials (BD Biosciences).
3.1. Instrument manufacturer	BD Biosciences
3.2. Instrument model	FACSCanto™ II

3.3. Instrument configuration and settings	Detector voltages 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 an FACS Canto II, BD Biosciences, equipped with a 405-nm laser, 488-nm laser, and 633-nm laser. The trigger threshold was set at FSC 200 arbitrary units. For FSC and SSC, the PMT voltages were 233 V and 373 V, respectively. APC signals were collected with the 638-D Red (Peak) detector (long pass 660/20 nm filter, PMT voltage 532 V). TruCount beads were used according to manufacturer's instructions to determine the flow rate.
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.19137194
4.2. Compensation description	No compensation was required because no fluorophore combinations were used that have overlapping emission spectra.
4.3. Data transformation details	Data were not transformed (or calibrated), because platelets were recognized and gated based on their relative fluorescence and light scattering intensity and a comparison with daily measured whole blood samples.
4.4.1. Gate description	A gate was applied manually using FlowJo (v 10.7.1, FlowJo, Ashland, OR, USA). To select platelets, the gate was set based on the measured CD61-APC fluorescence intensities and light scattering intensities. Platelets were identified based on a comparison with daily measured whole blood samples. The gate was set in a triangular shape around the platelet population. An example of the gating strategy can be found in Figure S3.1.
4.4.2. Gate statistics	The number of positive events was corrected for flow rate, measurement time and dilutions performed during sample preparation.
4.4.3. Gate boundaries	On overview of all gates can be found in the compressed data summary file via https://doi.org/10.6084/m9.figshare.19137194

APC: allophycocyanin; CD: cluster of differentiation; EDTA: Ethylenediamine tetraacetic acid; FSC: forward scattering; PMT: photomultiplier tube; SSC: side scattering.

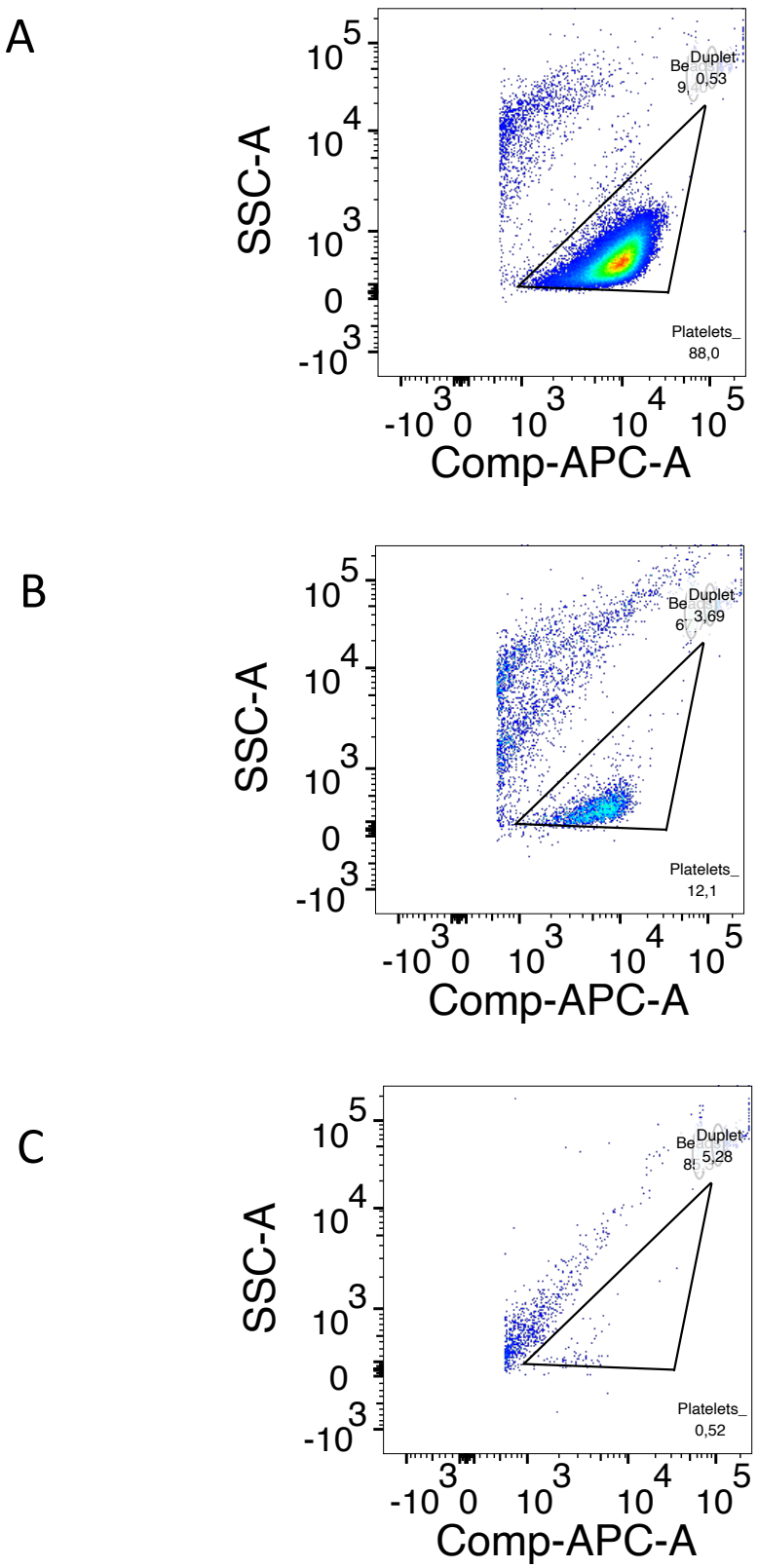

Figure S3.1 Gating strategy of platelets measured by the FACS Canto II. The plots show side scattering vs. APC fluorescence. (A) Platelet gate after the first centrifugation step. (B) Platelet gate after double centrifugation. (C) double centrifuged plasma after filtration.

Table S3.1: Overview of staining reagents. Characteristics being measured, analyte, analyte detector, reporter, isotype, clone, concentration, manufacturer, catalog number and lot number of used staining reagents. The antibody concentration during measurements was 11.3-fold lower than the antibody concentration during staining

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	YI-PL2	50	Invitrogen	17-0619-42	2062626

APC: allophycocyanin; IgG: Immunoglobulin G.