Supporting information 4: MIFlowCyt-EV checklist of Filtration experiments, "Removal of platelets from blood plasma to improve the quality of extracellular vesicle research"

1. Flow cytometry

1.1. Experimental design

The aim of the flow cytometry (A60-Micro, Apogee Flow Systems, Hemel Hempstead, UK) experiment was to compare the concentrations of extracellular vesicles (EVs) released from platelets (Cluster of differentiation (CD), CD61+) and erythrocytes (CD235a+) in double centrifuged human blood 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 without affecting the concentration of plasma EVs. Pre-analytical variables, such as blood collection and plasma preparation, are reported in the manuscript.

All 13 samples from one healthy volunteer were measured using an autosampler, which enables the measurement of samples at a 96-well plate. The 96-well plate contained a buffer-only control, antibody in buffer controls and isotype controls corresponding to the labels chosen for those experiments.

Scatter calibration and flow rate calibration were performed on the day of the experiment. Fluorescence calibration were performed three months before. To automatically process data, determine optimal samples dilutions, apply calibrations, determine and apply gates, generate reports with scatter plots and generate data summaries, custom-build software (MATLAB R2018b, Mathworks, Natick, MA, USA) was used.

1.2. Sample dilutions

The particle concentration in plasma differs between individuals, and therefore samples require different dilutions to avoid swarm detection (1) and to achieve statistically significant counts. Previously, a procedure to estimate to optimal sample dilution has been developed at our lab (2). In summary Buntsma et al. showed that for our flow cytometer and settings used,

a count rate ≤ $5.0 \cdot 10^3$ events per second unlikely results in swarm detection. To find the dilution resulting in a count rate ≤$5.0 \cdot 10^3$ events per second, we diluted the plasma sample 20-fold in Dulbecco's Phosphate-buffered saline (DPBS, 21-031-CV, Corning, Corning, NY) and measured the total concentration of particles for 120 seconds without staining. By pre-diluting the plasma samples 20-fold, followed by an additional 11-fold dilution post-staining, all samples had a count rate ≤$5.0 \cdot 10^3$ events per second.

1.3. EV staining

EVs in plasma were stained with antibodies. Prior to staining, antibodies were diluted in DPBS and centrifuged at 18,890 g for 5 min to remove aggregates. Table S1 shows an overview of the used reagents and antibody concentrations during staining. Each sample was double imuno-stained with CD61- allophycocyanin (APC) and CD235a- phycoerythrin (PE). To stain unfiltered plasma, 20 µL of 20-fold diluted plasma was incubated with 2.75 µL of antibodies or 2.5 µL of isotype control antibodies and kept in the dark for 2 h at room temperature. After staining, the unfiltered plasma was 11-fold diluted with DPBS. To stain filtered plasma, 100 µL of 20-fold diluted plasma was incubated with 13.75 µL of antibodies and kept in the dark for 2 h at room temperature. After staining, the filtered plasma was 10-fold diluted with DPBS.

1.4. Buffer-only control

The 96-well plate contained at least 1 well with DPBS, which was measured with the same flow cytometer and acquisition settings as all other samples. The mean count rate was 38 events per second, which is lower than the target count rate (2.5-5.0·10^3 events per second) for plasma samples.

1.5. Buffer with reagents control

The 96-wellplate contained a buffer with reagent control for each reagent (Table S1), which was measured with the same flow cytometer and acquisition settings as all samples. For CD61-APC and CD235a-PE the events per second were between 65 to 99, respectively, which is higher than in the buffer-only control (38 events per second). To investigate whether the relatively high background counts caused by CD61-APC and CD235a-PE affected the reported results, we applied the same calibrations and gates to CD61-APC and CD235a-PE in buffer as to the plasma samples stained with the corresponding antibody. On average, we obtained 3 CD61-APC+ events in buffer, which is acceptable compared to the 287 CD61-

APC+ EVs in plasma, and 27 CD235a-PE+ events in buffer, which is acceptable compared to the 897 CD235a-PE+ EVs in plasma.

1.6. Unstained controls

Unstained controls were measured at the same dilution and settings as the stained samples. Unstained controls were not used during data analysis.

1.7. Isotype controls

Table S1 shows an overview of the used isotype controls. For plasma control samples, we obtained 3 IgG1-APC+ events and 252 IgG1-PE+ events with a diameter ≤1,000 nm per measurement during 120 seconds. For comparison, on average 287 CD61-APC+, and 897 CD235a-PE+ events with a diameter ≤1,000 nm, were obtained in the experiments using plasma samples.

1.8. Trigger channel and threshold

Based on the buffer-only control (38 events per second), the acquisition software was set up to trigger at 14 arbitrary units SSC, which is equivalent to a side scattering cross section of 10 nm^2 (Rosetta Calibration, Rosetta Calibration, v1.13 Exometry, Amsterdam, The Netherlands).

1.9. Flow rate quantification

On the measurement day, we used 110 nm FITC beads with a specified concentration (Apogee calibration beads, Apogee Flow Systems, Hemel Hempstead, UK) to calibrate the flow rate of the A60-Micro. As the A60-Micro is equipped with a syringe pump with volumetric control, we assumed a flow rate of 3.01 µL/min for all measurements.

1.10. Fluorescence calibration

Calibration of the fluorescence detectors from arbitrary units (a.u.) to molecules of equivalent soluble fluorochrome (MESF) was accomplished using 2 µm Q-APC beads (2321-175, BD), and SPHERO PE Calibration Particle Kit, 3.0 -3.4 µm (ECFP-F2-5K, AK01, Spherotech Inc., Irma Lee Circle, IL, USA). Calibrations of the APC and PE detectors were performed on 2020-07-20. For each measurement, we added fluorescent intensities in MESF to the flow cytometry data files by custom-build software (MATLAB R2018a) using following equation:

$\mathrm{I(MESF)} = 10^{a \cdot \log_{10} \mathrm{I(a.u.)} + b}$	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 S4.2.

1.11. Light scatter calibration

We used Rosetta Calibration to relate scatter measured by forward scattering (FSC) and side scattering (SSC) to the effective scattering cross section and diameter of EVs. Figure S4.1 shows print screens of the scatter calibrations. We modelled EVs 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. For each measurement, we added the FSC and SSC cross sections and EV diameters to the flow cytometry datafiles by custom-build software (MATLAB R2018a). The SSC trigger threshold corresponds to a side scattering cross section of 10 nm^2.

1.12. MIFlowCyt checklist

The MIFlowCyt checklist is added to Table S4.3.

1.13. EV number concentration

The concentrations reported in the manuscript describe the number of particles (1) that exceeded the SSC threshold, corresponding to a side scattering cross section of 10 nm^2, (2) that were collected during time intervals, for which the count rate was within 50% of the median count rate, (3) with a diameter <1,000 nm as measured by SSC after light scatter calibration (section 1.11) and (4) are positive for APC, or PE, per mL of plasma.

1.14. Data sharing

Data is available via: https://doi.org/10.6084/m9.figshare.19137188

2. References

1. van der POL E, van GEMERT MJC, STURK A, NIEUWLAND R, van LEEUWEN TG. Single vs. swarm detection of microparticles and exosomes by flow cytometry. Journal of Thrombosis and Haemostasis TA - TT -. 2012;10(5):919–30.
2. MIFlowCyt-EV of "EDTA stabilizes the concentration of platelet-derived extracellular vesicles during blood collection and handling" 1 Flow cytometry.

Figures and tables

Figure S4.1.

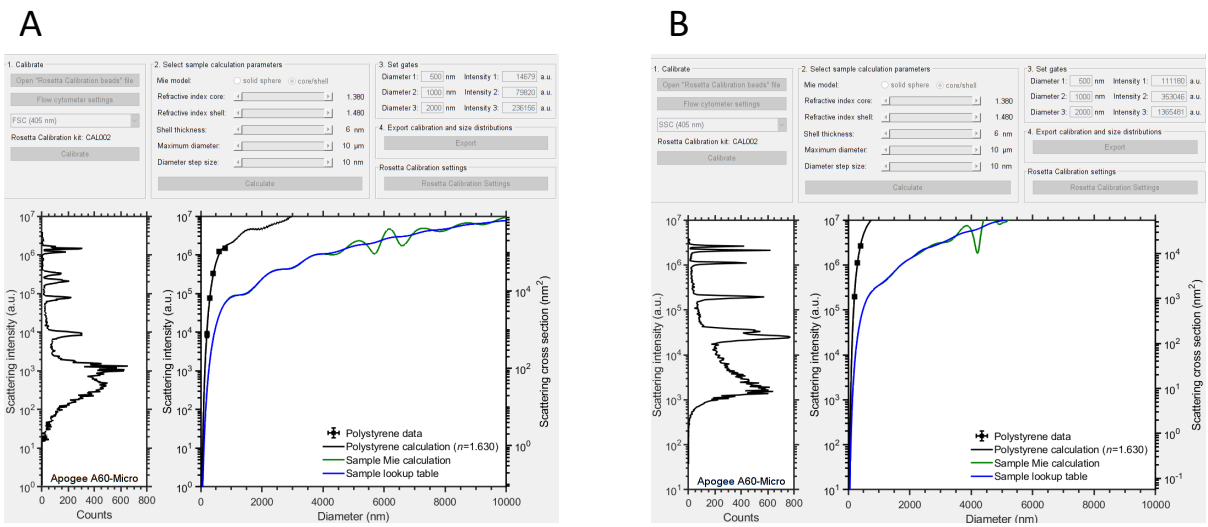

Figure S4.1 Forward scatter and side scatter calibration of the A60-Micro by Rosetta Calibration. To relate scatter to the diameter of EVs, we modelled EVs as core-shell particles with a core refractive index of 1.38, a shell refractive index of 1.48, and a shell thickness of 6 nm.

Table S4.1: 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
Glyco-protein	CD235a	Anti-human CD235a antibody	PE	IgG1	JC159	100	Dako	R7078	22079786
Affinity for Fc receptor	Fc receptor	IgG1	APC	n.a.	MPOC-21	200	Beckman Dickinson	554681	7075605
	Fc receptor	IgG1	PE	n.a.	DAK_G01	50	Beckman Dickinson	345816	9309643

Table S4.2: Overview of fluorescence calibrations.

	Calibration date	Slope	Intercept	R^2
APC	2020-07-20	1.2127	-2.0244	0.9942
PE	2020-07-20	1.0340	-1.6142	0.9993

APC: allophycocyanin; IgG: Immunoglobulin G; PE: phycoerythrin.

Table S4.3. MIFlowCyt checklist.

Requirement	Please Include Requested Information
1.1. Purpose	To compare the concentrations of extracellular vesicles (EVs) released from platelets (CD61+) and erythrocytes (CD235a+) in plasma before and after filtration with a polycarbonate membrane filter having a 0.8-µm pore diameter
1.2. Keywords	Extracellular vesicles, platelets, platelet-free-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	The concentration of CD61+ and CD235a+ EVs is not affected by double centrifugation combined with a polycarbonate membrane filter having a 0.8-µm pore diameter when compared with double centrifugation alone.
1.8. Quality control measures	All samples were measured using an autosampler, which facilitates subsequent measurements of samples in a 96-well plate. The well plate contained buffer-only controls (section S1.4), antibody in buffer controls (section S1.5), unstained controls (section S1.6) and isotype controls (section S1.7). The flow rate was calibrated with Apogee Calibration beads (Apogee Flow Systems, Hemel Hempstead, UK, section S1.9). Fluorescence detectors were calibrated (section S1.10) with 2 µm Q-APC beads (2321- 175, BD Biosciences, San Jose, CA) and SPHERO PE Calibration Particle Kit, 3.0 -3.4 µm (ECFP-F2-5K, AK01, Spherotech Inc.). FSC and SSC were calibrated with Rosetta Calibration (v1.13, section S1.11).
1.9 Other relevant experiment information	The entire experiment involved one 96-well plate that was measured within one day.
2.1.1.1. Sample description	Freshly prepared 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 in a 6-mLVacutainer® EDTA tube (367864, BD Biosciences) via antecubital vein puncture using a 21-gauge needle. The first 3.5 mL were discarded. Plasma was prepared by double centrifugation using a Rotina 380 R equipped with a swing-out rotor and a radius of 155 mm (Hettich Zentrifugen,

	Tuttlingen, Germany). The centrifugation parameters were: 2,500 g, 15 minutes, 20˚C, acceleration speed 9, deceleration 1. Whole blood was centrifuged, and one-time centrifuged plasma was collected 10 mm above the buffy coat. One-time centrifuged plasma was transferred into a 15-mL polypropylene centrifuge tube (Greiner Bio-One B.V., Alphen aan den Rijn, The Netherlands) and was centrifuged a second time at 2,500 g, 15 minutes, 20°C, acceleration speed 9, deceleration 1. Double centrifuged 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, a polycarbonate membrane filter having a 0.8-µm pore diameter (Isopore^{TM}, Merck Millipore, Darmstadt, Germany) with a 2.5-cm filter diameter was used. The concentration of platelets and EVs were measured in fresh plasma with and without applying the filters.
2.1.1.3. Biological sample source organism description	Healthy human volunteer.
2.2 Sample characteristics	Plasma is expected to contain detectable EVs, lipoproteins proteins, and platelets.
2.3. Sample treatment description	Plasma was either double centrifuged, or double centrifuged and filtered with a polycarbonate membrane filter having a 0.8-µm pore diameter (section 2.1.1.2).
2.4. Fluorescence reagent(s) description	Please see Table S1.
3.1. Instrument manufacturer	Apogee Flow Systems, Hemel Hempstead, UK
3.2. Instrument model	A60-Micro
3.3. Instrument configuration and settings	Samples were analysed for 120 seconds at a flow rate of 3.01 µL/min on an A60-Micro, equipped with a 405 nm laser (100 mW), 488 nm laser (100 mW) and 638 nm laser (75 mW). The trigger threshold was set at SSC 14 arbitrary units, corresponding to a side scattering cross section of 10 nm^2 (Rosetta Calibration). For FSC and SSC, the voltages were 380 V and 360 V, respectively. For all detectors, the peak height was analysed. APC signals were collected with the 638-D Red (Peak) detector (long pass 652 nm filter, PMT voltage 510 V). FITC signals were collected with the 488-Green (Peak) detector (525/50 nm band pass filter, PMT voltage 520 V). PE signals were collected with the 488-Orange (Peak) detector (575/30 nm band pass filter, PMT voltage 520 V).

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.19137188
4.2. Compensation description	No compensation was required because no fluorophore combinations were used that have overlapping emission spectra.
4.3. Data transformation details	Fluorescence detectors were calibrated (section S1.10) with 2 µm Q-APC beads (2321- 175, BD Biosciences) and SPHERO PE Calibration Particle Kit, 3.0 -3.4 µm (ECFP-F2-5K, AK01, Spherotech Inc.). FSC and SSC were calibrated with Rosetta Calibration (v1.13, section S1.11). The concentrations reported in the manuscript describe the number of particles that fulfil the gating criteria per mL.
4.4.1. Gate description	To automatically apply gates, generate pdf reports with scatter plots, and summarize the data in a table, custom-build software (MATLAB R2018b) was used. Please find below a description of the gates. First, only events that were collected during time intervals, for which the count rate was within 25% of the median count rate, were included. Second, platelets were excluded by applying a gate at the side scattering cross section (<2,000 nm^2) and, depending on the fluorescence label, at a fluorescence channel. Third, events with a diameter <1,000 nm as measured by SSC after light scatter calibration (section S1.11) were included. Fourth, events positive for either APC or PE were included. Fifth, fluorescence gates were automatically determined with a mathematical algorithm (MATLAB R2018b) and applied. Lower bounds of the fluorescent gates are 194 molecules of equivalent soluble fluorochrome (MESF) for CD61-APC and 230 MESF for CD235a-PE.
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 files https://doi.org/10.6084/m9.figshare.19137188

APC: allophycocyanin; CD: cluster of differentiation; EDTA: Ethylenediamine tetraacetic acid; FSC: forward scattering; MESF: Molecules of Equivalent Soluble Fluorochrome: PE: phycoerythrin; PMT: photomultiplier tube; SSC: side scattering.