MIFlowCyt-EV of "EDTA stabilizes the concentration of platelet-derived extracellular vesicles during blood collection and handling"

1 Flow cytometry

1.1 Experimental design

The aim of flow cytometry (A60-Micro, Apogee Flow Systems, Hemel Hempstead, UK) experiments was to measure the concentrations of extracellular vesicles (EVs) released from platelets ($CD61^+$), activated platelets ($CD61^+$/P-$selectin^+$), leukocytes ($CD45^+$) and erythrocytes ($CD235a^+$) in whole blood (WB) and platelet-depleted plasma (PDP) samples to compare between anticoagulated with citrate and ethylenadiaminetetraacetic (EDTA). EV concentrations are measured in different situations: i) both with and without platelet activation by thrombin receptor-activating peptide 6 (TRAP-6), ii) brought to the lab by manual or pneumatic tube transport, iii) before and after a freeze-thaw cycle and iv) after prolonged storage between blood collection and centrifugation either at room temperature or at 4 °C. We hypothesized that EDTA would be more effective in stabilizing platelet EV concentrations compared to citrate.

All samples were measured using an autosampler, which facilitates subsequent measurements of samples in a 96-well plate. For this study, samples were measured on sixteen different days between July 2019 and July 2021. Each day a buffer-only control was measured as well as antibody in buffer controls and isotype controls corresponding to the labels in the well plate. Flow rate and scatter calibrations were performed daily. Fluorescence calibration were performed twice during this time period. To automatically determine optimal sample dilutions, apply calibrations, determine and apply gates, generate reports with scatter plots and generate data summaries, we used custom-build software (MATLAB R2018b, Mathworks, Natick, MA, USA).

1.2 Sample dilutions

To avoid swarm detection in typical PDP samples, we measured serial dilutions of PDP pooled from 10 healthy male and 10 healthy female donors. Fig. S1A and S1B show the measured concentration and median side scattering signals versus dilution of pooled PDP, respectively. For measured concentrations $\leq 1.5 \cdot 10^8$ mL^{-1} and count rates $\leq 7.5 \cdot 10^3$ events per

second, the concentration decreases linearly with the dilution and the median side scattering signals are similar, confirming absence of swarm detection.

Based on the result in Fig. S1, and to be on the safe side, we aimed for count rates ≤ $5.0 \cdot 10^3$ events per second for all measurements. Measurements with count rates exceeding 10,000 events per second were excluded from analysis. Pre-staining, PDP samples were diluted 2 to 100-fold, followed by an additional 11.25-fold dilution post-staining, resulting in count rates between 1,513 and 7,872 events per second. WB samples were diluted 48-fold, resulting in count rates between 242 and 4,119 events per second. More research is required, however, to confirm that the count rate can be used as a benchmark to avoid swarm detection for a given sample type. To avoid potential obstruction of the flow cell, we further performed a fibrin generation test to confirm the absence of clots in 48-fold diluted WB samples.

1.3 EV staining

EVs 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 labelled with either CD61-APC and CD62p-PE (or CD62p-FITC) or CD45-APC and CD235a-PE. To stain, 20 µL of pre-diluted WB or PDP was incubated with 2.5 µL of antibodies or isotype controls and kept in the dark for 1 h at room temperature. Post-staining, samples were diluted 11.25-fold in 200 µL of DPBS to decrease background fluorescence from unbound reagents.

1.4 Buffer-only control

Each measurement day at least 1 well with DPBS was measured with the same flow cytometer and acquisition settings as the samples. The mean count rate was 34.0 events per second, which is substantially lower than the count rates obtained for WB and PDP samples (242 - $7.9 \cdot 10^3$ events per second).

1.5 Buffer with reagents control

Each 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 the samples. For all reagents but CD235a-PE, the mean count rate was between 31 and 62 events per second, which is in the same range as the buffer-only control. CD235a-PE in buffer resulted in a count rate of 228 events per second, which is substantially higher than the buffer-only control. To investigate whether the relatively high background counts caused by CD235a-PE affected the reported results, we applied the same calibrations and gates to CD235a-PE in

buffer as to the samples stained with CD235a-PE. On average per measurement, we obtained 99 CD235a-PE+ events in buffer, which is acceptable compared to the 506 CD235a-PE+ EVs in WB, on average, and an average of ≥ 647 CD235a-PE+ EVs in PDP experiments.

1.6 Unstained controls

Unstained controls were measured with the same flow cytometer and acquisition settings as the stained samples, resulting in 222 to 3,422 events per second for whole blood samples and 1,681 to 7,413 events per second for PDP.

1.7 Isotype controls

Table S1 shows an overview of the used isotype controls, which were added to a selection of samples. For four whole blood controls, this resulted in an average of 82 IgG1-APC+ events and 119 IgG1-PE+ events with a diameter ≤1,000 nm per measurement. For comparison, we obtained on average 798 CD61-APC+, 114 CD45-APC+, 506 CD235a-PE+ and 134 CD62p-PE+ events with a diameter ≤1,000 nm in whole blood per measurement. For 24 PDP control samples, we obtained an average of 140 IgG1-APC+ events, 34 IgG1-FITC+ and 207 IgG1-PE+ events with a diameter ≤1,000 nm per measurement. For comparison, on average 15,523 CD61-APC+, 355 CD45-APC+, 6,499 CD235a-PE+, 293 CD62p-PE+ and 248 CD62p-FITC+ events with a diameter ≤1,000 nm were obtained in the experiments using PDP samples.

1.8 Trigger channel and threshold

Based on the buffer-only control (34 events s^{-1}), the acquisition software was set up to trigger at 30 arbitrary units (a.u.) FSC or 14 a.u. units SSC, which is equivalent to an FSC cross section of 60 nm^2 and an SSC cross section of 10 nm^2 (Rosetta Calibration, v1.13, Exometry, Amsterdam, The Netherlands).

1.9 Flow rate quantification

Each measurement day, we used 110 nm FITC beads with a specified concentration (Apogee calibration beads, Apogee Flow Systems, Hemel Hempstead, UK) to check the flow rate of the A60-Micro. The measured flow rate per measurement day is presented in Fig. S2. The adjusted flow rate is 3.01 µL/min and the measured mean flow rate is 2.98 ± 0.18 uL/min (mean ± standard deviation). Because 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), SPHERO Easy Calibration Fluorescent Particles (AK01, Spherotech Inc., Irma Lee Circle, IL, USA) and QuantumTM MESF Kits (13734, Bangs Laboratories Inc., Fishers, IN, USA).

Calibrations of the APC and PE detectors were performed three times in the time period of these experiments, calibrations of the FITC detector was performed once. For each measurement, we added fluorescent intensities in MESF to the flow cytometry data files by custom-build software (MATLAB R2018a) using following equation:

$$\mathrm{I(MESF)} = 10^{a \cdot \log_{10} \mathrm{I(a.u.)} + b} \qquad \text{Equation S1}$$

where I is the fluorescence intensity, and *a* and *b* are the slope and the intercept of the linear fits, respectively, see table S2.

1.11 Light scatter calibration

We used Rosetta Calibration to relate scatter measured by FSC or SSC to the scattering cross section and diameter of EVs. Fig. S3 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 scattering cross sections and EV diameters to the flow cytometry datafiles by custom-build software (MATLAB R2018a).

1.12 MIFlowCyt checklist

The MIFlowCyt checklist is added to Table S3.

1.13 EV number concentration

The concentrations reported in the manuscript describe the number of particles (1) that exceeded the FSC or SSC threshold, corresponding to a forward scattering cross section of 60 nm^2 and 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, PE or FITC, per mL of WB or PFP.

1.14 Data sharing

Data is available via: https://doi.org/10.6084/m9.figshare.c.4753676

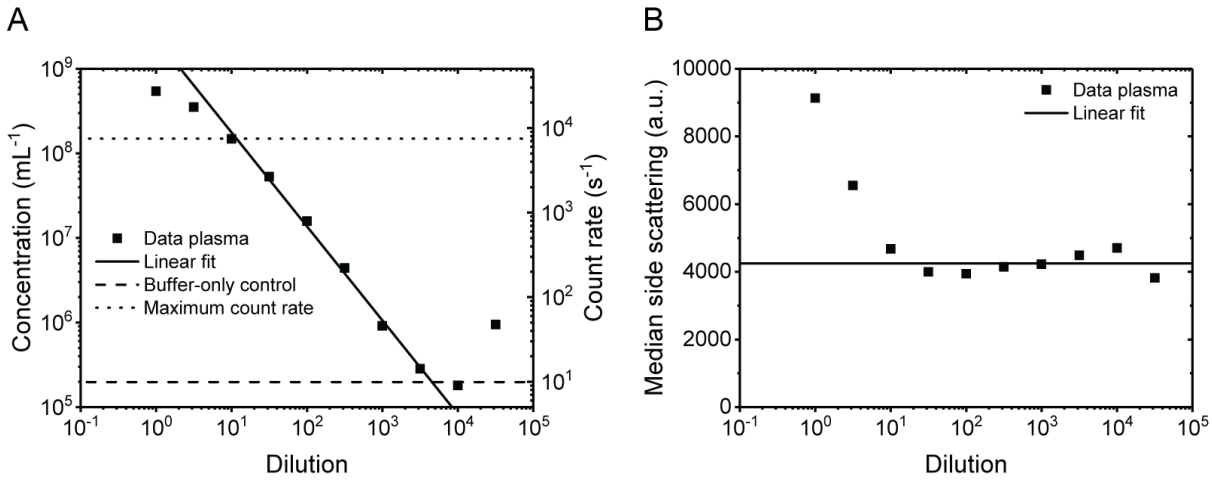

Figure S1. Estimation of the minimum dilution and maximum count rate to avoid swarm detection for particles in pooled platelet depleted plasma (PDP). (A) Particle concentration and count rate versus dilution measured (symbols) with the A60-Micro in pooled PDP and fitted with a linear function (solid line; slope -1.11, intercept 9.36, R^2=0.996) based on datapoints 3 to 8. As a reference, the concentration and count rate of the buffer-only control (dashed line) is shown. (B) Median side scattering versus dilution measured (symbols) with the A60-Micro in pooled PDP and fitted with a linear function (solid line) based on datapoints 3 to 10. For concentrations $\leq 1.5 \cdot 10^8$ mL^{-1} and count rates $\leq 7.5 \cdot 10^3$ events per second (dotted line in panel A), the measured concentration scales linearly with the reciprocal dilution and the median side scatter signals are similar.

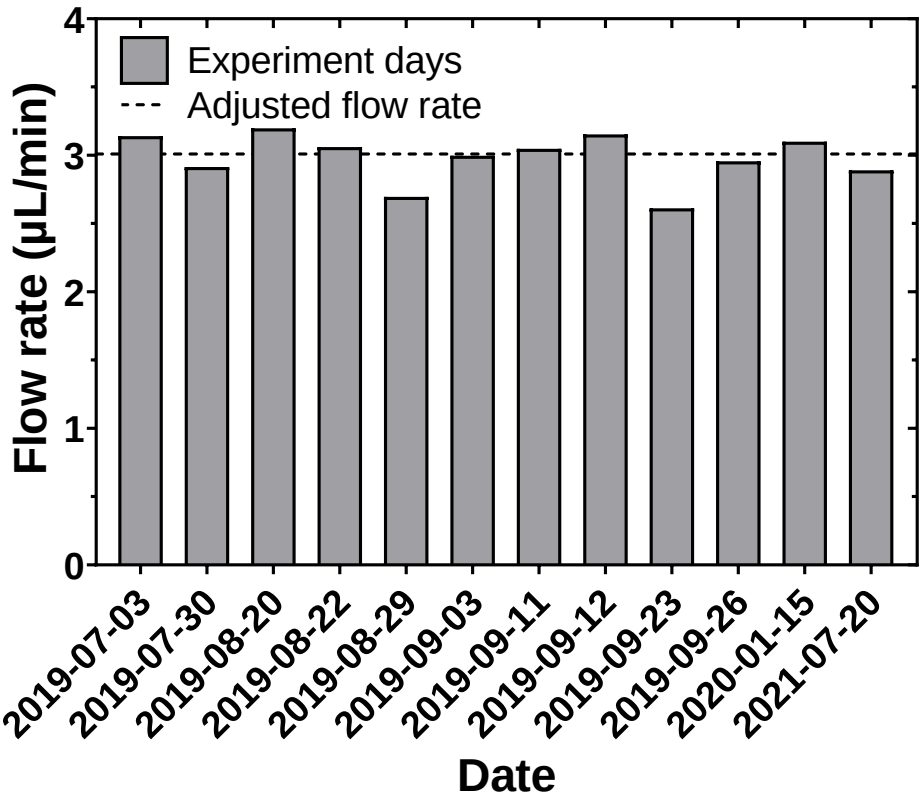

Figure S2. **Flow rate measured by A60-Micro versus date that experiments were performed.** The adjusted flow rate is 3.01 µL/min and the measured median flow rate is 3.02 µL/min. For all days the measured flow rate was within 13.2% of the adjusted flow rate.

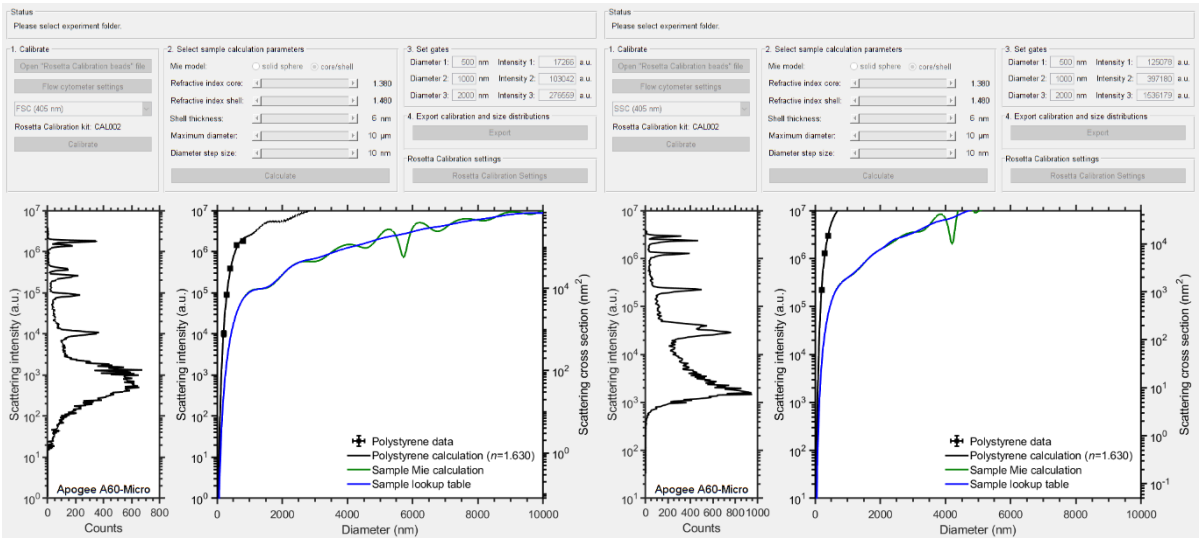

Figure S3. Scatter calibrations. (A) Forward scatter and (B) side scatter calibration of the A60-Micro by Rosetta Calibration. To relate scatter to the diameter of EVs, we modelled EVs as core-shell particles with a core refractive index of 1.38, a shell refractive index of 1.48, and a shell thickness of 6 nm.

Table S1: 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 11.25-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	VI-PL2	50	Invitrogen – by Thermo Fisher Scientific	17061942	2026494
									2062626
Adhesion molecule	Human CD62p	Anti-human CD62P antibody	PE	IgG1	CLBThrombo/6	6.25	Beckman Coulter	IM1759U	200049
									200050
									200051
			FITC	IgG1	AC1.2	50	Beckman Dickinson	550866	17
Leukocyte common antigen	CD45	Anti-human CD45 antibody	APC	IgG1	HI30	9.0	Biolegend	304037	B239297
									B272158
Glyco-protein	CD235a	Anti-human CD235a antibody	PE	IgG1	JC159	100	Dako	R7078	20056279
									20067598
									20079786
Affinity for Fc receptor	Fc receptor	IgG1	APC	n.a.	MOPC-21	200	Beckman Dickinson	554681	9059624
	Fc receptor	IgG1	PE	n.a.	X40	50	Beckman Dickinson	345816	7248665
									9035605
	Fc receptor	IgG1	FITC	n.a.	MOPC-21	50	Beckman Dickinson	555748	8287926

APC: allophycocyanin; CD: cluster of differentiation; FITC: fluorescein isothiocyanate; IgG: immunoglobulin G; PE: phycoerythrin.

Table S2: Overview of fluorescence calibrations.

	Calibration date	Slope	Intercept	R^2
APC	2019-08-14	1.2098	-2.0820	0.9948
	2019-12-11	1.2248	-2.1126	0.9934
	2021-07-15	1.1303	-1.8211	0.9977
PE	2019-07-22	1.0528	-1.7776	0.9995
	2019-12-11	1.0555	-1.7868	0.9997
	2021-07-15	1.0206	-1.5057	0.9998
FITC	2019-07-22	1.2369	-1.6590	0.9988

APC: allophycocyanin; FITC: fluorescein isothiocyanate; PE: phycoerythrin.

Table S3. MIFlowCyt checklist.

Requirement	**Please Include Requested Information**
1.1. Purpose	To compare the concentrations of EVs released from platelets (CD61+), activated platelets (P-selectin+), leukocytes (CD45+), and erythrocytes (CD235a+) between whole blood and platelet-depleted plasma samples anticoagulated with EDTA and anticoagulated with citrate.
1.2. Keywords	extracellular vesicles, platelets, anticoagulant
1.3. Experiment variables	EDTA, citrate TRAP-6, pneumatic tube transport, prolonged storage
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	Naomi C Buntsma, n.c.buntsma@amsterdamumc.nl
1.6. Date or time period of experiment	July 2019 – July 2021 Additional measurements for prolonged storage: July 2021
1.7. Conclusions	In WB anticoagulated with citrate, EV concentrations increased after platelet activation with TRAP-6. In WB anticoagulated with EDTA, EV concentrations remained stable after platelet activation with TRAP-6. Also after use of pneumatic tube transport and in PDP, EV concentrations were more stable in EDTA compared to citrate. EV concentrations remained stable in EDTA anticoagulated blood up to 6 hours of storage. No differences were observed in citrate- and EDTA-anticoagulated plasma after a freeze-thaw cycle.
1.8. Quality control measures	All samples were measured using an autosampler, which facilitates subsequent measurements of samples in a 96-well plate. Each 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 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) and SPHERO Easy Calibration Fluorescent Particles (AK01, Spherotech Inc., Irma Lee Circle, IL, USA). FSC and SSC were calibrated with Rosetta Calibration (v1.11, section S1.11).
1.9 Other relevant experiment information	Samples were measured on 11 different days, in a time period of seven months.
2.1.1.1. Sample description	TRAP-6: Whole blood samples (n=7). Pneumatic tube transport: Fresh platelet-depleted plasma samples (n=6). Freeze-thaw cycle: Fresh and thawed platelet-depleted plasma (n=10). Prolonged storage: Thawed platelet-depleted plasma (n=6). All samples were anticoagulated with citrate and with EDTA.
2.1.1.2. Biological sample source description	Blood samples were collected in plastic vacuum tubes (2.7 mL trisodium citrate; final concentration 0.109 mol/L, and 4.0 mL K2EDTA 7.2 mg, BD Vacutainer®, USA) via antecubital venepuncture using a 21-Gauge needle. The first 2 mL were discarded. Platelet-depleted 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, no brake. For freeze-storage, samples were transferred to 1.5 mL micro tubes (Sarstedt AG & Co., Germany), snap-frozen in liquid nitrogen and stored in -80˚C. Before staining, samples were thawed for 1 minute at 37 °C.
2.1.1.3. Biological sample source organism description	Healthy human volunteers.
2.2 Sample characteristics	1. Anticoagulated whole blood, which is expected to contain blood cells (erythrocytes, leukocytes and thrombocytes), EVs,

	lipoproteins and proteins. 2. Platelet-depleted plasma (PDP) is expected to contain EVs, lipoproteins and proteins.
2.3. Sample treatment description	TRAP-6: Whole blood was incubated at room temperature with or without the potent platelet activator TRAP-6 (Bachem, Switzerland; final concentration 30 µmol/L to activate platelets. Pneumatic tube transport: Blood tubes were transported to the lab either by manual (vertical position) or pneumatic tube transport. Freeze-thaw cycle: Samples of previous experiment were measured fresh and snap-frozen and stored at -80 ˚C. Samples were thawed in 1 minute at 37 ˚C for measurements after a freeze-thaw cycle. Prolonged storage: Blood tubes were kept at room temperature or in the fridge for a time period of 6, 24, 48 or 168 hours before centrifugation. Please see section S1.3 for staining procedure.
2.4. Fluorescence reagent(s) description	Please see Table S1.
3.1. Instrument manufacturer	Apogee, Hemel Hempstead, UK
3.2. Instrument model	A60-Micro
3.3. Instrument configuration and settings	Samples were analysed for 2 minutes 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; 150 mW for the four measurement days in 2021). The trigger threshold was set at FSC 30 a.u. or SSC 14 a.u., corresponding to an FSC cross section of 60 nm^2 and an SSC cross section of 10 nm^2 (Rosetta Calibration). In 2019 and 2020, for FSC and SSC, the PMT voltages were 380 V and 350 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). In the beginning of 2021 our A60-Micro was upgraded, after which we created settings mimicking the ones we used before the upgrade. For FSC and SSC, the PMT voltages were now 348 V and 350 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 423 V). FITC signals were collected with the 488-Green(Peak) detector (525/50 nm band pass filter, PMT voltage 476 V). PE signals were collected with the 488-Orange(Peak) detector (575/30 nm band pass filter, PMT voltage 459 V).
4.1. List-mode data files	Data is available via https://doi.org/10.6084/m9.figshare.c.4753676
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) and SPHERO Easy Calibration Fluorescent Particles (AK01, Spherotech Inc., Irma Lee Circle, IL, USA). 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, events that were collected during seconds for which the count rate was within 50% of the median count rate (in 2019/2020) or the count rate deviated less than 750 events/seconds from the median count rate (in 2021) were included. Second, events with a diameter <1,000 nm as measured by SSC after light scatter calibration (section S1.11) were included. Third, events positive for either APC, FITC or PE were included.

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	The lower boundaries of the fluorescent gates were automatically determined (MATLAB R2018b). For whole blood samples boundaries were 143 MESF for CD45-APC, 143 MESF for CD61-APC, 103 MESF for CD62p-PE and 133 MESF for CD235a-PE. For platelet-depleted plasma samples they were 149 MESF for CD45-APC, 150 MESF for CD61-APC, 370 MESF for CD62p-FITC, 105 MESF for CD62p-PE and 155 MESF for CD235a-PE.

a.u.: arbitrary units; EDTA: ethylenediaminetetraacetic acid; EVs: extracellular vesicles; FSC: forward scattering; PDP: platelet depleted plasma; SSC: side scattering; TRAP-6: thrombin receptor-activating peptide 6; WB: whole blood.