Supporting information 2: Material and Methods for Figure 2, *"Standardization of extracellular vesicles concentration measurements by flow cytometry: the past, present and future"*

1.1. Experimental design

The aim of the flow cytometry (FACSCanto™ (BD Biosciences)) experiment was to repeat an experiment performed by Nieuwland et al. in which three populations of particles exposing platelet-specific proteins were identified in diluted whole blood based on light scattering signals, of which one are platelet-derived EVs [1]. To repeat the experiment from Nieuwland et al. whole blood from one healthy volunteer was measured manually with a FACSCanto™. An FACSCanto™ instrument optimized for detection of cells was chosen for this experiment.

1.2. Blood collection

The collection of blood was performed according to the guidelines of the medical ethical committee of Amsterdam University Medical Centre, University of Amsterdam (W18_391#18.450). Blood was collected from one healthy individual who denied having a disease or using drugs and/or medication. Venous blood was collected using an 18-Gauge needle, and the first 3.5 mL of blood was discarded. Two tubes of Sodium Citrate blood (S-Monovette, 05.1071, Sarstedt) were collected, mixed gently, and processed within 15 minutes. One tube was used for the whole blood measurements, while the other one was used for activated platelet measurements.

1.3. Whole blood measurements by flow cytometry

Blood cells can be identified based on their light scattering intensity, when measured on a flow cytometer. Therefore, whole blood was pre-diluted 11-fold in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (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 NaH2PO4.H2O (6345, Merck Millipore), 2.7 mmol/L fc, KCl (24936, Merck Millipore), and 1

mmol/L fc MgCl2.6H2O (5833, Merck Millipore) in MiliQ (Baxter, TKF7114, Deerfield, IL). Whole blood samples were 45-fold post-diluted and fixated with 200 µL HEPES/0.3 % paraformaldehyde (PFA; 104005, Merck Millipore). To allow fixation, the HEPES/0.3 % PFA diluted samples were incubated for 1 hour in the dark at room temperature (RT). Afterward, the samples were measured with a FACSCanto™ II.

1.4. Staining platelets in whole blood for flow cytometry

To confirm that the identified populations are indeed platelets and/or platelet EVs whole blood was incubated with a platelet marker (CD61- Allophycocyanin; (APC)). Before staining, antibody aggregates were removed by centrifugation at 18,890 g for 5 minutes, at 20°C. The supernatant minus 10 µL of the starting volume was collected and used for staining. Whole blood was pre-diluted with 10-fold with HEPES buffer and stained with 5 µL anti-human CD61-APC antibody or mouse IgG1 isotype control at matching concentration and incubated for 30 minutes in the dark at RT. Afterward, samples were 45-fold post-diluted and fixated with 2.5 mL HEPES/0.3 % PFA. To allow fixation, the HEPES/0.3 % PFA diluted samples were incubated for 1 hour in the dark at RT. Afterward, the samples were measured with a FACSCanto™ II.

1.5. Staining platelets in washed platelet samples for flow cytometry

One of the collected Sodium Citrate anti-coagulated blood tubes was used to create a washed platelet sample. After 10 minutes of resting the blood tube was centrifuged for at 180 g for 15 minutes, at 20°C. Platelet rich plasma (PRP) was pipetted into a new tube and Acid-Citrate-Dextrose buffer (ACD; 2.5 g tri-sodium citrate, 2H2O (0.085 mol/L), 2.0 g Glucose (0.11 mol/L), 1.5 g citric acid, H2O (0.071 mol/L) in MiliQ, pH 4.4) at a ratio of 5:1 was added to the tube. The tube was centrifuged again at 800 g for 20 minutes, at 20°C. The supernatant was discarded and the pellet was first resuspended gently in 1 mL of buffer A (NaCl 1.6 g, NaHCO3 0.2 g, Glucose 0.2 g, MgCl2.6H2O 800 µL from the 250 mmol/L, KCL 835 µL from the 625 mmol/L stock in MiliQ, containing 100 mmol/L Ethylenediaminetetraacetic acid (EDTA)). Another 8 mL of buffer A was added and the tube was centrifuged again at 800 g for 20 minutes, at 20°C. The pellet was resuspended in buffer B (NaCl 1.6 g, NaHCO3 0.2 g, Glucose 0.2 g, MgCl2.6H2O 800 µL from the 250 mmol/L, KCL 835 µL from the 625 mmol/L stock in MiliQ, pH 7.35). The washed platelet sample was stained with anti-human CD61-

APC antibody or mouse IgG1 isotype as described previously. Afterward, samples were 18-fold post-diluted and fixated with HEPES/0.3 % PFA. To allow fixation, the HEPES/0.3 % PFA diluted samples were incubated for 1 hour in the dark at RT. Afterward, the samples were measured with a FACSCanto™ II.

1.6. Staining platelets in activated platelet samples for flow cytometry

To show that activated platelets secrete EVs that are found in R1 platelet-rich plasma samples (section 1.5), were incubated with calcium ionophore A23187 (Sigma-Aldrich, C7522, 2.5 µmol/ L final concentration) for 5 minutes at 37 °C. Activated platelets were stained (section 1.5) and measured with a FACSCanto™ II.

1.7. Flow cytometry

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 12 µL/min on an FACSCanto™ II, equipped with a 405-nm laser, 488-nm laser, and 633-nm laser. The trigger threshold was set at forward scattering (FSC) 1000 arbitrary units OR APC 200. For FSC and side scattering (SSC), the PMT voltages were 300 V and 420 V, respectively. APC signals were collected with the 638-D Red (Peak) detector (long pass 660/20 nm filter, PMT voltage 532 V). Besides the blood samples, a buffer-only control, and isotype controls corresponding to the labels chosen for those experiments were measured.

1.8. Software and statistics

Data was processes using FlowJo (v 10.7.1, FlowJo).

1.9. Data sharing

Data is available via: https://doi.org/10.6084/m9.figshare.21724244.v1; https://doi.org/10.6084/m9.figshare.21724139; https://doi.org/10.6084/m9.figshare.21724106

2. References

1 Nieuwland R, Berckmans RJ, Rotteveel-Eijkman RC, Maquelin KN, Roozendaal KJ, Jansen PGM, Have K ten, Eijsman L, Hack CE, Sturk A. Cell-Derived Microparticles Generated in Patients During Cardiopulmonary Bypass Are Highly Procoagulant. *Circulation* American Heart Association; 1997; **96**: 3534–41.

Table S2.1: 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	8.33	Invitrogen	17-0619-42	2062626
Affinity for Fc receptor	Fc receptor	IgG_1	APC	n.a.	MPOC-21	8.33	BC Bioscience	554681	7075605

APC: allophycocyanin; CD: cluster of differentiation; IgG: Immunoglobulin G