Supporting information 3: Material and Methods for Figure 3, *"Standardization of extracellular vesicles concentration measurements by flow cytometry: the past, present and future"*

1.1. Experimental design

The aim of the flow cytometry (Northern Lights, Cytek Biosciences) experiment was to repeat an experiment performed by Robert et al. in which Megamix beads were used to set size gates for EVs [1]. A Northern Lights instrument optimized for detection of EVs was chosen for this experiment.

1.2. Blood collection and preparation of blood plasma

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 21-Gauge needle, and the first 3.5 mL of blood was discarded. One tube of Sodium Citrate blood (363046, BD Biosciences) was collected, mixed gently, and processed within 15 minutes. Whole blood was centrifuged for 15 minutes at 1,500 g and 20°C, acceleration speed 9, without a brake using a Rotina 380 R equipped with a swing-out rotor and radius of 155 mm (Hettich Zentrifugen, Tuttlingen, Germany). Supernatant was collected and centrifuged for 2 minutes at 13,000 g and 20°C, acceleration speed 9, without brake. Plasma was collected afterwards.

1.3. Staining platelets in whole blood for flow cytometry

The plasma sample was 10-fold pre-diluted in Dulbecco's Buffered Saline (dPBS; 14190-144, Gibco). Platelet EVs present in plasma were immuno-fluorescently stained with CD61-Fluorescein isothiocyanate (FITC). Before staining, aggregates in the antibody 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. EVs were stained with anti-human CD61-FITC or mouse IgG1-FITC. Table S3 contains an overview of the staining reagents. 20

µL pre-diluted plasma were incubated for 2 hours at room temperature in the dark, with 2.5 µL of the antibody or isotype control. Afterward, 200 µL dPBS were added and the samples were measured by flow cytometry.

1.4. Flow cytometry

Stained samples were measured for 300 seconds at a flow rate of 15µL/min on a Northern Light equipped with a 405-nm laser, 488-nm laser, and 640-nm laser. Instruments settings were determined based on the instructions in the publication of Robert et al. [1] and the Megamix bead instructions (7801, BioCytex), which contain 0.5, 0.9, and 3 µm.
The trigger threshold was set at forward scattering (FSC) 2000 arbitrary units. For FSC and side scattering (SSC), the gain was set at 2, and the FITC gain at 20. Besides the blood samples, a buffer-only control, and isotype controls corresponding to the labels chosen for those experiments were measured.

1.5. Software and statistics

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

1.6. Data sharing

Data is available via: https://doi.org/10.6084/m9.figshare.21724283.v3

2. References

1 ROBERT S, PONCELET P, LACROIX R, ARNAUD L, GIRAUDO L, HAUCHARD A, SAMPOL J, DIGNAT-GEORGE F. Standardization of platelet-derived microparticle counting using calibrated beads and a Cytomics FC500 routine flow cytometer: a first step towards multicenter studies? *Journal of Thrombosis and Haemostasis* John Wiley & Sons, Ltd; 2009; **7**: 190–7.

Table S3: 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	FITC	IgG1	Y2/51	8.33	Dako	F0803	20027302
Affinity for Fc receptor	Fc receptor	IgG_1	FITC	n.a.	X40	8.33	BC Bioscience	345815	0148811

CD: cluster of differentiation; FITC: Fluorescein isothiocyanate; IgG: Immunoglobulin G