Supporting information 1: Material and Methods for Figure 1, *"Standardization of extracellular vesicles concentration measurements by flow cytometry: the past, present and future"*

1.1. Experimental design

The aim of the flow cytometry (Apogee A60-micro, Apogee Flow Systems) experiment was to illustrate challenges that complicate extracellular vesicle (EV) concentration measurements, such as i) the size distribution of EVs, ii) dim fluorescence signals of EVs, iii) the light scattering and iv) fluorescence distribution of EVs. For this figure one sample of pool plasma was labelled with lactadherin-Fluorescein isothiocyanate (FITC) to identify EVs with a flow cytometer (FCM). An Apogee A60-micro instrument optimized for detection of EVs was chosen for this experiment.

1.2. Blood collection and preparation of blood plasma

Blood was collected from 8 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 Ethylenediaminetetraacetic acid blood (9203871, 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) 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.) 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 tube and pooled.

1.3. Staining platelets in whole blood for flow cytometry

The plasma sample was 12-fold pre-diluted in Dulbecco's Buffered Saline (dPBS; 14190-144, Gibco). EVs present in plasma were fluorescently stained with Lactadherin-Fluorescein

isothiocyanate (FITC). Before staining, aggregates in the lactadherin reagent 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. Table S1 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 lactadherin reagent. Afterward, 200 µL dPBS were added and the sample was measured by flow cytometry.

1.4. Flow cytometry

Stained samples were measured for 120 seconds at a flow rate of 3.01 µL/min on a Apogge 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 side scattering (SSC) 14 arbitrary units, corresponding to a side scattering cross section of 10 nm^2 (Rosetta Calibration). For forward scattering (FSC) and SSC, the voltages were 470 V and 375 V, respectively. For all detectors, the peak height was analysed. FITC signals were collected with the 488-Green (Peak) detector (525/50 nm band pass filter, PMT voltage 560 V).

1.5. Software and statistics

Data was processes using OriginPro 2017 (OriginLab Corporation). Data was fitted with a power-law function at Concentration = 10^(4+986.05743/(sqrt(2*pi)*1.11431*(Diameter-30))*exp(-ln((Diameter 30)/229)^2/(2*1.11431^2))). Adjusted R^2 = 0.8976. The parabolic fit for fluorescence was fluorescence = 0.42226*diameter^2. Adjusted R^2 = 0.58045

1.6. Data sharing

Data is available via: https://doi.org/10.6084/m9.figshare.21749495.v1.

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.

Characteristic measured	**Analyte**	**Reporter**	**Isotype**	**Clone**	**Concentration during staining (µg mL^{-1})**	**Manufacturer**	**Catalog number**	**Lot number**
Glycoprotein	Human Lactadherin	FITC	n.a.	n.a.	41.5	Haematologic technologies	BLAC-FITC	#KK0804

FITC: Fluorescein isothiocyanate;