Removing blood platelets from human plasma to reveal extracellular vesicle-associated miRNA profiles

Supplementary File 1. Materials and Methods

Blood collection and plasma preparation

Blood collection was in accordance with the guidelines of the Medical Ethical Committee of the Amsterdam Medical Centre, University of Amsterdam (W19_271#19.421). Blood was collected from healthy individuals (n=3), of which 1 donor was overnight fasting and the other 2 donors were fasting for 2-3 hours. The donors denied having a disease or to use drugs and/or medication. Venous blood was collected using a 21-Gauge needle, and the first 2 mL of blood was discarded. Four tubes of Ethylene Diamine Tetra Acetic acid (EDTA) blood (6 mL; BD Biosciences) were collected from each donor, and the blood was processed within 15 minutes after withdrawal. Plasma was prepared by two centrifugation protocols, see **Figure 1**, using a Rotina 380 R centrifuge (Hettich, Tuttlingen, Germany).

In the first centrifugation protocol (ISTH protocol), plasma was prepared as recommended by the International Society on Thrombosis and Haemostasis (ISTH)[1]. Whole blood was centrifuged for 15 minutes at 2,500 x g and 20 °C, with the centrifuge acceleration set at 9, and deceleration set at 1. Single centrifuged plasma was collected to exactly 10 mm (using a lego brick) above the buffy coat, and was then transferred to a 15 mL polypropylene tube (Greiner Bio-One B.V., Alphen aan den Rijn, The Netherlands). The plasma was then centrifuged for 15 minutes at 2,500 x g and 20 °C, with the centrifuge acceleration set at 9, and deceleration set at 1. Double centrifuged plasma was collected to exactly 10 mm above the cell pellet.

In the second centrifugation protocol (non-ISTH protocol)[2], whole blood was centrifuged for 7 minutes at 900 x g and 20 °C, with the centrifuge acceleration and deceleration set at 9. Single centrifuged plasma was collected to approximately 10 mm (by eye) above the buffy coat, and

transferred to a 15 mL polypropylene tube (Greiner Bio-One B.V., Alphen aan den Rijn, The Netherlands). The obtained plasma was then centrifuged for 10 minutes at 2,500 x g and 20 °C, with the centrifuge acceleration and deceleration set at 9. Double centrifuged plasma was collected to approximately 10 mm (by eye) above the cell pellet.

We deliberately included two centrifugation protocols to prepare plasma, to emphasize that the presence of platelets and ery-ghosts is a common phenomenon that requires more attention and investigation.

Size-exclusion chromatography

Sepharose CL-2B (GE Healthcare, Uppsala, Sweden) was washed with Dulbecco's Phosphate-Buffered Saline (DPBS; Gibco, Waltham, MA). Subsequently, the double 20 µm polyethylene frit on the bottom of a 15 mL Kinesis TELOS Chromatography Filtration Column was degassed (Cole-Parmer, St Neots, UK), and the column was stacked with 10 mL washed sepharose CL-2B. Then, a separate 20 µm Kinesis TELOS polyethylene frit was degassed (Cole-Parmer), and the frit was placed on top of the stacked sepharose. Columns were washed with DPBS containing 0.05% sodium azide and stored at 4 °C. Prior to use, columns were washed with DPBS to remove sodium azide. One mL of plasma was loaded onto a SEC2B column and 0.5 mL fractions (F) were collected after sample loading. DPBS was used as elution buffer. The EV-enriched fractions (F8-10) were pooled and used for further analyses.

Flow cytometry measurement of platelets and erythrocyte ghosts

The concentration of platelets (anti-human CD61-allophycocyanin [CD61-APC, >6,000 MESF] and anti-human CD41-phycoerythrin [CD41-PE, >3,200 MESF]), and activated platelets (anti-human CD61-APC, >6,000 MESF and anti-human CD62p-PE, > 2,800 MESF) with a diameter between 500 nm and 6,000 nm (Rosetta Calibration v1.28, Exometry, The Netherlands) were measured on a

calibrated FACSCanto II flow cytometer (BD Biosciences, San Jose, CA). Ery-ghosts (anti-human CD235a-fluorescein isothiocyanate [CD235a-FITC, >287,000 MESF]) with a diameter between 6,000 nm and 10,000 nm (Rosetta Calibration v1.28) were also measured on a calibrated FACSCanto II flow cytometer (BD Biosciences). Flow cytometry experiments were reported in accordance with MIFlowCyt[3]. Details can be found in the MIFlowCyt document added to the supplements.

Flow cytometry measurement of extracellular vesicles

The concentration of platelet- (anti-human CD61-FITC, > 99 MESF), erythrocyte- (anti-human CD235a-FITC, >140 MESF), and leukocyte-derived (anti-human CD45-APC, >43 MESF) EV subpopulations with a diameter between 150 nm and 1,000 nm (Rosetta Calibration v1.28) were measured on a calibrated A60-Micro flow cytometer (Apogee flow systems, Hemel Hempstead, UK). Flow cytometry experiments were reported in accordance with MIFlowCyt-EV[4]. Details can be found in the MIFlowCyt-EV document added to the supplements.

Transmission electron microscopy

A 200-mesh formvar and carbon coated copper grid (Electron Microscopy Science) was placed on a 10 µL droplet of EV-enriched SEC fractions to allow adherence of particles to the grid (7 minutes, room temperature). Thereafter, the grid was transferred onto drops of 1.75% uranyl acetate (w/v) for negative staining. Each grid was studied using a single TEM instrument (Fei, Tecnai-12; Eindhoven, the Netherlands) at 80 kV. Illumination was performed with e-beam spot size 2 and filament 30. Images result from the average of two 700 ms acquisitions on a Veleta 2,048 × 2,048 side-mounted CCD camera and Imaging Solutions software (Olympus, Tokyo, Japan).

Removal of platelets and erythrocyte ghosts

For platelet and ery-ghost removal from samples, polycarbonate track-etched membrane filters were used with a pore diameter of 0.8 µm and a filter diameter of 25 mm (Isopore™, Merck Millipore, Darmstadt, Germany), as reported previously[8].

miRNA isolation and qRT-PCR analysis

Total RNA was isolated from 200 µL plasma or EV-enriched SEC2B fractions using the miRNeasy serum/plasma kit (QIAgen, Hilden, Germany) according to the protocol of the manufacturers. RNA was eluted in 14 µl nuclease free water, and reverse transcribed using the TaqMan® MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA) in a multiplex reaction containing Reverse Transcription primers for hsa-let7a-5p (Thermo Fisher Scientific; Assay ID 000377), hsa-miR-21-5p (Thermo Fisher Scientific; Assay ID 000397) and hsa-miR-223-3p (Thermo Fisher Scientific; Assay ID 002295). After cDNA synthesis, nuclease free water was added up to a final volume of 50 µl. 3 µl of cDNA was subjected to 40 cycles of 95°C for 15 seconds and 60°C for 1 minute on an ABI 7500 Fast Real-Time PCR System (Thermo Fisher Scientific). All samples were measured in duplicate and data was analyzed using 7500 Software v2.0.6. (Thermo Fisher Scientific).

Data analysis and statistics

Flow cytometry data were processed using FlowJo (v 10.8.1, FlowJo, Ashland, OR) and MATLAB software (R2018b, Mathworks, Natick, MA). Statistical analyses were performed using Prism 9.0 (GraphPad, La Jolla, CA). We applied a Student's t-test to test for statistical differences in platelet-, ery-ghost- and EV concentrations, and to test for statistical differences in miRNA quantities pre- and post-filtration. Least square linear regression was used to study the relationship between the quantity of miRNAs and the concentration of platelets, ery-ghosts and EVs. A p-value < 0.05 was considered significant.

References

1. Lacroix R, Judicone C, Poncelet P, et al. Impact of pre-analytical parameters on the measurement of circulating microparticles: towards standardization of protocol. *J Thromb Haemost.* 2012;10(3):437-446.
2. van Eijndhoven MA, Zijlstra JM, Groenewegen NJ, et al. Plasma vesicle miRNAs for therapy response monitoring in Hodgkin lymphoma patients. *JCI Insight.* 2016;1(19):e89631.
3. Lee JA, Spidlen J, Boyce K, et al. MIFlowCyt: the minimum information about a Flow Cytometry Experiment. *Cytometry A.* 2008;73(10):926-930.
4. Welsh JA, Van Der Pol E, Arkesteijn GJA, et al. MIFlowCyt-EV: a framework for standardized reporting of extracellular vesicle flow cytometry experiments. *J Extracell Vesicles.* 2020;9(1):1713526.