Supplementary figures of: "Platelet removal from human blood plasma improves detection of extracellular vesicle-associated miRNA"

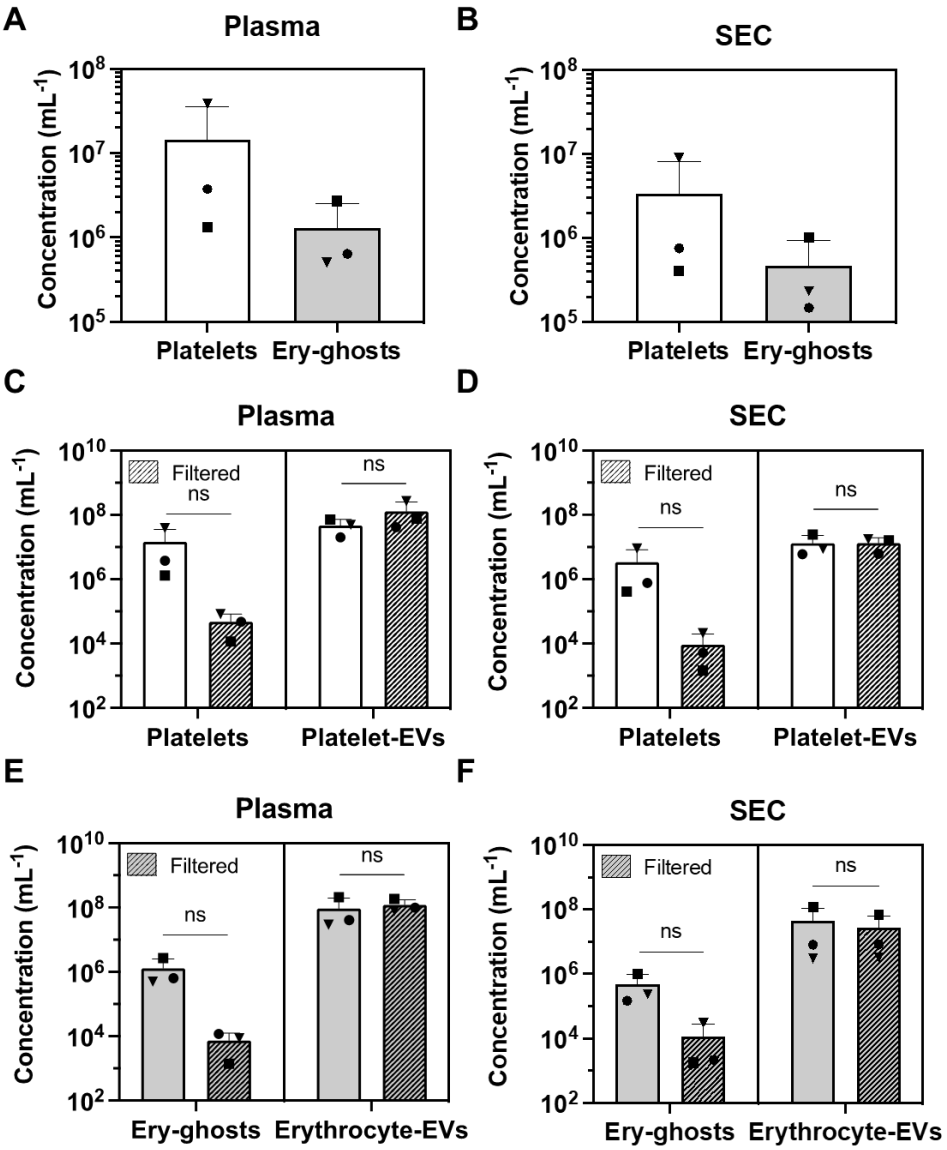
Jillian W.P. Bracht[1,2,3*], Mandy Los[1], Monique A.J. van Eijndhoven[2,4], Britta Bettin[1,5], Edwin van der Pol[1,2,3,5], D. Michiel Pegtel[2,4], Rienk Nieuwland[1,2,3]

1. Amsterdam UMC location University of Amsterdam, Vesicle Observation Centre, Laboratory of Experimental Clinical Chemistry, Department of Clinical Chemistry, Meibergdreef 9, Amsterdam, The Netherlands;
2. Cancer Centre Amsterdam, Imaging and Biomarkers, Amsterdam, The Netherlands;
3. Amsterdam Cardiovascular Sciences, Atherosclerosis and Ischemic Syndromes, Amsterdam, the Netherlands.
4. Amsterdam UMC location Vrije Universiteit Amsterdam, Department of Pathology, Boelelaan 1117, Amsterdam, The Netherlands;
5. Amsterdam UMC location University of Amsterdam, Department of Biomedical Engineering and Physics, Meibergdreef 9, Amsterdam, The Netherlands.

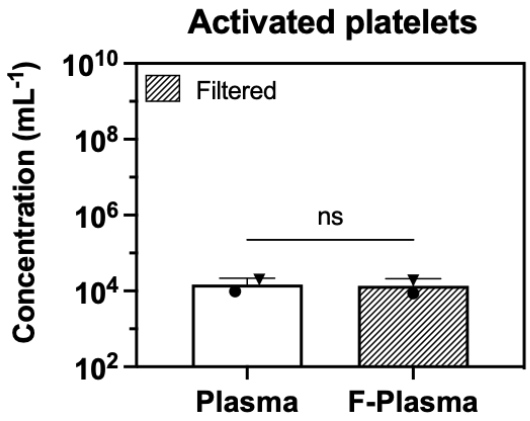
*** Corresponding author**

Jillian W.P. Bracht
Laboratory of Experimental Clinical Chemistry
Amsterdam University Medical Centers, Location University of Amsterdam
Meibergdreef 9, Room: B1-238
1105 AZ, Amsterdam,
The Netherlands

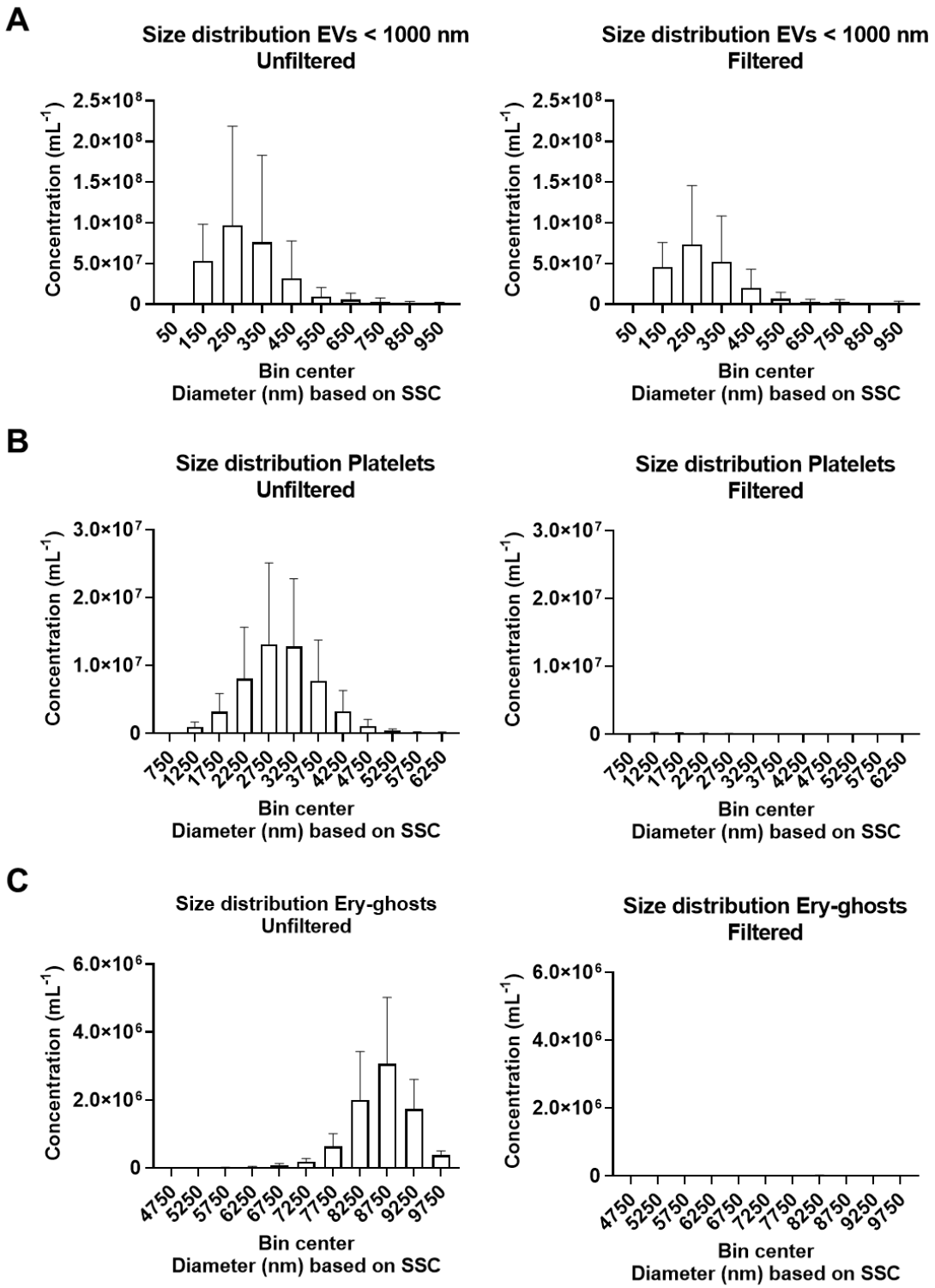
Phone: +31 (6) 53921071
E-mail: j.w.p.bracht@amsterdamumc.nl
ORCID: 0000-0001-9552-3960



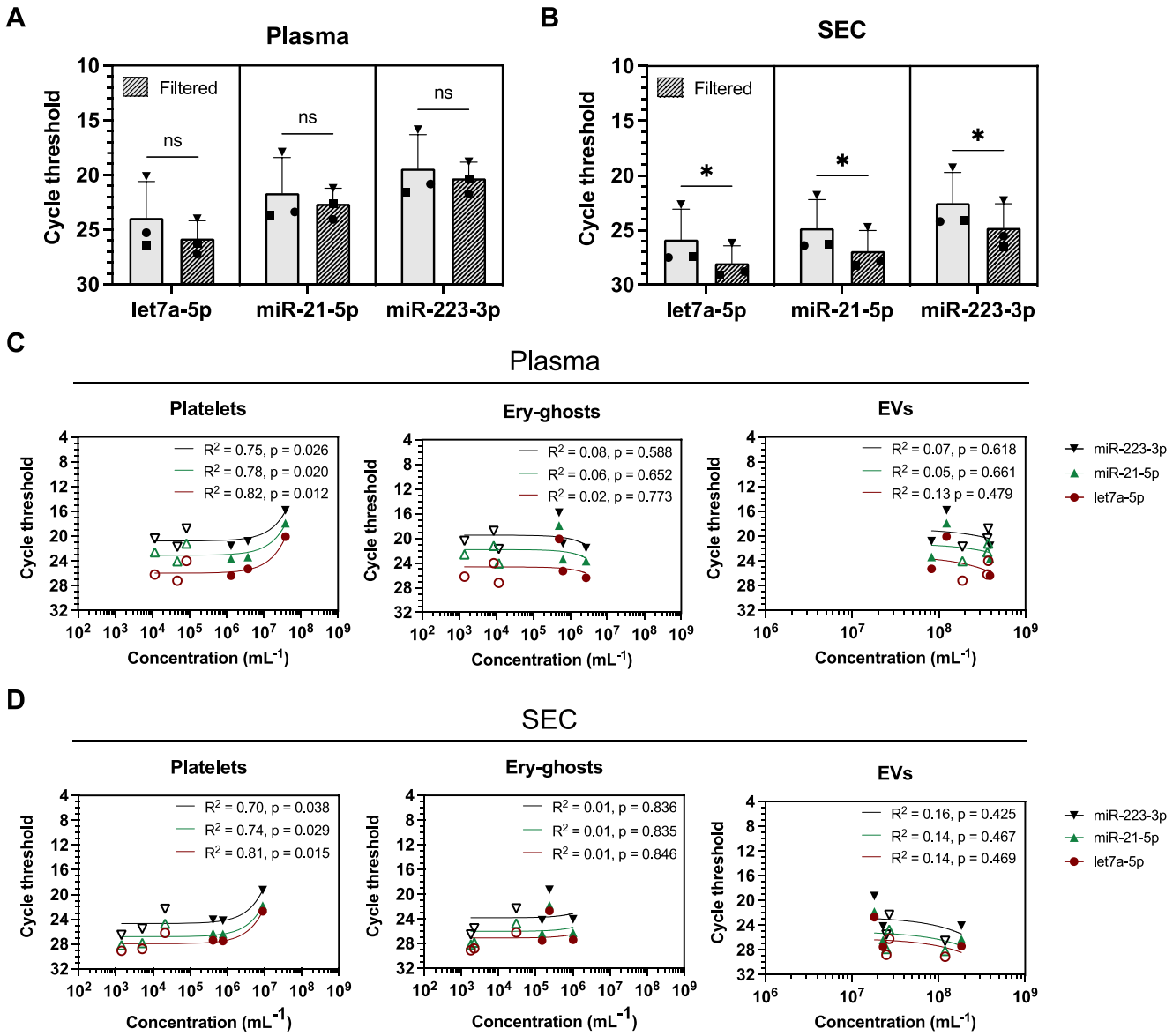
Supplementary Figure 1. *Plasma, prepared using the non-ISTH centrifugation protocol, still contains platelets (CD61+, CD41+) and ery-ghosts (CD235a+) as measured by flow cytometry **(A)**. Platelets and ery-ghosts are larger than the cut-off of Sepharose CL-2B, and thus co-migrate with EVs during SEC2B **(B)**. Filtration (0.8 µm polycarbonate filters) of plasma **(C)**, or corresponding EV-enriched SEC2B fractions 8-10 **(D)**, reduces the platelet concentration > 97%, without affecting the concentration of platelet-derived EVs (CD61+)*. Filtration also reduces the ery-ghost concentration > 97% **(E)**, while the concentration of erythrocyte-derived EVs (CD235a+)* remains unaffected **(F)**. Experiments were performed using plasma obtained from 3 healthy controls. A one-tailed, paired Student's t-test was used to test for statistical differences in platelet-, ery-ghost and EV concentrations, pre- and post-filtration (panel C-F). A p-value ≤ 0.05 was considered significant. Ery-ghost: erythrocyte ghost; EV: extracellular vesicle; ns: not significant; SEC2B: size-exclusion chromatography CL-2B; *: EVs were measured using flow cytometry (Apogee A60) with a size detection range of 150-1,000 nm).*



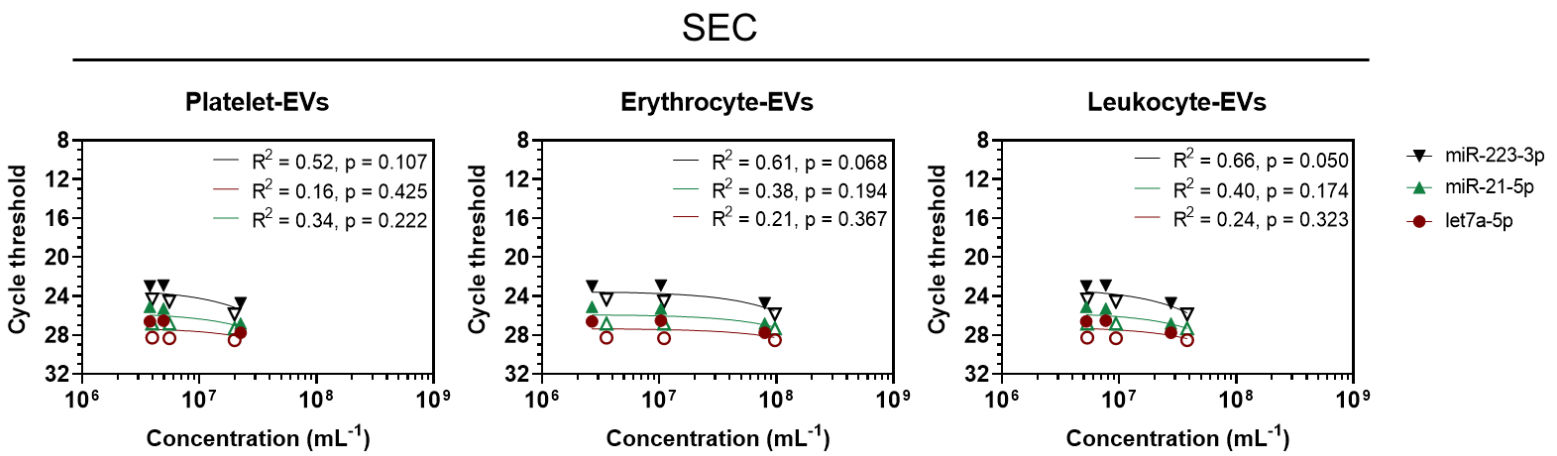
Supplementary Figure 2. *Plasma prepared using the ISTH centrifugation protocol contains activated platelets (CD61+, CD62p+) as measured by flow cytometry. The concentration of activated platelets did not change upon filtration of the plasma, indicating that filtration did not cause additional platelet activation, but also did not remove the activated platelets from plasma. Experiments were performed using plasma obtained from 2 healthy controls. A one-tailed, paired Student's t-test was used to test for statistical differences in activated platelet concentrations, pre- and post-filtration. A p-value ≤ 0.05 was considered significant. F-plasma: filtered plasma; ns: not significant.*



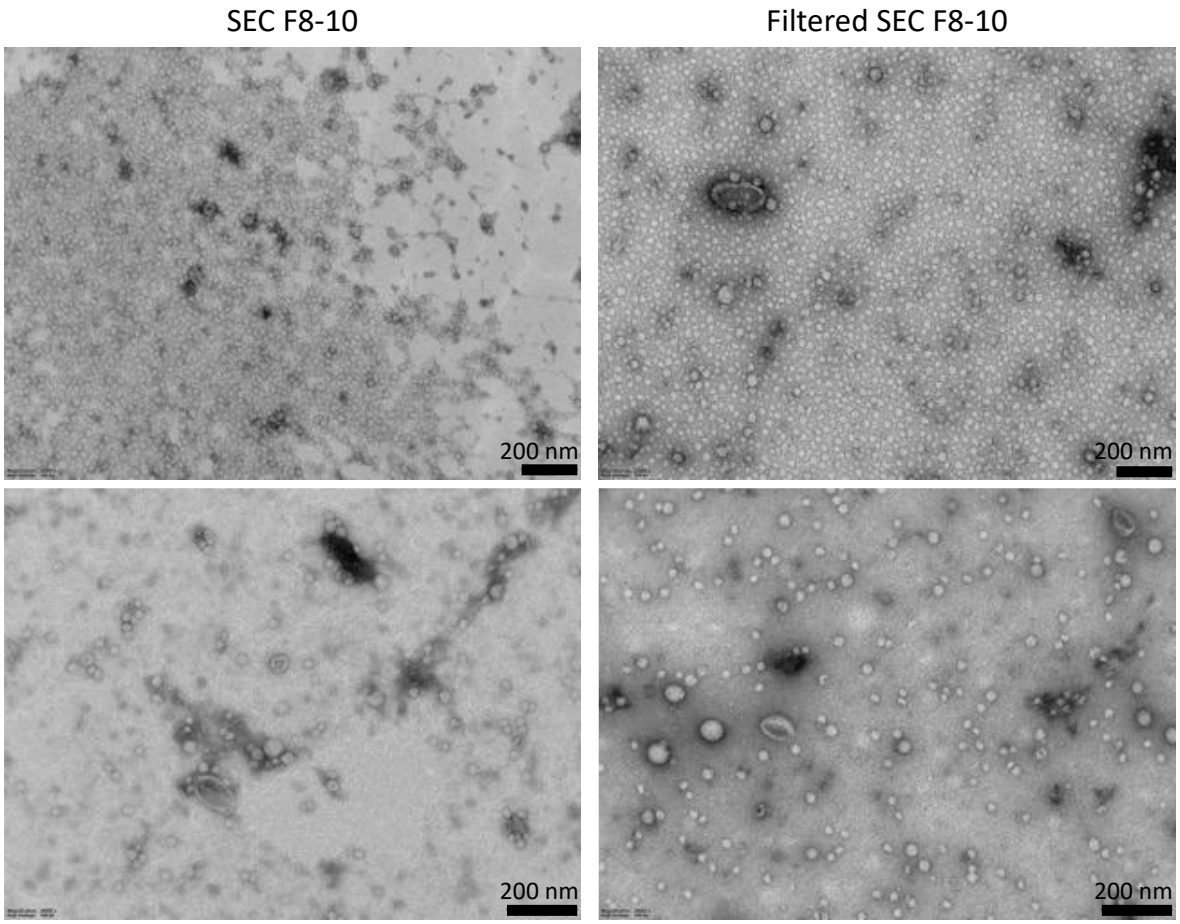
Supplementary Figure 3. *The size distribution of EVs* (CD235a+, CD45+ and CD61+)* ***(A)*** *platelets (CD61+, CD41+)* ***(B)*** *and ery-ghosts (CD235a+)* ***(C)*** *before (left) and after (right) filtration of plasma prepared according to the ISTH centrifugation protocol, as measured by flow cytometry. Experiments were performed using plasma obtained from 3 healthy controls. Ery-ghost: erythrocyte ghost; EV: extracellular vesicle; *: EVs were measured using flow cytometry (Apogee A60) with a size detection range of 150-1,000 nm).*



Supplementary Figure 4. *Filtering out remaining cells from plasma prepared using the non-ISTH centrifugation protocol **(A)** or corresponding EV-enriched SEC2B fractions **(B)** leads to a qRT-PCR cycle threshold increase of 1 to 2, and thus in a decrease of miRNA quantity. The quantity of let7a-5p, miR-21-5p and miR-223-3p miRNAs detected by qRT-PCR correlates with the concentration of platelets in plasma **(C)** especially in unfiltered samples (filled symbols), but not with the concentration of ery-ghosts or EVs**. For the EV-enriched SEC2B fractions **(D)** miRNA quantities also correlate with the concentration of platelets, but not with the concentration of ery-ghosts or EVs**. Experiments were performed using plasma obtained from 3 healthy controls. A one-tailed, paired Student's t-test was used to test for statistical differences in miRNA quantities pre- and post-filtration (panel A-B). A linear regression analysis was used to quantify the relationship between the quantity of miRNAs and the concentration of platelets, ery-ghosts, and EVs** (panel C-D). A p-value ≤ 0.05 was considered significant. Ery-ghost: erythrocyte ghost; EV: extracellular vesicle; ns: not significant; SEC2B: size-exclusion chromatography CL-2B; qRT-PCR: quantitative real-time PCR; *: p ≤ 0.05; **: EVs were measured using flow cytometry (Apogee A60) with a size detection range of 150-1,000 nm).*



Supplementary Figure 5. *The quantity of let7a-5p, miR-21-5p and miR-223-3p miRNAs detected by qRT-PCR does not correlate with the concentration of measured platelet-derived (CD61+), erythrocyte-derived (CD235a+), or leukocyte-derived (CD45+) EVs* in the EV-enriched SEC2B fractions (obtained from plasma prepared using the ISTH protocol). Experiments were performed using plasma obtained from 3 healthy controls. A linear regression analysis was used to quantify the relationship between the quantity of miRNAs and the concentration of EVs. EV: extracellular vesicle; SEC2B: size-exclusion chromatography CL-2B; qRT-PCR: quantitative real-time PCR; *: EVs were measured using flow cytometry (Apogee A60) with a size detection range of 150-1,000 nm).*



Supplementary Figure 6. *TEM images of unfiltered (left) and 0.8 μm filtered (right) EV-enriched SEC2B fractions. EV- and lipoprotein particles pre- and post-filtration have a similar size range, indicating that while particles > 0.8 μm are removed, particles < 0.8 μm (including EVs and lipoprotein particles) remain unaffected. The shown images are representative examples. Scale bars are 200 nm.*