# The proteome of circulating extracellular vesicles and their functional effect on platelets vary with the isolation method – Supplementary Information

Eva M Fernández-Sáez[1], Susana B. Bravo[2], Carmen Pena[2], and Ángel García[1*].

[1]Platelet Proteomics Group, Center for Research in Molecular Medicine and Chronic Diseases (CiMUS), Universidade de Santiago de Compostela, and Instituto de Investigación Sanitaria de Santiago (IDIS), Santiago de Compostela, Spain.

[2]Proteomics Unit, Instituto de Investigación Sanitaria de Santiago de Compostela (IDIS), Santiago de Compostela, Spain.

*Corresponding author.

**Corresponding author:**

Dr. Ángel García,

Platelet Proteomics Group,

Center for Research in Molecular Medicine and Chronic Diseases (CIMUS), Universidade Santiago de Compostela, Avda de Barcelona s/n, 15782 Santiago de Compostela, Spain,

Tel.: +34 881 815429;

e-mail: angel.garcia@usc.es

# Supplementary Methods

## Proteomic analysis by LC-MS/MS

For LC-MS/MS protein and peptide analysis using both DDA and SWATH-DIA method, dried peptides were resuspended in 0.1% formic acid in water (phase A) at a final concentration of 1μg/μl.

## Protein Identification by DDA Acquisition Method

Digested peptides (4 μg) of individual samples (UC-EVs and SEC-EVs) were separated by reverse phase chromatography using a micro-LC system (Eksigent Technologies nano-LC 400, Sciex) coupled to a high-speed Triple TOF 6600 mass spectrometer (Sciex) with a micro flow source using a DDA mode. The analytical column was a silica-based reversed phase column Eksigent C18 150 x 0.30 mm, with 3 mm particle size and 120 Å pore size (Eksigent, Sciex). The trap column was a YMC-TRIART C18 (YMC Technologies, Teknokroma), with a 3 mm particle size and 120 Å pore size, switched on-line with the analytical column. The loading pump delivered a solution of 0.1% formic acid in water at 10 μL/min. The micro-pump generated a flow rate of 5 μL/min and was operated under gradient elution conditions, using 0.1% formic acid in water as mobile phase A, and 0.1% formic acid in acetonitrile as mobile phase B. Peptides were separated using a 90 min gradient ranging from 2% to 90% mobile phase B.

Data acquisition was performed using a TripleTOF 6600 System (Sciex, Foster City, CA, USA) using a DDA workflow. Source and interface conditions were the following: ionspray voltage floating (ISVF) 5500 V, curtain gas (CUR) 25, collision energy (CE) 10 and ion source gas 1 (GS1) 25. Instrument was operated with Analyst TF 1.7.1 software (Sciex). Switching criteria was set to ions greater than mass to charge ratio (m/z) 350 and smaller than m/z 1400 with charge state of 2-5, mass tolerance 250 ppm and an abundance threshold of more than 200 counts per second (cps). Previous target precursor ions were excluded for 15 s. The instrument was calibrated automatically every 4 hours with tryptic peptides from PepCalMix as external calibrant.

Peptide and protein identifications were performed with ProteinPilot™ 5.0.1 software (Sciex), which uses Paragon™ algorithm for database searching and Progroup™ for data grouping. Data were searched using a Human specific uniprot database with iodoacetamide at cysteine alkylation as a variable modification and methionine oxidation as a fixed modification. Global false discovery rate (FDR) was set to 1% for both peptides and proteins.

Scaffold (version 5.2.2 Proteome Software Inc, Portland, OR, USA) was used to validate MS/MS based peptide and protein identification and to perform a semiquantitative

analysis by spectral counting. Peptides identifications were accepted if they could be established with more than 97% probability to achieve a FDR < 1% by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established with more than 99% probability to achieve a FDR < 1% and with a minimum requirement of 2 peptides for each protein. Protein probabilities were assigned by the Protein Prophet algorithm and Fisher's Exact test was used for quantification and fold change (FC) determination. Proteins were considered differentially expressed when $p < 0.05$. Proteins with a FC = 0 were considered present only in SEC-EVs and proteins with a FC = Infinity were considered present only in UC-EVs.

## Protein Quantification by SWATH Acquisition Method (DIA)

Prior to protein quantification, a MS/MS spectral library was constructed analyzing peptides by a shotgun DDA approach by micro-LC-MS/MS. To ensure adequate representation of the peptides and proteins present in all samples, pooled vials of samples from each group (SEC and UC) were prepared using equal volumes from the original samples. For each pool, 4 µL of digested peptides were separated into a micro-LC system Ekspert nLC425 (Eksigent, Dublin, CA, USA) using the same conditions as before. In this assay, the gradient run consisted of 5-95% of B for 30 min, 90% of B for 5 min, and 5% of B for 5 min to column equilibration (total run time of 40 min). When the peptides eluted, they were directly injected into a hybrid quadrupole-TOF mass spectrometer Triple TOF 6600 (Sciex) operated with a DDA system in positive ion mode. A Micro source (Sciex) was used for the interface between microLC and MS, with application of 2600 V. The acquisition mode consisted of a 250 ms survey MS scan from 400-1250 m/z, followed by an MS/MS scan from 100-1500 m/z (25 ms acquisition time) of the top 65 precursor ions from the survey scan, with a total cycle time of 2.8 s. The fragmented precursors were then added to a dynamic exclusion list for 15 s, any singly charged ions were excluded from the MS/MS analysis. Peptide and protein identification were performed with ProteinPilot™ 5.0.1 software (Sciex) as previously described.

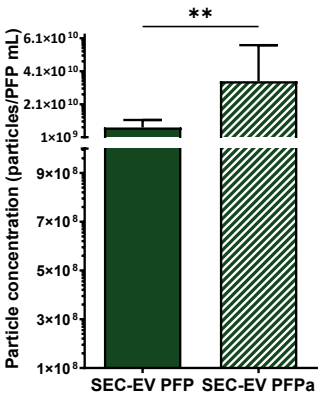
SWATH-MS method was performed on a TripleTOF 6600 LC-MS/MS system (Sciex). Each sample was analyzed using a data-independent acquisition (DIA) method. The method involved repeating a cycle that consisted of the acquisition of 100 TOF MS/MS scans (400-1500 m/z, high sensitivity mode, 50 ms acquisition time) of overlapping sequential precursor isolation windows of variable width (1 m/z overlap), covering the 400-1250 m/z mass range with a previous TOF MS scan (40-1500 m/z, 50 ms acquisition time) for each cycle (total cycle time was 6.3 s). The 100 variable windows width was optimized according to the ion density found in the DDA runs using a SWATH variable window calculator worksheet from Sciex.

Targeted data extraction of the fragment ion chromatogram traces from the SWATH runs was performed by PeakView (version 2.2, Sciex) using the SWATH Acquisition MicroApp

(version 2.0). This application processed the data using the spectral library generated from the shotgun data. Up to 10 peptides per protein and 7 fragments per peptide were selected, based on signal intensity; any shared and modified peptides were excluded from processing. Five-minute windows and 30 ppm widths were used to extract the ion chromatograms. The retention times from the peptides that were selected for each protein were realigned in each run according to the calibration curve retention time (iRT). The chromatograms of the extracted ions were then generated for each selected fragment ions. Peak areas for the protein were obtained by summing the peak areas from 10 peptides (MS scan) and 7 corresponding fragment ions (MS/MS scan) from each peptide. PeakView 2.2 computed a FDR and a score for each assigned peptide according to the chromatographic and spectra components. Only peptides with a FDR < 5% were used for protein quantization. Protein quantization was performed by adding the peak areas of the corresponding peptides.

The integrated peak areas were directly exported to the MarkerView software (version 1.3.1, Sciex) for relative quantitative analysis. The export generated 3 files containing quantitative information about individual ions, the summed intensity of different ions for a particular peptide, and the summed intensity of different peptides for a particular protein. MarkerView 1.3.1 uses processing algorithms that accurately find chromatographic and spectral peaks directly from the raw SWATH-MS data. Data alignment by MarkerView compensated for minor variations in both mass and retention time values, ensuring that identical compounds in different samples are accurately compared to one another. Unsupervised multivariate statistical analysis using principal component analysis (PCA) was performed to compare the data from the different samples. A most-like ratio (MLR) normalization was performed after statistical analysis to control for possible uneven sample loss across the different samples during the sample preparation process. The average MS peak area for each protein was derived from each sample, followed by analysis using a Student's t-test (MarkerView 1.3.1 software, Sciex) to compare between samples based on the averaged total area of all transitions for each protein. The t-test result ($p$ value) indicates how well each variable distinguishes between the two groups. Candidate proteins were selected for each library based on $p < 0.05$ and FC > 2.

# Supplementary Figures




**Supplementary Figure 1**. Particle concentration (particles/mL of PFP) in samples isolated by SEC from PFP and PFPa. ** $P < 0.001$.