

Comparison Of Plasma-derived Small Extracellular Vesicle Isolation Methods Using LC-MS/MS Proteomics

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Background

- Extracellular vesicles (EVs) are a promising source of biomarkers for neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), and progressive supranuclear palsy (PSP). For PSP, the lack of reliable biomarkers makes diagnosis especially difficult [1].
- Plasma extracellular vesicles (EVs) originate from multiple cell types and carry molecular cargo that reflects their cells of origin. Together, the circulating EV population may capture both peripheral and central nervous system alterations associated with neurodegenerative diseases.
- Although numerous commercial methods are available for isolating extracellular vesicles (EVs) from plasma, identifying an approach that is robust, scalable, and suitable for routine clinical implementation while remaining compatible with downstream proteomic analyses remains a major challenge [2].
- Mass spectrometry is the gold-standard method for protein quantification and enables unbiased biomarker discovery beyond predefined targets.

Aim

To compare plasma EVs isolation methods and evaluate their performance and reproducibility using mass spectrometry-based proteomics

Materials and Methods

1. Sample collection and preprocessing

Plasma samples of participants with subjective cognitive impairment were obtained from the Neurology Department at Hospital del Mar and used for this study (n=3; 4 technical replicates).

2. Sample preprocessing



3. sEV isolation

Three different methods were used and compared:

a) Size Exclusion Chromatography (SEC)

Required sample volume: 100 µL

A qEV single column 70 nm (ICS-70; IZON) per sample was used for each sample. Each column was pre-washed with filtered PBS following manufacturer's instructions. After the sEV isolation, the sample was enriched using an Amicon 30 kDa filter.

b) Immunoprecipitation - Thermo Fisher (THE)

Required sample volume: 100 µL

The plasma sample was incubated with beads coated with CD63 antibody obtained from the Exosome-Human CD63 Isolation/Detection Reagent of Thermo Fisher.

c) Immunoprecipitation – Miltenyi (MIL)

Required sample volume: 250 µL

The plasma sample was Incubated with beads coated with CD63 antibody obtained from the EV isolation Kit CD63 of Miltenyi.

4. Sample digestion and desalting

- EVs were lysed with 6M Urea. After reduction (DTT) and alquilation (IAM) samples were digested with trypsin and Lys-C. Peptide mixtures were analysed in an Orbitrap Astral mass spectrometer by data-independent-acquisition. Protein quantification was obtained with DIA-NN 2.2.

Results

Technical replicability

- Technical variability in protein abundances differed between methods, with higher mean CV% for MIL (41%), followed by THE (26.3%) and SEC (22.7%) (Figure 1).

↑ TECHNICAL VARIABILITY: Miltenyi

↓ TECHNICAL VARIABILITY: Thermo Fisher

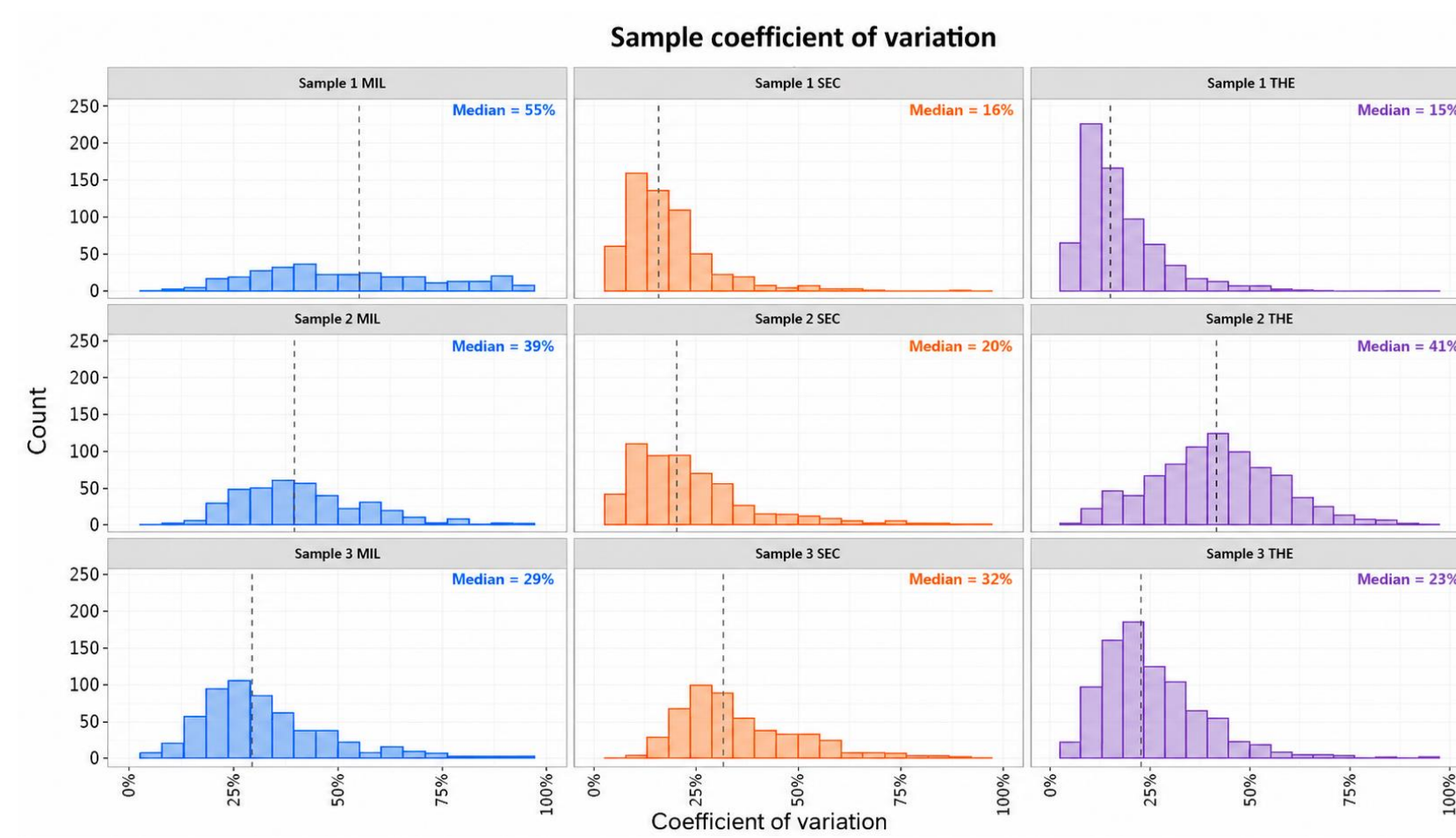


Figure 1. Protein coefficient of variation (CV) across technical replicates, shown as individual histograms with the median indicated by the dashed line.

sEV markers

- Despite lower protein abundance, MIL provided more consistent detection of canonical sEV markers [3] across technical and biological replicates (Figure 2).
- Further comparisons, based on Alamar (NULISA) panels and CNS studies [4], enabled the detection of proteins that are hallmark for neurodegenerative disease identification (Clusterin, SOD1, α -synuclein, and SMOC1) specially in THE- and MIL-isolated samples.

↑ ENRICHMENT IN sEV-ASSOCIATED PROTEINS: Miltenyi

↓ ENRICHMENT IN sEV-ASSOCIATED PROTEINS: Size Exclusion Chromatography

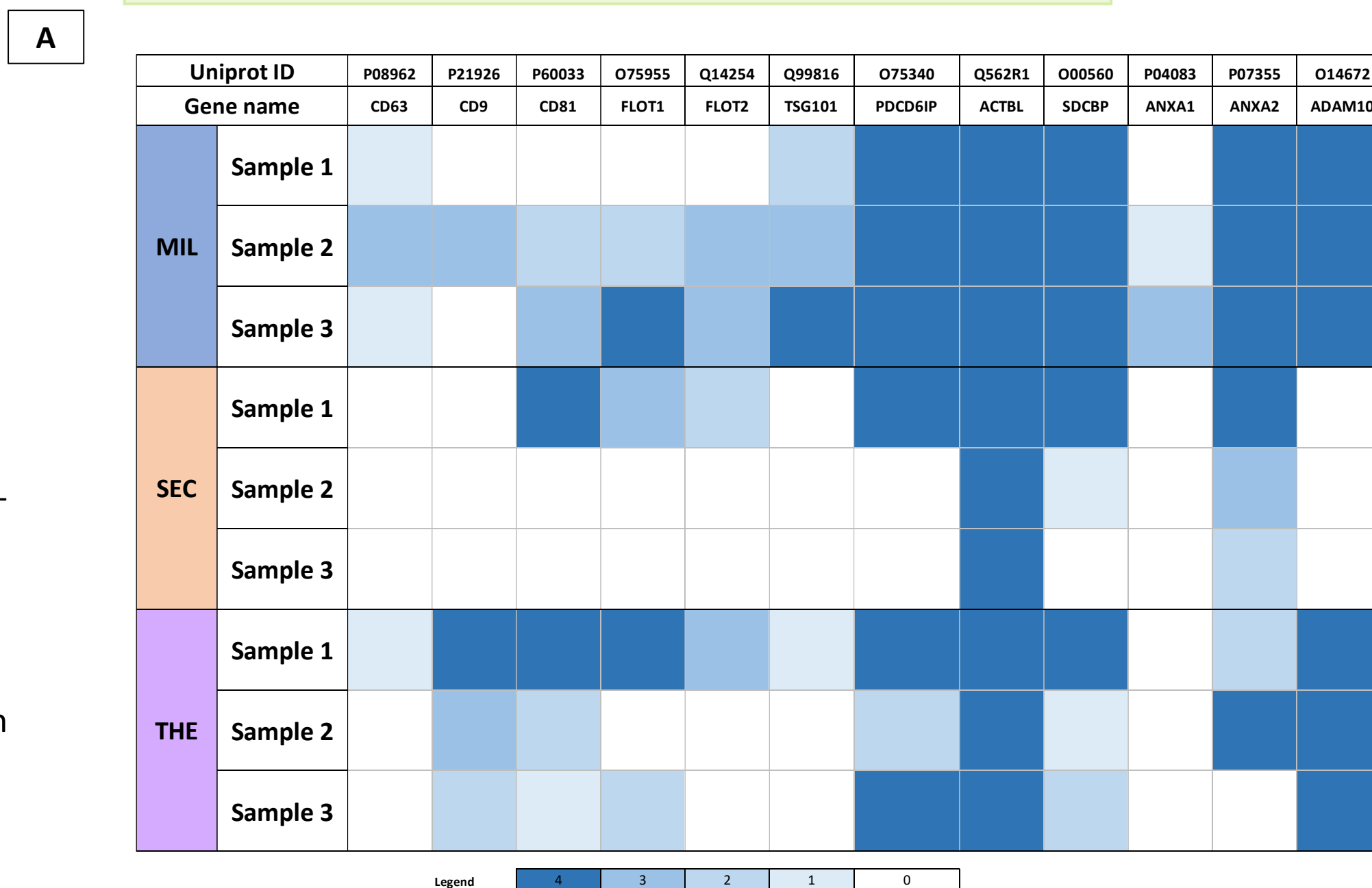


Figure 2. (A) Presence/absence heatmap of sEV marker proteins across isolation protocols and samples. The blue color intensity shows how many technical replicates are detected in each marker. (B) Gene Ontology (GO) Cellular Component terms significantly enriched among proteins detected in at least three of the four technical replicates for each biological sample, stratified by isolation method.

Detected proteins

- THE identified the most protein groups (mean: 938; CV 11%), followed by SEC (643; CV 7%) and MIL (597; CV 16%) (Figure 3).

↑ NUMBER OF DETECTED PROTEINS: Thermo Fisher

↓ NUMBER OF DETECTED PROTEINS: Miltenyi

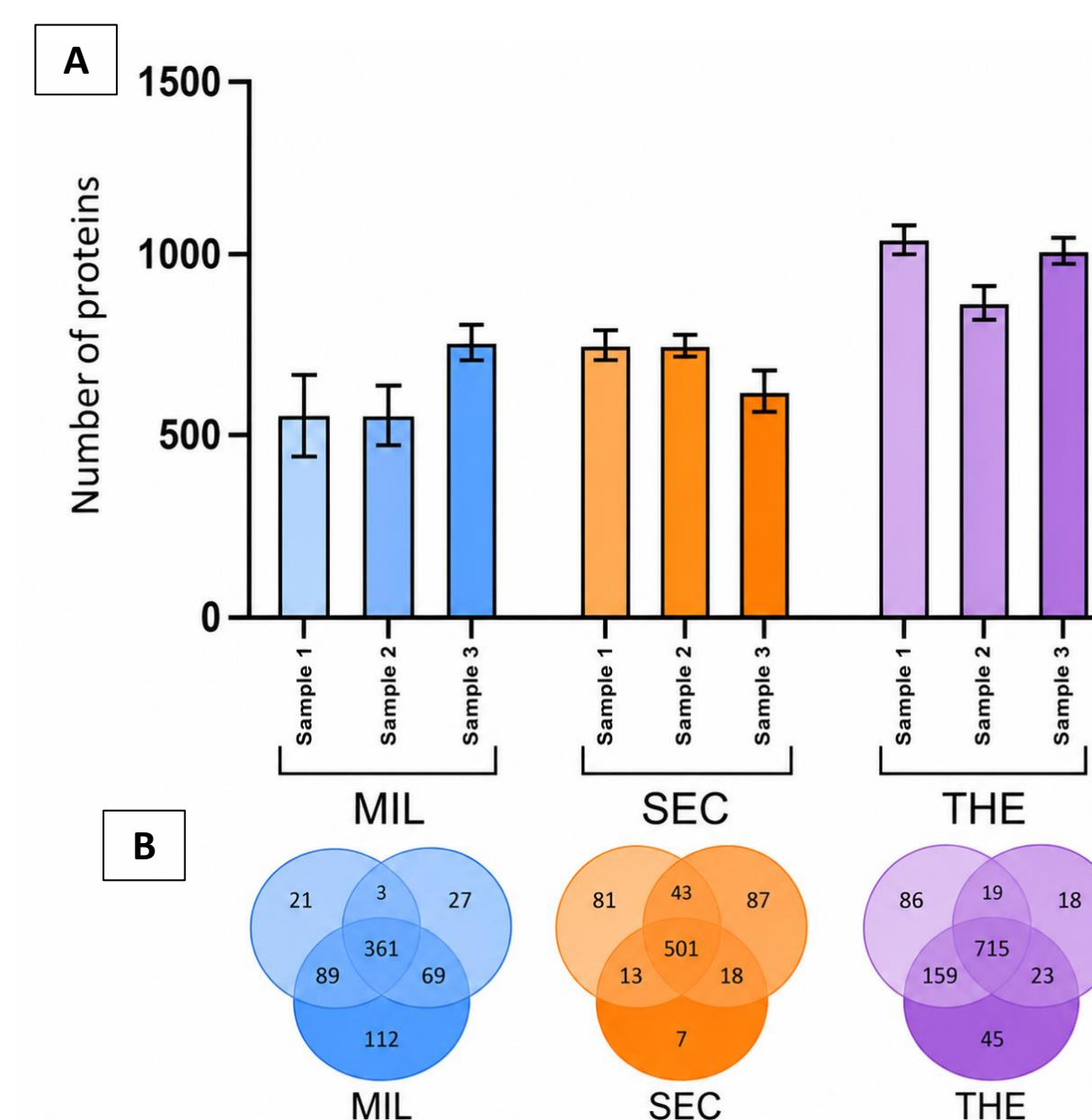
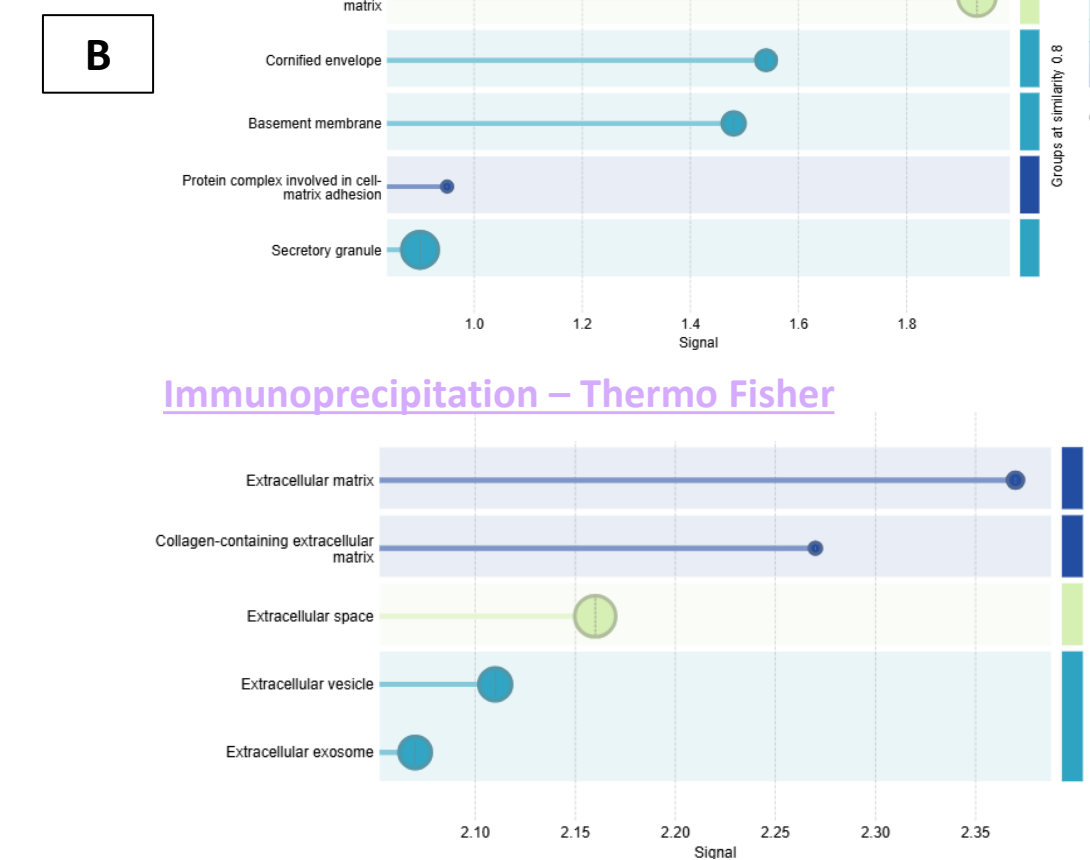
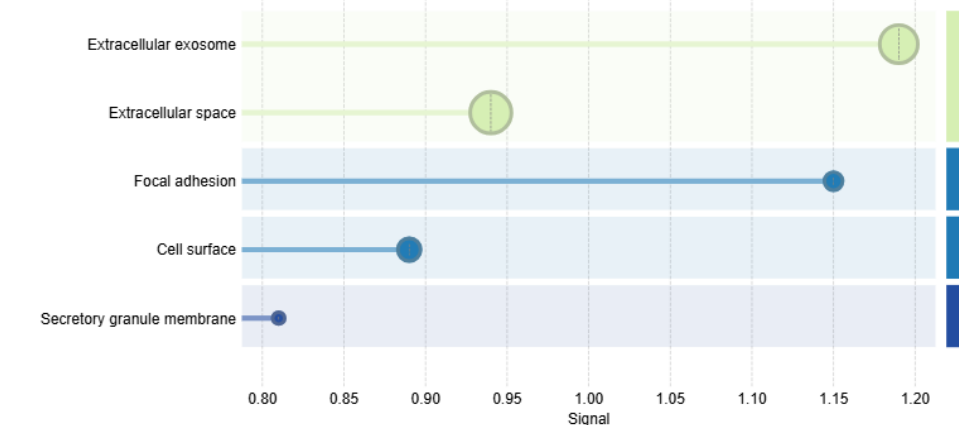


Figure 3. (A) Bar plots show the total number of protein groups detected by LC-MS/MS per sample and isolation method (mean $\pm$ SD of technical replicates). (B) Venn diagrams show the overlap and method-specific protein groups for each isolation protocol specifically for those human proteins that are detected in at least 3 technical replicates.

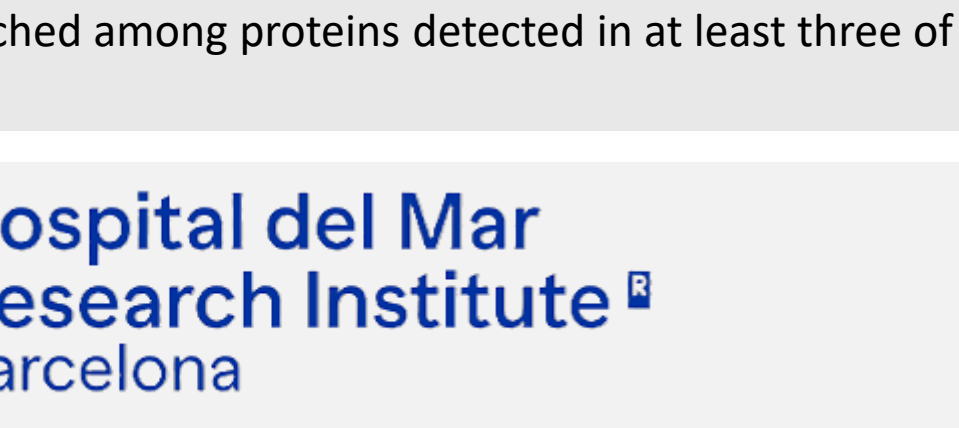
Size Exclusion Chromatography



Immunoprecipitation – Thermo Fisher



Immunoprecipitation – Miltenyi



Conclusions

- The Thermo Fisher immunoprecipitation kit demonstrated superior technical reproducibility across replicates, and enabled the detection of a higher number of proteins by LC-MS/MS.
- The Miltenyi immunoprecipitation kit enables the identification of a higher number of sEV-related markers and reference proteins.
- This project represents a first step toward characterizing new biomarkers of common neurodegenerative diseases such as AD, PD, or PSP. A subsequent step would be to study the observed markers in plasma samples from clinically diagnosed patients with these pathologies.
- In the future, there will also be an interest in further exploring proteomic panels, enabling comparisons across different platforms and even allowing the selection of proteins that help characterize specific extracellular vesicle markers directly associated with neurodegenerative diseases.

→ References

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