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Impact of preanalytical factors on plasma extracellular vesicles and human plasma proteome

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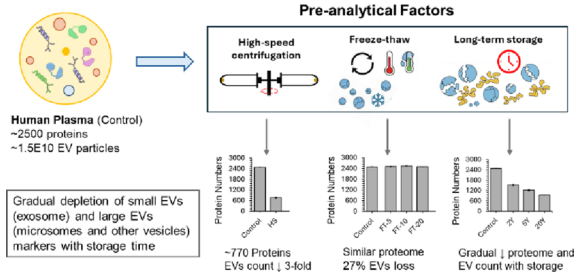
Abstract

Using human plasma and its protein-rich extracellular vesicles (EVs) for proteomic biomarker discovery requires an in-depth understanding of the influences of pre-analytical procedures. This study systematically investigates the ramifications of high-speed centrifugation, freeze-thaw cycles, and prolonged storage on the plasma proteome and abundance of plasma EVs. Employing a Mag-Net-assisted workflow for high-throughput EV-based plasma proteomics, we find that high-speed centrifuging plasma samples at 8,000 g prior to analysis reduces both EV and protein numbers to only 30% of the original plasma, rendering numerous established EV markers undetectable. In contrast, multiple freeze-thaw cycles within a week have very minimal effect on proteome coverage, albeit with a reduced number of EVs and subtle shifts in the enrichment and depletion patterns of specific proteins. Long-term storage of plasma in freezer for years gradually and significantly diminishes proteome coverage with reduced EV counts and altered size distributions. These results caution the use of biobank samples for retrospective studies, particularly longitudinally collected blood specimens that have been preserved for years for EV-focused biomarker research, and underscore the imperative for stringent standardization of pre-analytical protocols to enhance the reliability and reproducibility of plasma EV proteomics.

Keywords Human plasma, Extracellular vesicles, Mag-Net plasma proteomics, EV proteomics, Long-term plasma storage

Graphical Abstract



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Introduction

Plasma, a highly accessible biofluid, holds immense promise for the discovery of protein biomarkers crucial for disease monitoring, therapeutic evaluation, and early diagnosis [1, 2]. However, the clinical utility of plasma proteomics is inextricably linked to the stability of its constituent biomolecules. Pre-analytical variables, such as high-speed centrifugation, freeze-thaw cycles, and storage time, are recognized as significant sources of variation that can compromise the accuracy and reproducibility of downstream measurements [3, 4, 5]. Clinical biobanks serve as invaluable repositories of biospecimens for retrospective biomarker research, yet the susceptibility of plasma biomolecules to degradation under various processing and storage conditions poses a substantial challenge. Such degradation can introduce confounding variability, obscure genuine biological signals, and ultimately undermine research integrity. Therefore, a comprehensive understanding of how these factors impact the plasma proteome is paramount for establishing robust sample handling guidelines and preserving the fidelity of invaluable clinical specimens [6, 7].

The inherent complexity of the plasma proteome is characterized by a dynamic range spanning over ten orders of magnitude [1]. Often it impedes the detection of low-abundance, tissue-specific proteins critical for deciphering pathological states [8] However, plasma extracellular vesicles (EVs), including exosomes and microvesicles, offer a compelling solution to these analytical limitations. These lipid-bilayer-enclosed structures, released by cells into circulation, encapsulate cellular cargo and represent the physiological or pathological state of their cells of origin [9]. Consequently, plasma EVs are well-positioned as prime candidates for biomarker discovery and translational medicine.

The already complex plasma proteome is further complicated by pre-analytical variables that directly affect the integrity of EVs and their cargo, contributing another layer of complexity to plasma EV-based biomarker research [10]. High-speed centrifugation (>5000 g) is applied to prepare platelet-free plasma, which is known to lead to vesicle (apoptotic bodies, microvesicles, and EVs) aggregation and protein loss [11]. Similarly, freeze-thaw cycles can disrupt EV structural integrity, leading to the release of intravesicular proteins and a concomitant loss of vesicle-associated proteoforms [12, 13]. Furthermore, prolonged storage is known to trigger protein degradation and diminish EV integrity, impacting downstream proteomic analyses [14, 15]. Given these challenges, the implementation of robust and reproducible analytical workflows is critical for monitoring individual variability in EV proteomes, particularly in longitudinal studies.

Traditional EV isolation methods, such as ultracentrifugation and size-exclusion chromatography, often involve extensive processing steps that can introduce variability and compromise EV proteome quality [16]. Recent technological advancements in EV enrichment have paved the way for streamlined workflows that enhance the reproducibility and sensitivity of plasma proteomics. In particular, a high-throughput Mag-Net protocol was developed that integrates high-abundance protein depletion, EV enrichment, and proteolytic digestion into a single-step workflow [17]. By minimizing the time from EV isolation to digestion, this approach effectively reduces variability and improves the reproducibility of proteomic analyses. This methodology is particularly well-suited for high-throughput investigations, facilitating reliable characterization of EV proteomes and overcoming key limitations associated with conventional workflows [18].

In the present work, we aimed to systematically evaluate the impact of pre-analytical factors on the plasma EV proteome. Such assessments are crucial for improving our understanding of the robustness of EV-based biomarkers and for informing best practices in sample processing and handling within biobanks supporting retrospective clinical studies. Moreover, elucidating the effects of pre-analytical variables on EV integrity and proteome composition is essential for standardizing EV research and ensuring inter-study reproducibility [19]. In this context, using an ultracentrifugation-based EV isolation approach and the Mag-Net-based plasma EV proteomics workflow, we meticulously examined the effects of high-speed centrifugation, freeze-thaw cycles, and prolonged storage time on plasma. The results provided insights into how common pre-analytical factors influence plasma EV heterogeneity and its proteome.

Materials and methods

Plasma preparation

Fresh blood sample was obtained *via* venipuncture from a volunteer using EDTA Vacutainer Tubes (BD Biosciences), then it was processed immediately to isolate plasma through centrifugation at 2,000 g for 10 min. The supernatant was collected for further centrifugation at 8,000 g for 25 min to obtain high-speed processed plasma. For freeze-thaw treatment, plasma was aliquoted into 1 mL tubes and subjected to a controlled freeze-thaw process. Each cycle involved freezing samples at -80 °C for at least 2 h and thawing at room temperature. Plasma samples undergoing 0 (Control), 5, 10, and 20 freeze-thaw cycles were prepared to evaluate the impact of repeated freezing and thawing on EV proteome coverage. Archived human plasma samples that have been stored for 2-years (Clinical Trial NCT05907135), [20] 5-years (Clinical Trial NCT03445234) [21], and 20-years (Clinical Trial NCT00004984) [22] were transferred to

the study laboratory under dry ice and stored at -80 °C until further analysis. The details about blood collection and processing are included in Table S1. To mitigate individual variability, the archived samples were pooled from multiple donors according to each study. The fresh blood sample collection was approved by Appalachian State University's Institutional Review Board (IRB), and all other samples from clinical trials were approved under each clinical trial's IRB.

Isolation and characterization of plasma EVs

Plasma samples (100 µL) were diluted with 11.9 mL of phosphate-buffered saline (PBS) and ultracentrifuged at 150,000 g for 3 h at 4 °C using a Sorvall™ WX 80 Ultracentrifuge (Thermo Scientific). The resulting pellets were carefully resuspended in 100 µL of PBS, then diluted 1,000-fold in PBS for particle analysis using a ZetaView® Quatt instrument (Particle Metrix) in scatter mode in triplicate. Measurement settings included a camera sensitivity of 80, shutter speed of 100, particle minimum brightness of 30, trace length of 7, and a particle size range of 10 nm to 1,000 nm, as used previously [23].

Mag-Net-assisted digestion of plasma EV

MagReSyn® strong anion exchange (SAX) beads (MR-SAX005, ReSyn Biosciences, 5 µL) were equilibrated and washed with 100 µL of equilibration/wash buffer (50 mM Bis-Tris Propane, pH 6.5, 150 mM NaCl). This step was performed twice on a 96-well plate for 30 s using a magnetic separator. Subsequently, 5 µL of plasma was mixed with 45 µL of EV binding buffer (100 mM Bis-Tris Propane, pH 6.3, 150 mM NaCl) and combined with the pre-equilibrated SAX beads. This mixture was gently agitated at room temperature for 20 min. Following this incubation, the flow-through containing unbound plasma proteins was removed using a magnetic separator. Three additional washes with 100 µL of equilibration/wash buffer were performed, mixed gently for 5 min, to deplete any additional high-abundance proteins. The bound vesicles were lysed by 50 µL of a lysis buffer (1% SDS, 10 mM TCEP, and 15 mM IAA in 50 mM Tris-HCl, pH 8.5) at 37 °C for 60 min with gentle mixing. Then, 200 µL of acetonitrile was added to the lysis buffer-beads mixture which was pipette-mixed ~ 10 times and incubated at room temperature for 10 min without further mixing to induce on-bead protein precipitation. The washing steps were performed twice, each using 100 µL of 100% acetonitrile, with the beads kept captured on a magnetic separator and avoiding mixing during the washes. Finally, for protein digestion, 100 µL of 25 mM Tris-HCl (pH 8) containing 1 µg of trypsin/LysC was added and incubated at 37 °C overnight with gentle mixing. The digestion was quenched by adding 5 µL of 10% trifluoroacetic acid.

LC-MS/MS analysis

Peptides from digestion were loaded onto EvoTip trap columns (Evosep, cat# EV2013) following a standardized protocol. Initially, the EvoTips were washed with 20 µL of solvent B (acetonitrile, 0.1% formic acid), conditioned with 2-propanol, and equilibrated using 60 µL of solvent A (water, 0.1% formic acid). After sample loading, the tips were washed twice with 60 µL of solvent A, followed by a brief final rinse with 100 µL of solvent A for 10 s to prevent drying. All steps were centrifuged at 800 g for 60 s.

Peptide separation was carried out on an Evosep One LC system (Evosep, Denmark) using a 15 cm × 150 µm column packed with 1.5 µm C18 beads (Evosep, cat# EV1109). A flow rate of 1 µL/min and the 21-min gradient method (30 samples per day) were employed, with peptide elution achieved at a maximum of 35% solvent B. Eluted peptides were analyzed in positive ion mode using an Orbitrap Ascend Tribrid Mass Spectrometer (Thermo Fisher).

Mass spectra were acquired across the 400–900 *m/z* range at a mass resolution of 60k (200 *m/z*), followed by data-independent acquisition (DIA) MS/MS. DIA scans utilized a mass isolation window of 20 *m/z* with a mass resolution of 30k (200 *m/z*). The quadrupole was used for isolation, with higher-energy collisional dissociation (HCD) for activation. Detection was performed using an Orbitrap detector. Instrument settings included an RF lens value of 45%, normalized HCD collision energy of 28, a normalized AGC target of 225% for MS1 and 800% for DIA, and ion injection times of 123 ms for MS1 and 59 ms for DIA scans.

Proteomics data analysis

The LC-MS/MS raw data were analyzed using DIA-NN software (version 1.8.1) [24]. Protein identification was performed against the Human protein database retrieved from UniProt on June 7, 2024. The workflow incorporated a peptide spectrum library-free search against a FASTA in silico digest and advanced deep learning algorithms to predict spectral and retention time profiles. Key analysis parameters included a mass accuracy of 15.0 ppm, an MS1 accuracy of 20.0 ppm, and a scan window width of 4. Trypsin/P was specified as the proteolytic enzyme, allowing for a single missed cleavage site. Carbamidomethylation of cysteine residues was designated as a fixed modification. The match-between-runs feature was activated to enhance data alignment across samples. Protein inference was carried out at the gene level using a neural network classifier in single-pass mode, while quantification was optimized for high-accuracy LC workflows. Additionally, cross-run normalization accounted for retention time variations, and library profiling was implemented with smart profiling strategies. All unspecified parameters were left at their default values.

Statistical and bioinformatics analysis

The output files generated by DIA-NN were subjected to statistical analysis using Perseus software (version 2.0.11.0) [25]. Proteomic data were filtered to include proteins with ≥70% valid values across all samples. The data were then log2-transformed and normalized using the width adjustment algorithm. Missing values were imputed from a normal distribution. Statistically significant protein alterations were identified using a multiple-sample test with a permutation-based false discovery rate (FDR) threshold of $q < 0.05$ and an S_0 value of 0.05. Further data exploration included hierarchical clustering (using Z-score normalization), volcano plot visualization, and principal component analysis (PCA).

Bioinformatic analyses were performed using Metascape (version 3.5.20240901) to identify enriched functional pathways within distinct plasma samples [26] The in silico Human Surfaceome platform (https://wlab.ethz.ch/surfaceome/, version 1.0) [27] was employed to characterize storage time-related alterations in the plasma surfaceome. Protein-protein interaction networks, Gene Ontology enrichment, functional pathway analyses, and subcellular localization were further investigated using the STRING database (version 12.0, https://string-db.org/) [28].

Results

Pre-analytical conditions alter plasma proteome coverage

We conducted a comprehensive proteomic analysis to assess the consequences of high-speed centrifugation, freeze-thaw cycles, and storage time on plasma. Utilizing a 5 µL plasma input, EVs were concurrently enriched and digested *via* the Mag-Net-assisted workflow, followed by LC-MS/MS analysis (Fig. 1A). Approximately 2,500 protein groups (FDR < 0.05%) were identified, in fresh and freeze-thawplasma samples.

in fresh and freeze-thaw plasma samples, while only 770 were identified in high-speed centrifuged plasma and 1525, 1219, and 949 in plasma stored for 2, 5, and 20 years (Fig. 1B). A detailed summary of identified proteins per plasma sample type is provided in Table S2. The Mag-Net workflow demonstrated effective enrichment and digestion of EVs or EV-like vesicles, with approximately 71% of identified proteins among all samples associated with EVs according to the ExoCarta database (Fig. 1B). Notably, 82 proteins were linked to the top 100 ExoCarta-listed EV markers, including Alix (PDCD6IP), Annexin A2, CD9, TSG101, Flottilin-1, HSPA8, and HSP90AA1. STRING enrichment analysis for subcellular localization revealed a predominant association of enriched proteins with extracellular membrane-bound organelles, extracellular vesicles, and exosomes (Fig. 1C).

PCA plots illustrate the distinct impacts of high-speed centrifugation, freeze-thaw cycles (Fig. 1D), and storage time (Fig. 1E), on the plasma proteome, along with the Control, which has no high-speed processing and zero freeze-thaw cycles. For high-speed centrifugation and freeze-thaw cycles, principal components (PC1 and PC2) collectively explained 77.3% of the total variance, revealing clear clustering differences. Plasma subjected to high-speed centrifugation exhibited significant divergence along PC1 from fresh and freeze-thaw plasma, indicative of substantial proteomic alterations. Conversely, plasma exposed to multiple freeze-thaw cycles clustered closely together, diverging along PC2 from fresh plasma, suggesting a minimal overall impact on the proteomic profile. In the context of plasma under long storage time, PC1 and PC2 accounted for 80.9% of the variance, with prolonged storage inducing gradual and systematic shifts along PC1, likely reflecting time-dependent protein degradation. In all PCA plots, replicates within each group consistently clustered closely, affirming high technical reproducibility. These results underscore the sensitivity of the plasma proteome to pre-analytical variables and highlight the critical need for standardized sample handling and storage protocols in plasma proteomics. Statistical analysis further validated the significant proteomic changes induced by high-speed processing, freeze-thaw cycles, and extended storage.

High-speed centrifugation reduces proteome coverage by depleting plasma EV populations

High-speed centrifugation primarily sediments cellular debris and larger EVs, while retaining a subset of smaller EVs and other vesicular and non-vesicular particles, including protein aggregates. The 8,000 g speed used in the current work is typically used for the collection of high-speed processed plasma; however, this processing resulted in a substantial reduction in plasma proteome, leading to the detection of approximately 770 proteins, nearly a 3-fold decrease compared to unprocessed plasma. Analysis of EV markers revealed that most canonical small EV markers, such as Alix, TSG101, FLOT2, and SDC4, became undetectable following high-speed centrifugation (Fig. 2A). In contrast, ANXA2, CD9, FLOT1, and HSPA8 remained detectable, albeit at significantly reduced levels. Similarly, traditional large EV markers, such as AXTN3, VAMP3/7, and VPS35, were not detected after high-speed centrifugation, whereas ATP5F1A/B, HSP90AA1, THBS1/4, and ITGB1/6 showed marked depletion. Notably, some large EV/microvesicle-specific markers, including ANXA1, APOE, and C3 demonstrated enrichment after high-speed centrifugation (Fig. 2A).

High-speed centrifugation significantly reduced the plasma EV population, also showing approximately a 3-fold depletion compared to unprocessed plasma (Fig. 2D). Size distribution analysis confirmed that large

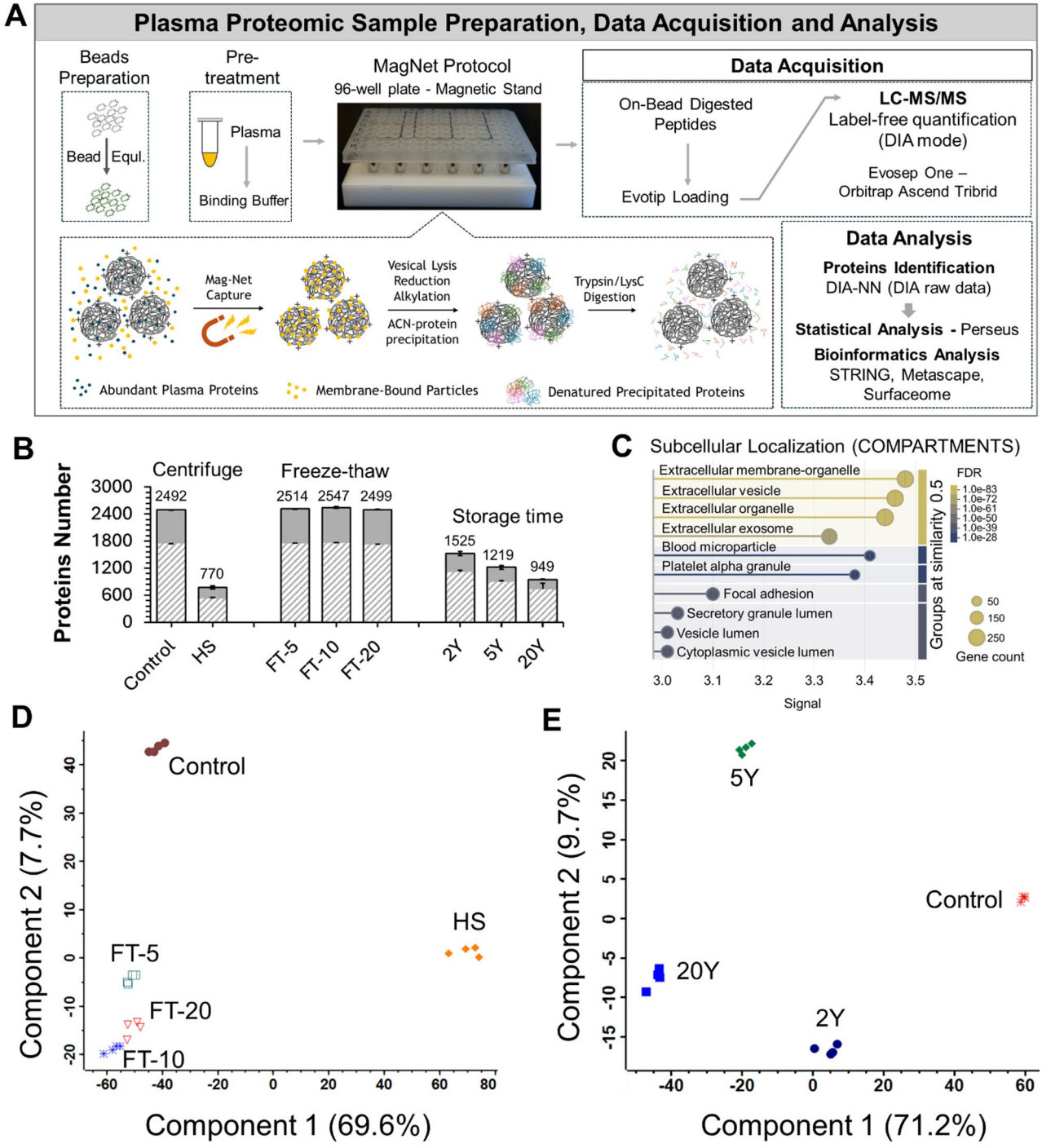

Fig. 1 Plasma proteomics workflow and overview of results. (**A**) Schematic representation of the workflow. Plasma EVs were simultaneously enriched and depleted of high-abundant proteins, vesicle-lysed, and proteolytic digested using the Mag-Net protocol (adopted from Wu et al., 2025) in a 96-well plate. Subsequently, peptides were loaded into Evotips and further analyzed by the 30SPD method with Evosep One - Orbitrap Ascend with DIA mode. Data analysis was performed with DIA-NN, Perseus, and various open-access bioinformatics databases. (**B**) The number of proteins identified in different plasma samples, with the number of proteins associated with EVs (Based on ExoCarta database), is shown in diagonal stripes. (**C**) The subcellular localization of the proteins enriched through the Mag-Net protocol in all plasma samples were analyzed using STRING. (**D**) The PCA scores plot shows clustering and separation in the plasma proteome between high-speed centrifugation and the number of freeze-thaw cycles. The control is fresh human plasma without high-speed centrifugation and a zero freeze-thaw cycle. (**E**) The PCA scores plot shows clustering and separation between plasma samples from different storage times. Fresh human plasma is the control. (HS, high-speed; FT, freeze-thaw; Y, years)

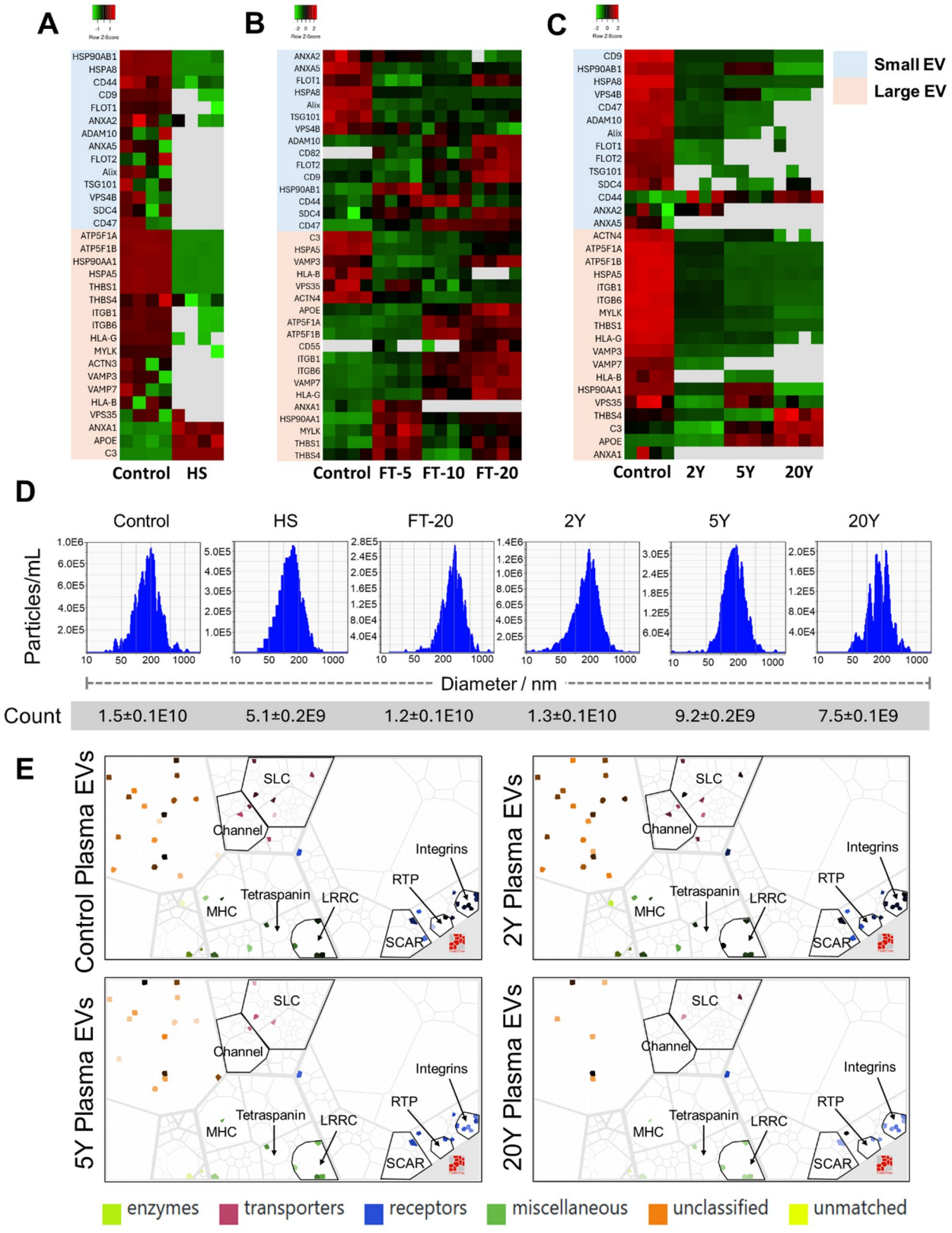

Fig. 2 (See legend on next page.)

(See figure on previous page.)

Fig. 2 Impact of high-speed centrifugation, number of freeze-thaw cycles, and storage time on the integrity of plasma EVs. Hierarchical clustering analysis of plasma samples illustrates the impact of pre-analytical factors on the detection of specific markers associated with small EV (exosomes) and large EV (microsomes): (**A**) High-speed centrifugation, (**B**) Freeze-thaw cycles, and (**C**) Storage time. Proteins shown in grey within the heatmap are not observed. (**D**) The impact on plasma EV counts and size distribution profile by high-speed centrifugation, freeze-thaw cycle, and storage time. The NTA analysis was performed in triplicate, and the EV count is denoted in mean ± sd. (**E**) Voronoi treemaps illustrate the variation of surface proteins on EVs by different storage times, generated using wlab.ethz.ch/surfaceome. Light colors signify low expression levels, while dark colors indicate high expression levels. Genes shown in white within Voronoi treemaps are not expressed. (HS, high-speed; FT, freeze-thaw; Y, years)

EV particles were depleted in high-speed processed plasma. Furthermore, high-speed centrifugation considerably altered the overall protein composition of plasma. Certain protein families, including heat shock proteins, tubulins, HLAs, and integrins, were depleted, while apolipoproteins and complement components were enriched after high-speed processing (Fig. 3). This enrichment likely reflects the inefficiency of high-speed centrifugation in sedimenting all apolipoproteins and complement complexes, while relatively enriching (1.5-2 fold) some abundant plasma proteins, such as albumin and immunoglobulins. A detailed list of depleted and enriched protein families is provided in Table S3.

Limited Freeze-Thaw cycles influence specific protein group abundance but barely affect plasma proteome coverage

Limited freeze-thaw cycles did not significantly alter the total number of detectable proteins, with ~2,500 proteins consistently observed across various freeze-thaw cycles. However, subtle variations in proteomic profiles were observed, particularly in protein abundance. Two distinct protein clusters emerged with opposite patterns with increasing number of freeze-thaw cycles: 49 proteins associated with intermediate filament organization and secretory vesicle pathways were depleted, while 124 proteins linked to the tricarboxylic acid (TCA) cycle, respiratory electron transport, cellular localization, and organelle organization showed enrichment (Figure S1).

Additionally, freeze-thaw cycles influenced the abundance of EV markers. Canonical small EV markers such as ANXA2, FLOT1, Alix, HSPA8, and TSG101, along with large EV markers like ACTN4, C3, VAMP3, and VASP35, display depletion trends. Conversely, other small EV markers (CD9, SDC4, FLOT2) and large EV markers (APOE, ANXA1, ATP5F1A/B, HSP90AA1) demonstrated enrichment in response to freeze-thaw cycles (Fig. 2B). Notably, CD82 (small EV) and CD55 (large EV) were undetectable in non-freeze-thaw (FT-0) plasma but became evident after multiple freeze-thaw cycles. These trends likely stem from freeze-thaw-induced EV degradation, as NTA analysis indicated a loss of approximately 27% EVs following 20 freeze-thaw cycles (Fig. 2D). Lysed EVs lead to the release of EV cargo into plasma, and possibly the lysed EV membranes are released into plasma, which was subsequently enriched by the SAX beads. The depleted EV markers are primarily cytosolic or intracellular membrane-interacting proteins, while enriched markers such as CD9, CD82, FLOT2, and SDC4 are transmembrane proteins (Fig. 2B). The lysis of EVs induced by freeze-thaw allows transmembrane protein-containing membranes to remain enriched by SAX beads and digested by proteases. Remarkably, a subset of large EVs persisted in plasma even after 20 freeze-thaw cycles, highlighting their relative resistance to the freeze-thaw cycle-mediated lysis.

Freeze-thaw cycles did not result in complete depletion or high degree enrichment of any particular protein family. However, several protein families showed a distinct trend of abundance over increasing freeze-thaw cycles. Most individual proteins within the families of, including alpha-actinins, annexins, heat shock proteins, Rab-associated proteins, and Ras-related proteins, were depleted. In contrast, most transmembrane and structural protein families, such as apolipoproteins, integrins, kinesin-like proteins, tetraspanins, and transmembrane proteins (TMEM), were enriched (Fig. 3). This enrichment suggests that even short-term multiple freeze-thaw cycles promote EV lysis, releasing intravesicular cargo into plasma while retaining transmembrane proteins on EV membranes, which robustly interact with the SAX beads used in the Mag-Net proteomics workflow. A detailed list of protein families affected by freeze-thaw cycles is provided in Table S3.

Extended plasma storage time progressively reduces proteome coverage and depletes EV populations and canonical EV markers

Storage time has a significant influence on the plasma proteomic profile and over time systematic reductions in proteome coverage were observed. Compared to the fresh plasma, protein counts decreased by 39%, 49%, and 62% in plasma stored for 2, 5, and 20 years, respectively (Fig. 1B). The number of proteins identified in plasma stored for 20 years was very close to that of fresh plasma processed with high-speed centrifugation, underscoring the detrimental effects of prolonged storage on EV populations.

Volcano plots revealed that among the commonly detected proteins, approximately 418 were depleted and 130 were enriched relative to fresh control plasma (Figure S2A). Depleted proteins are primarily associated with hemostasis, wound healing, RHO GTPase signaling, vesicle transport, and cytoskeletal functions, while enriched

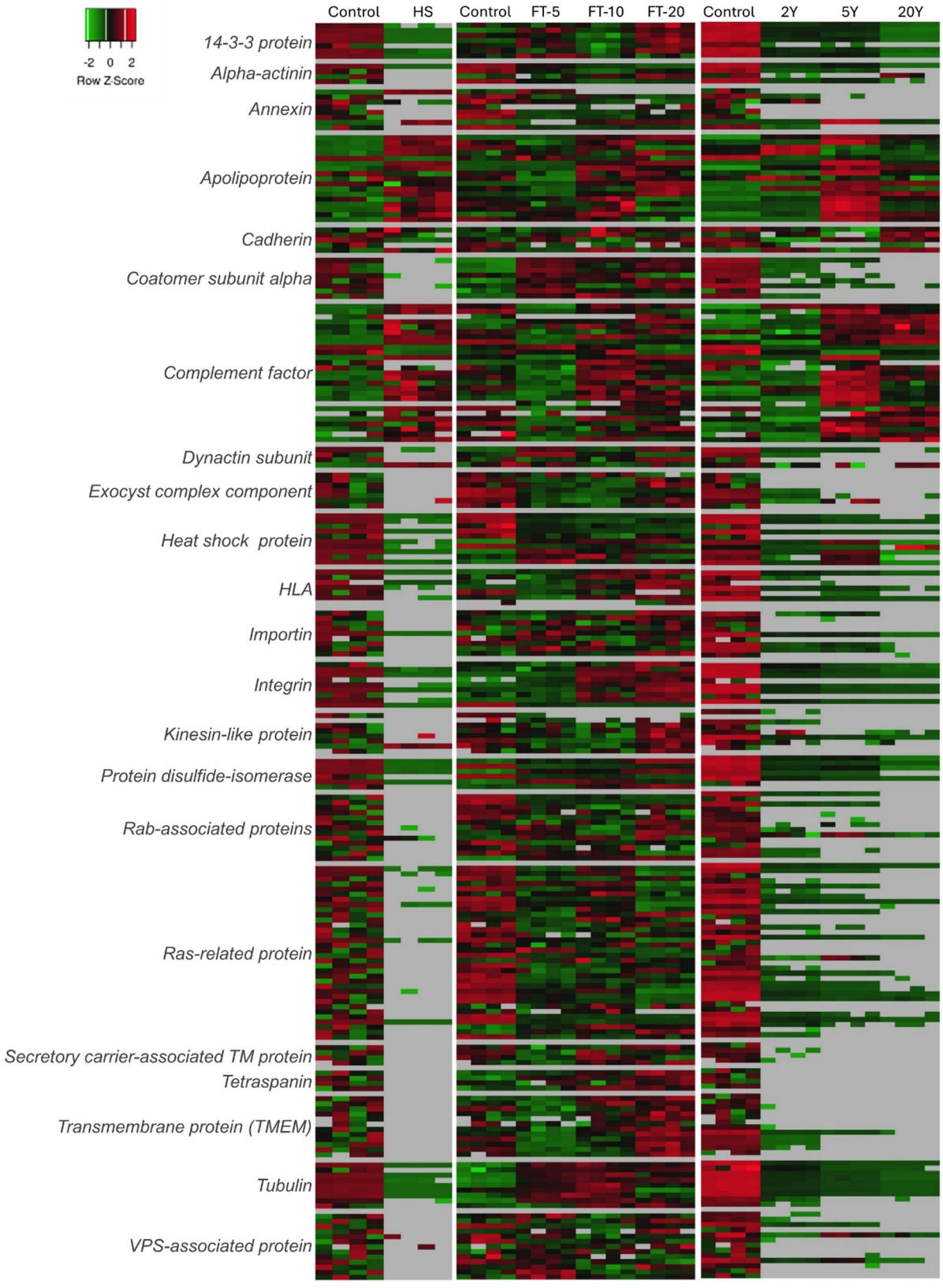

Fig. 3 The influence of high-speed centrifugation, number of freeze-thaw cycles, and storage time on some plasma protein families

proteins are linked to immune processes (Figure S2B). Depleted proteins predominantly are of intracellular, whereas enriched proteins are from extracellular (Figure S3).

Prolonged storage resulted in the progressive depletion of canonical markers for both small and large EVs. Small EV markers, including CD9 and HSPA8, were progressively depleted, while markers such as Alix, FLOT1, and TSG101 were sporadically detected in older plasma. Large EV markers, like HSPA5, ITGB1, and ITGB6, were gradually depleted, while ACTN4, VAMP3, and HLA-B were irregularly identified after 20 years (Fig. 2C). Conversely, increasing storage time gradually enriched markers such as CD44, ANXA2, C3, and APOE. The decline in the EV marker abundance correlated with reduced EV counts, with approximately 50% of EV populations lost in plasma stored for 20 years, possibly due to EV degradation over time (Fig. 2D). Storage at -80 °C may lead to complete degradation of the membranes of some EVs, preventing the enrichment of transmembrane EV markers. In-depth surfaceome analysis of stored plasma EVs revealed significant depletion of integrins, receptor tyrosine phosphatases, leucine-rich repeat-containing proteins, scavenger receptors, solute carriers, channels, and MHCs (Fig. 2E).

Similarly, key plasma protein families, such as 14-3-3 protein, annexins, coatomer subunit alpha, HLAs, importins, integrins, proteasomes, protein disulfide-isomerase, RAS-related proteins, and tubulins were sequentially depleted with prolonged storage (Fig. 3). Enrichment of apolipoproteins and complement factors was observed, indicating only non-EV-associated proteins were enriched with storage time and suggesting intense vesicle degradation under prolonged storage. A detailed list of protein families affected by storage time is provided in Table S3.

Discussion and conclusion

The Mag-Net workflow [17] can also isolate out EVs for characterization, our previous work showed that EVs isolated by this approach had a narrow size distribution but low yield [23], which may not truly reflect the impact of preanalytical factors on EV population, therefore, in this work, the standard ultracentrifugation method was used to isolate EVs for particle size and yield characterization, while the Mag-Net on-bead EV enrichment and digestion workflow was used to obtain peptides for proteomic analysis.

We investigated the impact of pre-analytical variables, including high-speed centrifugation, freeze-thaw cycles, and storage duration, on plasma EVs and the plasma proteomes. High-speed centrifugation, used in high-speed processed plasma preparation, emerged as a primary factor influencing EV proteome coverage, resulting in an almost 3-fold decrease in protein quantity compared to regular plasma. This reduction is primarily attributed to the sedimentation of large EVs and cellular debris during centrifugation, a finding corroborated by NTA analysis. Previous studies have also highlighted the enrichment and depletion of specific protein groups during centrifugation-based EV isolation protocols [29, 30]. These results underscore the necessity for standardizing centrifugation protocols to minimize artifacts and preserve biologically relevant EV populations.

Multiple freeze-thaw cycles can occur during plasma sample processing, especially if the samples are obtained from biobanks. The physical disruption caused by repeated freeze-thaw cycles, including ice crystal formation, leads to the subsequent lysis of EVs and the disruption of protein structures [31]. We found a significant reduction in EV counts after 20 freeze-thaw cycles, consistent with earlier reports of marked decreases in EV concentration [32, 33] and of membrane disruption induced by freeze-thaw cycles [34]. Despite these effects on EVs, freeze-thaw cycles had a minimal impact on the total number of detectable proteins. However, subtle variations in protein abundance were observed, specifically the depletion of intravesicular cargo and the enrichment of vesicular transmembrane proteins, suggesting that EV lysis under the freeze-thaw conditions that we tested was noticeable, but not severe. Therefore, whenever possible, freeze-thaw cycles should be minimized in plasma proteomics.

Keeping the specimen in -80 °C freezer is a common practice for long term storage of plasma by most research laboratories and biobanks, this condition is also recommended for storage of isolated EVs [30]. However, deep freezing conditions can change the contents of lipoprotein fractions and potentially induce sublimation, recrystallization, and alter the biochemical components of plasma [35]. We found prolonged storage of human plasma consistently diminished the EV population and proteome coverage, resulting in a loss of approximately 50% of EV populations, depleted many canonical transmembrane EV markers, and a 60% reduced plasma proteome coverage over 20 years. Despite inherent protein abundance variations among blood donors, plasma samples collected under similar protocols should have similar proteome coverage as demonstrated by several studies the small inter-individual deviation in the coverage of the majority of the plasma proteome [36, 37, 38], then the observed decline in proteome coverage likely resulted from the reduced EV populations affected by the storage time [39, 40, 41]. Study on short-term storage (up to six months) showed that -80 °C storage time-dependently reduces plasma EV concentration, increases the particle size variability, and alters zeta potential [33]. Our results suggest that long-term storage conditions for plasma

need to be limited to ensure the reliability of plasma EV proteomics.

This study was constrained by some inherent limitations that warrant careful consideration. The samples used for control, high-speed centrifugation, and freeze-thaw cycles were from the same donor, while the 2-, 5-, and 20-year samples were pooled from individual, archived samples. The storage time study samples presumably reflect impacts from both storage time and freeze-thaw cycles. Limited freeze-thaw cycles occurred to the 2- and 5-year samples as they were transferred to our laboratory shortly after collection, but we do not have detailed knowledge about the history of the 20-year samples, although we received them frozen from the biobank repository, when or how they were shipped from clinical centers to the repository was not well documented. In addition, despite EDTA was the anticoagulant in all plasma samples used in this study and similar protocols were used in blood collection, donors in each cohort were different, and those clinical differences (age, gender, and health status, etc.) would affect the protein abundance but unlikely could result in majority of plasma proteins being below the limit of detection were the samples all acquired fresh, therefore the results presented in this work mostly reflect impact of the studied preanalytical factors on the EV-enriched vesicle population/plasma proteome, but we cannot rule out the inherent variations introduced by each clinical study, despite pooling somewhat normalized the biological variation between individual donors. A comprehensive evaluation of preanalytical variables was also not fully realized in this study. While we investigated the effects of high-speed centrifugation, freeze-thaw cycles, and extended storage, we overlooked other pivotal factors known to influence plasma and EV integrity. These include the choice of anticoagulant, the duration between venipuncture and processing, and precise centrifugation parameters (e.g., different g-forces and spin times), all of which can introduce significant variability to the plasma proteome or EV population. Further, the analytical scope of the study was primarily descriptive, focusing on proteome coverage and shifts in EV populations without delving into the functional ramifications of these alterations. Furthermore, although 82 out of the top 100 ExoCarta-listed EV markers, including canonical EV marker proteins such as Alix, Annexin A2, CD9, TSG101, Flottilin-1, HSPA8 were identified using the Mag-Net proteomics workflow and further being used to assess the impact of preanalytical factors, by no means this EV-enriched proteomics workflow only identifies EV-related proteins as many other proteins were identified as well, including the greatly depleted abundant plasma proteins. However, given that there is no method that can isolate pure EVs from human plasma, we chose the Mag-Net approach to profile the proteomic changes of the EV population impacted by preanalytical factors. This is because, to our knowledge, it is one of the most reproducible and comprehensive plasma proteomics methods that are tailored for the EVs [17, 23]. Finally, the employment of the Mag-Net protocol, which enriches a broad population of EVs or EV-like vesicles, lacks the specificity required to differentiate between distinct EV subtypes (e.g., exosomes vs. microvesicles). Given the differential biogenesis, cargo, and potential clinical utility of these subpopulations, this lack of resolution in particle size prevents a nuanced interpretation of how specific pre-analytical variables affect distinct EV classes, a critical detail for the development of EV-based diagnostics and therapeutics.

In conclusion, this study underscores the importance of standardizing sample handling protocols to ensure reliable plasma EV proteomic analysis and interpretation. It demonstrates that preanalytical factors in plasma proteomics, such as high-speed centrifugation, freeze-thaw cycles, and storage, have a significant impact on the heterogeneity of plasma EVs and their proteomic profile. Among these, high-speed centrifugation is the most disruptive factor, markedly altering proteome coverage and EV characteristics. Freeze-thaw cycles, conducted within a relatively short amount of time (days), have a minimal impact on overall protein coverage but can have subtle alterations in specific EV markers. Prolonged storage time (years to decades) can drastically reduce the number of EV particles and proteome coverage. While these factors might be less significant for whole plasma proteomics, they are vital in plasma EV-focused studies, as preanalytical discrepancies can alter the EV cargo, including proteins and nucleic acids. This concern is particularly pressing in retrospective, longitudinal cohort studies, where longer storage time can reduce plasma EV population for samples collected at early time points if all samples are analyzed collectively post-collection.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

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Author contributions

P.S.S. performed the experiments, analyzed data and wrote the manuscript; Q.Z. designed and supervised the study, acquired funding, and wrote the manuscript.

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Data availability
The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium ***via*** the PRIDE 42 partner repository with the dataset identifier PXD065458.

Declarations

Competing interests
The authors declare no competing interests.

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