Journal of Proteomics 150 (2017) 109–120

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Multicentric study of the effect of pre-analytical variables in the quality of plasma samples stored in biobanks using different complementary proteomic methods

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ARTICLE INFO

Article history:
Received 11 July 2016
Received in revised form 6 September 2016
Accepted 7 September 2016
Available online 09 September 2016

Keywords:
Plasma
Proteomics
Biobank
Sample
Pre-analytical variables

ABSTRACT

Analytical proteomics has experienced exponential progress in the last decade and can be expected to lead research studies on diagnostic and therapeutic biomarkers in the near future. Because the development of this type of analysis requires the use of a large number of human samples with a minimum of quality requirements, our objective was to identify appropriate indicators for quality control of plasma samples stored in biobanks for research in proteomics. To accomplish this, plasma samples from 100 healthy donors were obtained and processed according to the pre-analytical variables of: a) time delay for the first centrifugation of the original blood sample (4 or 24 h) and b) number of freeze/thaw cycles (1, 2 or 3) of the processed plasma samples. The analyses of samples were performed by different and complementary methods such as SPE MALDI-TOF, DIGE, shotgun (iTRAQ, nLC MALDI TOF/TOF) and targeted nLC MS/MS proteomic techniques (SRM). In general, because the distribution of proteins in all samples was found to be very similar, the results shown that delayed processing of blood samples and the number of freeze/thaw cycles has little or no effect on the integrity of proteins in the plasma samples.

Significance: The results of the present work indicate that blood proteins in plasma are broadly insensitive to such preanalytical variables as delayed processing or freeze/thaw cycles when analyzed at the peptide level. Although there are other studies related to protein stability of clinical samples with similar results, what is remarkable about our work is the large number of plasma samples examined and that our analyses assessed protein integrity by combining a wide set of complementary proteomic approaches performed at different proteomic platform participating laboratories that all yielded similar results. We believe our study is the most comprehensive performed to date to determine the changes in proteins induced by delayed sample processing and plasma freeze/thaw cycles.

1. Introduction

The remarkable proliferation in biomedical research taking place is driving the need for increased quality of storage and handling of large numbers of biological samples, including tissues, tumors, cells, proteins,

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http://dx.doi.org/10.1016/j.jprot.2016.09.003
1874-3919/

DNA, blood, serum, and urine, to insure suitability for further studies [1]. Biobanks house collections of biological samples intended for diagnostic or biomedical research and have the proper infrastructure and technology to ensure high quality of the samples [2]. To facilitate establishment of collaborations and joint projects among different researchers, biobanks collect samples from donors with a specific pathology and from healthy controls to reduce turnaround time and validation of investigations [3]. However, the sizeable storage of biological samples raises complex technical issues that affect different procedural steps, including, among others, sample collection, transport, identification, traceability, storage at different temperatures, recovery of the stored sample and processing of the data. Because it is essential that information on the handling of biological materials be complete and accurate, it is necessary to develop standardized protocols [4].

Heterogeneity in sampling and storage procedures can introduce significant variability in the molecular composition of biological samples, thus interfering with or affecting reproducibility [5]. This issue becomes even more evident in research studies in which biological samples from different origins are employed. In fact, some authors have shown that differences detected in retrospective collections of biological samples are more dependent on pre-analytical factors (e.g., processes taking place at different points of the experimental workflow) than on the inherent differences among the samples [6]. For example, the Biospecimen Science Working Group of the ISBER (International Society for Biospecimen Science) [7] retrospectively reviewed several research studies using stored biological samples. They found that the heterogeneity in the protocols for collection and storage made it very difficult to draw reliable conclusions about the usefulness of the samples for the identification of biomarkers. Therefore, the identification of clinically relevant biomarkers can only be successful if biological samples are obtained, processed and stored according to strict standard protocols [8,9].

Furthermore, in parallel with the definition of a standard protocol for collection and storage of biological samples, it is essential to identify specific biomarkers that can be used as quality controls to assess the effects of pre-analytical factors on variability in the results [10]. However, quality criteria cannot be specifically defined because they are dependent both on the sample type and the experimental purpose.

Plasma from human subjects is a promising source for biological markers of disease [11], it is a complex biological fluid containing various biomolecules (lipids, peptides, amino acids, cytokines, proteases, enzymes, antibodies, etc.) with very different physicochemical properties. Despite progress made in the study of the plasma proteome, to date, no usable protein markers for monitoring the quality of these samples have been described. Thus, it is necessary to define a panel of protein markers that establishes minimal quality standards for the analytical phase and excludes those samples whose integrity is compromised by a defective pre-analytical phase [12]. Among the variables involved in the collection, processing and storage procedures, we must first examine those that potentially compromise the quality of the samples and establish a set of parameters aimed at reducing variability at critical sampling points.

Following blood sample extraction, the critical variables to consider include the time between sample collection and processing, the freezing protocol and freezing temperature, the storage duration and number of freeze/thaw cycles. In the time lapse between sample collection and processing, modifications of peptides and proteins or release of cell-derived substances may occur, suggesting that shorter processing times correlate with more reliable samples [13,14]. For example, serum values of the CD40 ligand increased with delayed processing and prolonged storage of serum samples or decreased after prolonged storage of serum samples at elevated temperature [15]. The effect of repeated freeze-thaw cycles on sera was also analyzed in an inconclusive attempt to find a marker for the quality of serum cryopreservation [16]. Another important factor potentially affecting quality and stability of samples is storage time [17] and temperature [18]. It is known that some biomarkers are not sensitive to prolonged or inappropriate temperature storage, but others may degrade spontaneously. In spite of all the abovementioned studies, we conclude that, thus far, the search for potential quality control indicators for plasma proteomics is far from completion.

Analytical proteomics has experienced extensive progress in the last decade because of the emergence of MS-based techniques (MALDI-TOF, iTRAQ) that combined with classical techniques for protein separation (e.g., chromatography, two-dimensional gel electrophoresis, etc.) facilitate the identification and characterization of proteins. Proteomics is expected to be the tool of choice for the search for diagnostic or therapeutic biomarkers and for the identification and characterization of the proteins encoded by the genome [19]. One of the main objectives of proteomics is the identification of markers of disease by comparing the protein status in normal and pathological conditions. It is noteworthy that the control of pre-analytical effects, which can produce a significant influence on the results of analyses, is a critical point for proteomic studies [5,20].

By using different complementary proteomic techniques in separate coordinated laboratories, the aim of this study was to search for quality markers that would allow assessment of the integrity and purity of plasma proteome samples. Thus, recommendations could be established to include or exclude samples of plasma for proteomic studies according to their quality parameters.

2. Materials and methods

2.1. Design of the study and donors

The study was carried out using prospectively obtained peripheral blood samples from a cohort of healthy donors through participation of seven different Spanish Biobanking Facilities. The sample population ($n = 100$) was comprised of 50 men and 50 women. Distribution by age of the cohort was 20–40 ($n = 40$), 40–60 ($n = 40$), and 60–80 ($n = 20$) years. This study was approved by the Ethics Committee of Clinical Investigation of Galicia (Spain).

2.2. Collection of plasma samples from peripheral blood

EDTA-Blood samples (40 mL) from each donor were obtained and processed by the participating biobanks applying the same standard operating procedure (SOP) for collection and storage of platelet-poor plasma. The SOP was based on the recommendations of the Human Proteome Organization (HUPO) [12], but including some variables detailed in the next section. Once obtained, the plasma samples were distributed into 200 μL aliquots (ProteinLoBind Tube 0.5 mL, Eppendorf AG©, Germany) and stored at −80 °C for later analysis.

2.3. Variables studied

Briefly, EDTA-blood tubes were subjected to different pre-analytical conditions according to the following variables:

1) Time between blood collection and processing, to evaluate the effect of the delay in the first centrifugation step in plasma protein composition. Two conditions were considered: a) Delay of less than 4 h at room temperature after blood extraction ($t \leq 4$ h) (representative of the daily routine of a laboratory of clinical analysis that process local samples) or b) 24 h delay ($t = 24$ h) at 4 °C (reflecting those samples arriving from external laboratories).

2) Repeated freeze/thaw cycles, to evaluate the influence of this variable on plasma protein integrity. To that end, plasma samples ($t \leq 4$ h) were subjected to 1, 2 and 3 cycles of freezing and thawing. Thawing was performed leaving the samples for 1 h at room temperature. Thawing was for 1 h at room temperature with any subsequent freeze/thaw cycle done after 24 h.

For proteomics analyses, samples were pooled and compared as follows: a) Mix of 100 plasma samples ($t \leq 4$ h) that were subjected to one

(P4.D1); two (P4.D2); or three freeze/thaw cycles (P4.D3); or b) mix of 100 plasma samples (t = 24 h) subjected to one cycle of freezing and thawing (P24.D1). Fig. 1 summarizes the whole work-flow.

2.4. Immunodepletion of abundant proteins

Prior to the proteomic analysis, pooled plasma samples were depleted of the most abundant plasma proteins using commercial immunoaffinity columns. Briefly, 30 μL of each sample pool was diluted to a final volume of 240 μL with mobile phase A (Agilent Technologies©, Santa Clara, CA, USA) and filtered through a 0.22 μm cellulose acetate membrane (CLS810, Costar Spin-X, Corning©, Tokyo, Japan) at 5000 ×*g* for 15 min to remove insoluble material and debris. The filtered flow-through was collected and processed to deplete the fourteen most abundant plasma proteins using a M.A.R.S. Hu-14 (Agilent Technologies) in a 1200 HPLC system (Agilent, Wilmington, DE, USA), following the manufacturer's instructions. Individual 150 μL injections were performed and the flow-through fractions (non-binding proteins) containing the lower-abundance proteins were collected in a final volume of approximately 600 μL. The protein concentration was determined by direct absorbance measurement at 280 nm. Samples were then aliquoted and stored at −80 °C until delivery to the participating laboratories.

2.5. Solid phase extraction (SPE) and maldi-tof-tof ms analysis

After delivery, the depleted plasma samples were stored at −80 °C. Protein concentrations were normalized at 1 mg/mL (±5%) by the addition of 0.1% trifluoroacetic acid (TFA) from Pierce (Thermo Fisher Scientific Inc.©, Waltham, MA, USA) in water. The samples were divided into two sets, one set for each of the two SPE methods, using two different ZipTip (EMD Millipore©, Billerica, MA) affinities: C4 and C18 reverse phase resins. 4 μL of each sample were added for each ZipTip affinity, and the flow-through was discarded. Following this step, samples were washed with 4 μL 0.1% TFA, and finally, the peptides were eluted from the microcolumns with 4 μL of HCCA matrix (Alfa-Aesar© Karlsruhe, DE), 5 mg/mL in 70:30 ACN:0.1% TFA, onto a 0.5 mL Eppendorf tube (Eppendorf AG©, Germany). Four technical replicates of 1 μL for each sample were spotted, 1 μL each, onto an Anchorchip 800/384 (Bruker Daltonics©, Bremen, DE) MALDI target.

An Autoflex III MALDI-TOF mass spectrometer was used in linear geometry for spectra acquisition up to 20.000 Th as previously described [21,22]. Spectral representation was made using ClinProt Tools 2.2 (Bruker Daltonics©, Bremen, DE) software. Spectra were mathematically processed by the following: baseline correction with Top Hat algorithm (using 5.0% of minimal linear base); smoothing with Savitsky-Golay algorithm (using peak width of 2.0 (*m*/*z*) and 5 processing cycles); and recalibration using up to 10 of the most intense peaks.

2.6. Differential gel electrophoresis (dige) and LC-MS/MS analysis

2.6.1. Two-dimensional (2D)-DIGE

Fifty micrograms of each depleted pooled-plasma sample was labeled with 400 pmol of CyDye DIGE Fluor minimal dyes (GE Healthcare©, Little Chalfont, UK) according to the manufacturer's instructions (Cy3 and Cy5 for samples and Cy2 for an internal control using equal parts of all samples). Pairs of samples were reverse-labeled to eliminate the possibility of dye-labeling bias and then resolved by 2D-DIGE as previously described [23]. Following Decyder analysis, differential spots were selected for protein identification when the p-value after ANOVA or t-test was <0.05. Proteins were visualized by staining with SYPRO Ruby Protein Gel Stain (Bio-Rad, Hercules, CA, USA) and differentially identified spots were excised manually. In-gel

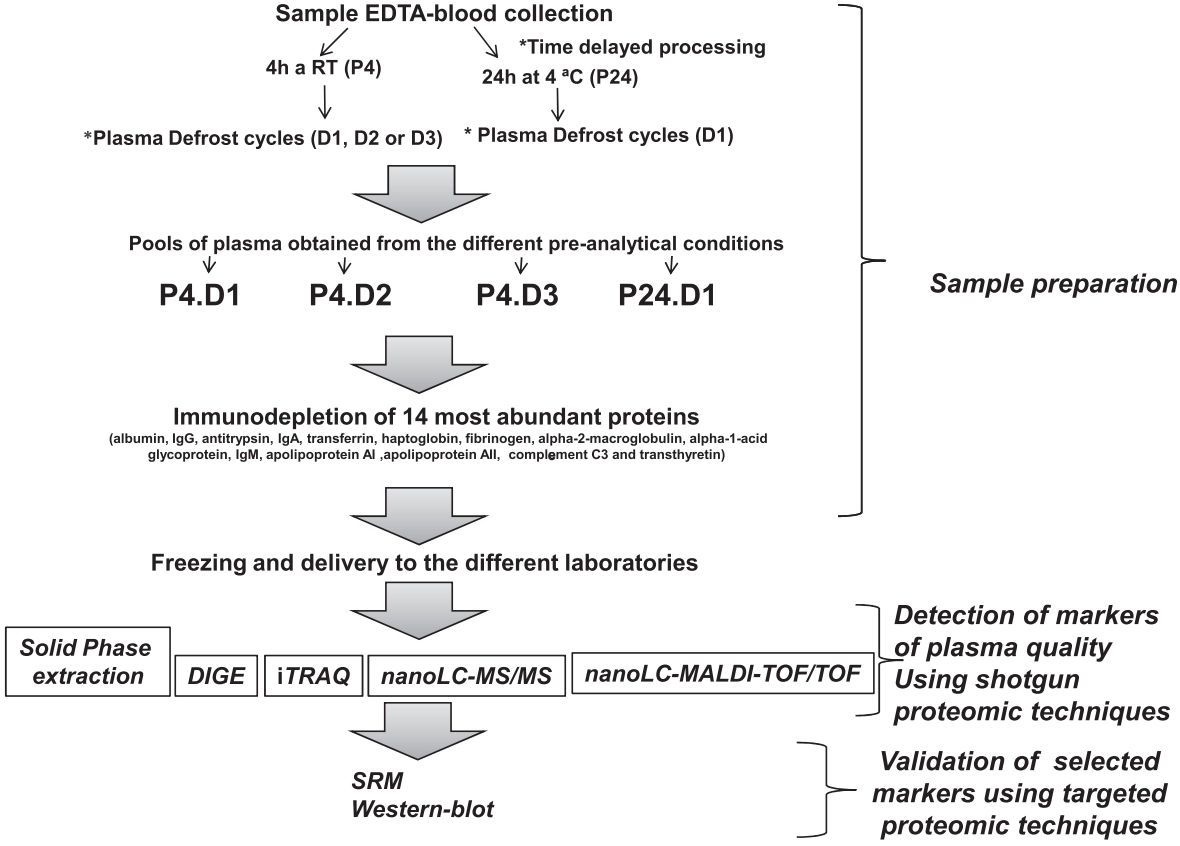


Fig. 1. Work-flow for sample preparation and analysis. Blood samples were processed following standard procedures with less than 4 h of delay after extraction (P4: t ≤ 4 h) and subjected to 1, 2 or 3 freeze/thaw cycles. Alternatively, blood samples were processed following a 24-hour delay (P24) at 4 °C. Samples were pooled and immuno-depleted to obtain the flow-through fraction enriched in lower-abundance proteins. Frozen samples were then distributed to the participating laboratories.

tryptic digestion was performed using 10 ng/μL sequencing grade modified trypsin (Promega Corporation©, Madison, WI, USA) in 50 mM ammonium bicarbonate overnight at 37 °C, after denaturation with DTT (10 mM, 30 min, 40 °C) and an alkylation step with iodoacetamide (25 mM, 30 min, room temperature). The resulting peptides were extracted with 1% formic acid, 50% ACN and evaporated to dryness prior to LC-MS/MS analysis.

2.6.2. LC-MS/MS analysis

For each spot, a total volume of 5 μL of the solution of tryptic digestion peptides was injected with a flow rate of 300 nL/min in a nanoLC Ultra1D plus (ABSciex©, Framingham, MA, USA). A trap column, the Acclaim PepMap100 (100 μm × 2 cm; C18, 2 μm, 100 Å) and an analytical column, the Acclaim PepMap RSLC (75 μm × 15 cm, C18, 5 μm, 100 Å) from Thermo Scientific (Thermo Fisher Scientific Inc.©, Waltham, MA, USA) were used following the next gradient: 0–1 min (5% B), 1–50 min (5–40% B), 50–51 min (40–98% B), 51–55 min (98% B), 55–56 min (5% B), 56–75 min (5% B). (Buffer B: 100% ACN, 0.1% formic acid, Buffer A: 0.1% formic acid). MS analysis was performed on a Q-TRAP 5500 system (ABSciex, Sciex©, Framingham, MA, USA) with a NanoSpray® III ion source (ABSciex) using Rolling Collision Energy in positive mode. MS/MS data acquisition was performed using Analyst 1.5.2 (AB Sciex) and submitted to Protein Pilot software (ABSciex) against UniprotKB/Swiss-Prot database, using the Paragon™ Algorithm and the pre-established search parameters for 5500 QTRAP.

2.7. ITRAQ analysis

2.7.1. Trypsin digestion and iTRAQ labeling

12 μg of protein from each depleted plasma sample were precipitated using the methanol/chloroform method. Protein pellets were resuspended and denatured in 20 μL 7 M urea, 2 M thiourea, 100 mM triethylammonium bicarbonate (TEAB) buffer, pH 7.5, reduced with 2 μL of 50 mM TCEP ((tris-(2-carboxyethyl)phosphine; ABSciex©, Framingham, MA, USA), pH 8.0, at 37 °C for 60 min. Cysteines were then blocked by incubation with 1 μL of 200 mM methyl methanethiosulfonate (MMTS; Pierce, Thermo Fisher Scientific Inc.©, Waltham, MA, USA). for 10 min at room temperature. Samples were diluted up to 140 μL to reduce urea concentration with 25 mM TEAB. Trypsin (Sigma-Aldrich Co©, Saint Louis, MO, USA) was added at a 1:20 ratio and digestion was left to proceed overnight at 37 °C. The resulting peptides were subsequently labeled using the iTRAQ 4-plex kit (ABSciex©, Framingham, MA, USA) following manufacturer's instructions and identified: P4.D1, tag-114; P4.D2, tag-115; P4.D3, tag-116 and P24.D1, tag-117. After labeling, the samples were pooled, speedvac dried and stored at −20 °C for further analysis.

2.7.2. Reversed phase HPLC

The iTRAQ labeled peptides were fractionated by basic rpHPLC in a SmartLine (Knauer) HPLC system using an XBridge C18 column (100 × 2.1 mm, 5 μm particle diameter; Waters). Solvent A was 10 mM ammonium hydroxide (pH 9.4); and solvent B was 80% methanol, 10 mM ammonium hydroxide (pH 9.3). Peptides were fractionated using a linear gradient of 0–100% buffer B at 150 μL/min for 90 min. Thirty fractions were collected and pooled into 5 fractions using the fraction mixing strategy n + 1 (i.e., fractions 1 + 6 + 11 + 16 + 21 + 26). The peptide fractions were vacuum-dried, desalted using a SEP-PAK C18 Cartridge (Waters) and stored at −20 °C before LC-MS analysis.

2.7.3. LC-ESI-MS/MS and data analysis

A 2 μg aliquot of each resulting fraction was subjected to LC-ESI-MS/MS analysis as described elsewhere [24], using a nanoLC Ultra 1D plus (ABSciex©, Framingham, MA, USA) coupled online to a 5600 Triple TOF mass spectrometer (ABSciex©, Framingham, MA, USA). The MS and MS/MS data obtained were analyzed as a previously described workflow [24]. The confidence interval for protein identification was set to ≥95% ($p < 0.05$) and peptides were filtered at a false discovery rate (FDR) ≤ 1%. For the quantitative analysis a 5% FDR threshold was applied.

2.8. SDS-PAGE followed by LC-ESI-MSMS

2.8.1. Protein separation by SDS/PAGE and trypsin digestion

Individual depleted pooled plasma samples containing 50 μg of protein were loaded onto and separated by pre-cast tris-gly gels of 4–20% polyacrylamid (Thermo Fisher Scientific Inc.©, Waltham, MA, USA). After electrophoresis, the gels were stained with Coomassie blue and each lane was sliced just below the 20 kDa marker. Samples were digested with sequencing grade trypsin (Promega Corporation©, Madison, WI, USA) as described elsewhere [25]. The digestion was stopped with TFA (1% final concentration) and a double peptide extraction was done with ACN. The peptides were dried and dissolved with 9 μL of 2% ACN; 0.1% TFA.

2.8.2. LC-MS/MS analysis

The peptides were then analyzed by LC-MS/MS by loading 5 μL of each sample onto a trap column (NanoLC Column, 3 μ C18-CL, 75 μm × 15 cm; ABSciex©, Framingham, MA, USA) and desalted with 0.1% TFA at 2 μL/min for 10 min. Afterwards, the peptides were transferred onto an analytical column (LC Column, 3 μ C18-CL, 75 μm × 25 cm, ABSciex©, Framingham, MA, USA) equilibrated in 5% ACN 0.1% formic acid (FA). Elution was carried out with a linear gradient of 5–35% B (ACN, 0.1% FA) in A (0.1% FA) for 60 min at a flow rate of 300 nL/min. Peptides were analyzed in a mass spectrometer nanoESI qQTOF (5600 TripleTOF, ABSciex©, Framingham, MA, USA). The tripleTOF was operated in an information-dependent acquisition mode, in which a 0.25-s TOF MS scan from 350 to 1250 *m/z*, was performed, followed by 50 ms product ion scans from 100 to 1500 m/z on the 50 most intense 2–5 charged ions.

Protein identification was performed with ProteinPilot v4.5 search engine (ABSciex©, Framingham, MA, USA). ProteinPilot default parameters were used to generate a peak list directly from 5600 TripleT of wiff files. The Paragon algorithm of ProteinPilot was used to search the Expasy protein database with the following parameters: trypsin specificity, cys-alkylation, taxonomy restricted to human, and the search effort set to "thorough". Identified proteins were grouped in quartiles according to their expected molecular weights. The number of proteins and the coverage in each quartile were determined.

2.9. LC-MALDI-TOF/TOF

2.9.1. Protein separation by SDS-PAGE

Individual depleted pooled-plasma samples of 30 μg of protein for each pool, in triplicate, were loaded and separated by SDS-PAGE in 10% acrylamide in-house gels in a Protean® mini-gel system (Bio-Rad, Hercules, CA, USA). Following Coomassie blue staining to check protein integrity, each lane was size-fractioned into 6 sections below the 65 KDa molecular weight marker. Sections were subsequently processed independently. Each section was de-stained with methanol, diced into small pieces and in-gel digested following standard procedures [24]. Peptides were then extracted, vacuum-dried, reconstituted in 0.1% TFA and de-salted using home-made stage-tips [26].

2.9.2. LC-MALDI-TOF/TOF analysis

Peptide fractions were separated using reversed phase chromatography in a nanoLC system (Tempo, Eksigent, Dublin, CA, USA) by loading through a trapping column into a C18 silica based column (New Objective, Woburn, MA, USA) with an internal diameter of 75 μm and a pore size of 300 Å. Peptides were eluted at a flow rate of 0.35 μL/min during a 1 h linear gradient from 2 to 36% ACN (mobile phase A: (0.1% TFA, 2% ACN; mobile phase B: 0.1% TFA, 95% ACN), mixed with α-cyano matrix (4 mg/mL at a flow rate of 1.2 μL/min) and deposited onto a MALDI plate

using an automatic spotter (SunCollect, Sunchrome, Friedrichsdorf, Germany). Chromatograms corresponding to each gel section were composed of 240 spots, each one comprising a 15 s deposition. The MS runs for each chromatogram were acquired and analyzed in a MALDI-TOF/TOF instrument 4800 (ABSciex, Sciex©, Framingham, MA, USA) using a fixed laser intensity of 3400 kV and 1000 shots/spectrum. Automated precursor selection was done using a Job-Wide interpretation method (up to 20 precursors per fraction with a signal-to-noise lower threshold of 50) with a laser voltage of 4400 and 2000 shots/spectrum at medium CID collision energy range.

The complete MS and MS/MS raw data were processed and peptide identification was performed against uniprot_sprot database (Release 2015_07 with 548,872 entries) using the Mascot server on-line (www.matrixscience.com). Search parameters included carbamidomethylation of cysteines (fixed modification), oxidation of methionine (variable modification, ≤4), up to one trypsin miscleavage, precursor ion mass range 800–4000 Da, ±50 ppm tolerance on precursor and ±0.3 Da on fragmentation of ions. A Mascot server was used to calculate the exponentially modified protein abundance index (emPAI) [27] as an estimation of the absolute protein amount. The individual emPAI indexes from each of the three experimental replicates were then used to calculate an average emPAI index for each protein.

2.9.3. Selected reaction monitoring (SRM)

Proteins from depleted plasma samples were precipitated (Ready Prep Cleanup Kit-BioRad) and the pellet was resuspended in 7 M urea and diluted 1:10 with 50 mM ammonium bicarbonate. 20 μg of protein was digested overnight as indicated previously. Peptide desalting, concentration and purification were performed using C18 ZipTip® pipette tips (Millipore), according to the manufacturer's instructions. The peptides were resuspended in 2% ACN/0.1% formic acid. Each sample was spiked with an exogenous peptide (LVIDEPTK from bovine hepatoma-derived growth factor (HDGF)), which was used as an internal standard for chromatographic performance. Proteotypic peptides were selected by in silico digestion using MRM Pilot software (ABSciex©, Framingham, MA, USA). Peptides containing cysteine or methionine residues were excluded. Three to five transitions were programmed for each peptide: EFTPPVQAAYQK, LHVDPENFR, LLVVYPWTQR, SAVTALWGK, VLGAFSDGLAHLDNLK and VNVDEVGGEALGR for Hemoglobin B-Human. SRM experiments were performed on a 5500 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer (ABSciex©, Framingham, MA, USA) as described elsewhere [28]. Data analysis was performed using Analyst 1.5.2 and MultiQuant 2.0.2 software (ABSciex©, Framingham, MA, USA). The mean area of all transitions was obtained for each peptide. The value of area ratio (analyzed peptide/exogenous peptide) was calculated.

Variations in the levels of 33 peptides using the corresponding standards [29] that were spiked into each trypsin digested plasma sample (Supplementary Table 1), were monitored in a different laboratory using similar conditions (Supplementary material 1). Data analysis was performed with Skyline [30].

2.10. Western blot

20 μg of non-depleted pooled samples were loaded into pre-cast tris-gly gels AnyKD (Bio-Rad Laboratories Inc.©, Hercules, CA, USA). After electrophoresis, the gels were incubated in transfer buffer (Bio-Rad Laboratories Inc.©, Hercules, CA, USA) for 10 min. Transfer was

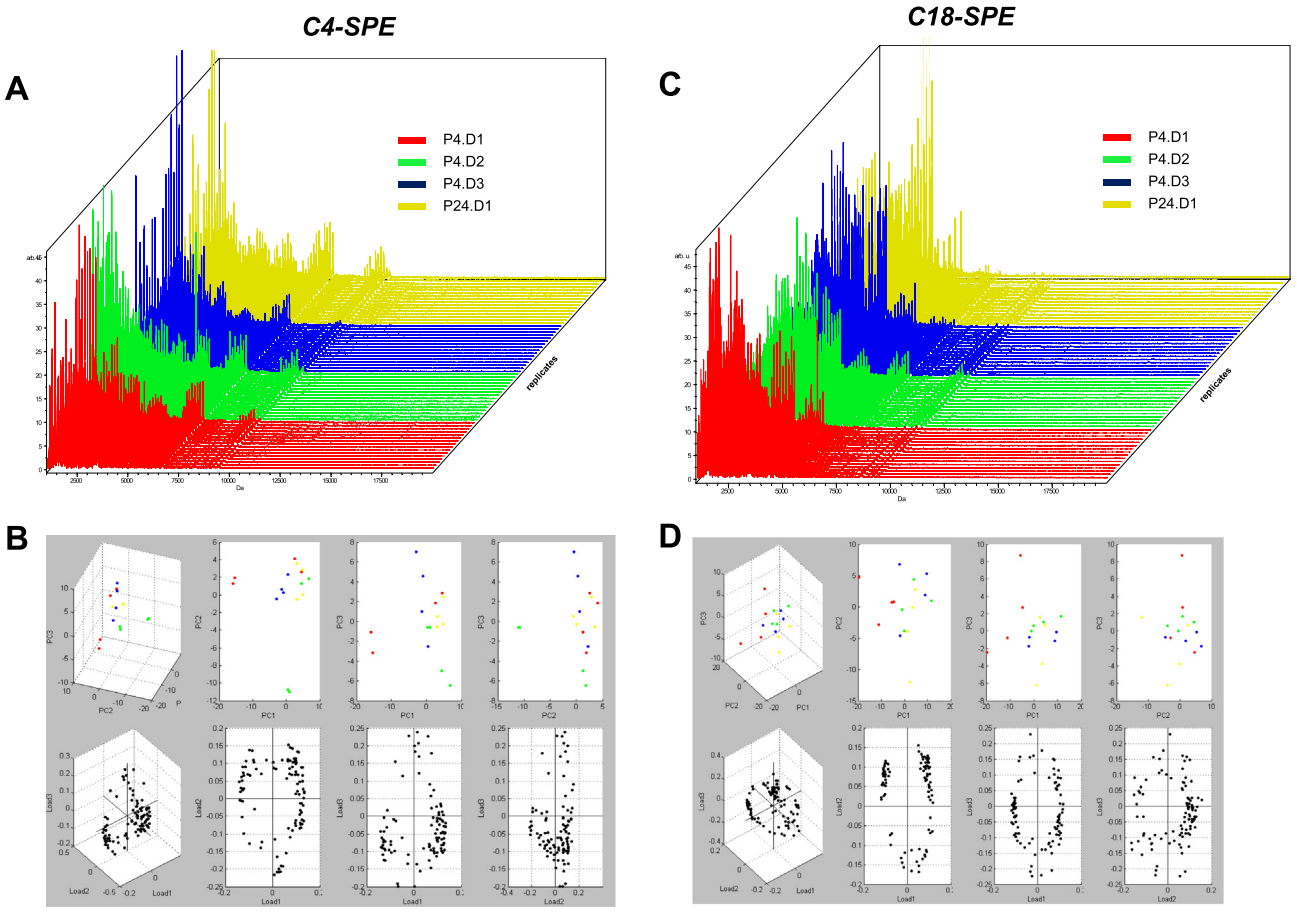


Fig. 2. Solid phase-extraction (SPE) and MALDI-TOF MS analysis of C4 and C18-extracted depleted samples. A, C) MALDI profiling of (A) C4- and (C) C18-extracted pools. The diagrams represent the intensity of the signal (Y axis, arbitrary units) along the correspondent mass window (X axis, Th) of the different technical and experimental replicates (Z axis). B, D) Principal component analysis (PCA) of (B) C4- and (D) C18-extracted pools to reveal putative segregation and hierarchical clustering of the samples.

performed in a Bio-Rad minitransfer system using a PVDF membrane previously activated with methanol for 1.5 h at 200 mA. After transfer, the gels were fixed in 40% ethanol/10% acetic acid and stained. Membranes were blocked overnight with 5% BSA (Roche Diagnostics©, S.L, Basilea, Switzerland) in TBS buffer (Bio-Rad Laboratories Inc.©, Hercules, CA, USA) at 4 °C. Membrane washing was performed with TBS buffer–Tween20 (5%). The primary antibodies were monoclonal antibodies anti-Apo A1 (Santa Cruz Biotechnology, Inc.©, Dallas, TX, USA), anti-kininogen 1 (Abcam©, Cambridge, UK) and anti-vitronectin (Abcam©, Cambridge, UK) all diluted in 5% TBS-Tween20.

3. Results

3.1. Immunodepletion of plasma samples prior to proteomic analysis

Factors in handling samples that we assessed by proteomic analyses were the influence of time from blood collection to plasma isolation, <4 h at RT or after a delay of 24 h at 4 °C, and the effect if 1, 2 or 3 freeze/thaw cycles. Prior to proteomic analysis, we subjected the plasma pools to chromatographic immunodepletion because this extra step permits wider coverage of the proteome of serum and other physiological fluids [31,32]. Initial preparatory experimental proofs showed a highly reproducible pattern of the depletions with no carry-over between injections (data not shown). Analytical injections were done randomly and blanks were inserted between samples.

3.2. Solid phase extraction (SPE) and MALDI-TOF MS analysis

MALDI-profiling was used to find alterations in the mass patterns of the four pooled plasma samples studied. In all cases, the coefficient of variation (CV) during the extraction process and between the technical replicates did not exceed 21% and 6%, respectively (data not shown); this is in agreement with previous reports [21,33].

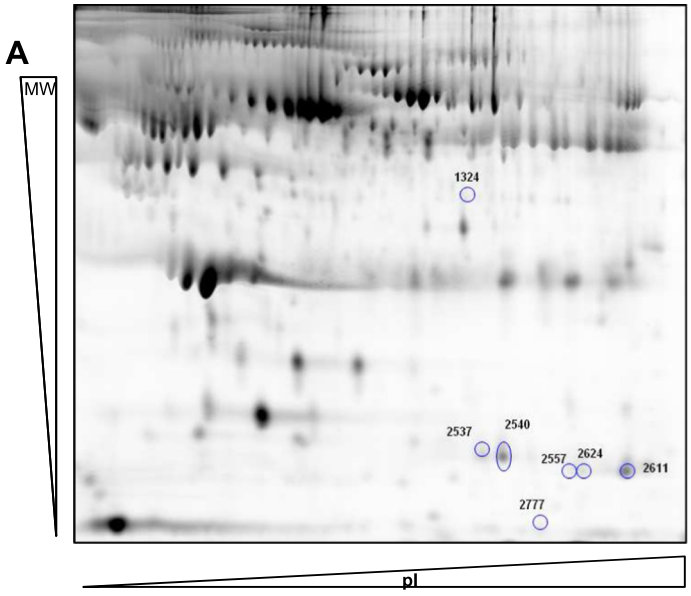
The results, corresponding to a 1000–30,000 Th. window, are represented in Fig. 2. The spectral representation of the C4-enriched fraction showed no visible differences among the four samples (Fig. 2A). A principal component analysis (PCA) (Fig. 2B) did not reveal any significant segregation or hierarchical clustering, although some subtle differences were observed for the P24.D1 sample.

The spectral representation of the C18-enriched fractions demonstrated, as expected, a decrease in the number of peaks in the 10,000–30,000 Th. range and an increase in the 1000–10,000 Th. range (Fig. 2C). Again, no significant differences were detected among the four samples and no segregation or hierarchical clustering was observed after PCA analysis (Fig. 2D).

To summarize, defrost cycles in the samples tested using the MALDI-Profiling SPE with prior enrichment by reverse phase C4 or C18 resins showed no significant differences in the observed spectra profiles. Slight non-significant differences were detected in the P24.D1 samples, suggesting that minor alteration in the proteins present in the plasma might occur after a 24-h delay in processing.

3.3. Differential in gel electrophoresis (DIGE) and selected reaction monitoring (SRM) analysis suggest that hemolysis occurs after delay in the processing of the initial blood samples

The DIGE analysis was expected to detect plasma protein degradation, through the detection of increasing or decreasing spot intensities in the bi-dimensional gel electrophoresis protein map. The image analysis of the results (Fig. 3A) showed several low molecular weight



B

Spot #	Name	SwissProt ID	Mascot score	% Seq. Cov.	# of matched peptides
2537	Hemoglobin subunit beta	HBB_HUMAN	452	87	11
2540	Hemoglobin subunit beta	HBB_HUMAN	912	76	9
2557	Hemoglobin subunit alpha	HBA_HUMAN	72	25	3
2611	Hemoglobin subunit alpha	HBA_HUMAN	337	64	6

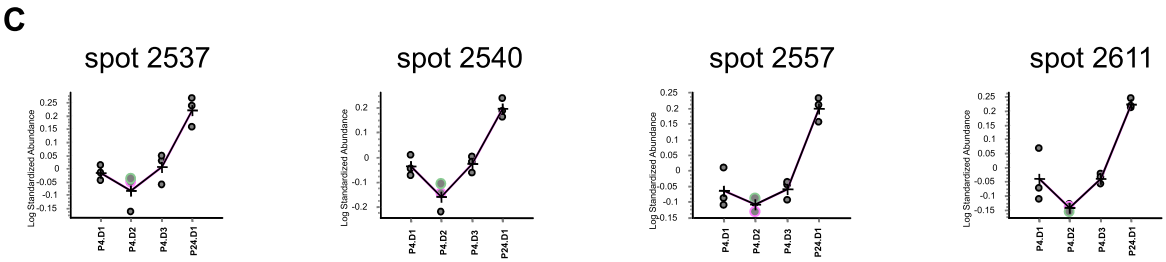


Fig. 3. Differential in gel electrophoresis (DIGE) of depleted samples. A) Representative two-dimensional DIGE image of the samples showing spots designated as modulated following software analysis. B) Table summarizing the characteristics of the four identified spots. C) Relative abundance of the four identified spots comparing the different analyzed samples.

isoforms appearing to be significantly more abundant in P24.D1 samples. Only four of these spots were identified by mass spectrometry (LC-MS/MS), corresponding to different isoforms of hemoglobin chains (Fig. 3B and C). These results would suggest that there is no proteolytic degradation associated with freeze/thaw cycles, although a certain degree of hemolysis can occur after a 24-h delay in the sample processing.

To validate these results, we performed SRM assays for hemoglobin B, which was the hemoglobin-related protein detected in the DIGE experiment with the highest MS/MS score. Six out of 13 putative proteotypic peptides were observed (Fig. 4A) in the enhanced resolution mode, and were subsequently used for quantification in the MRM mode. Quantification of the selected peptide areas (Fig. 4B and C) indicate that hemoglobin B is more abundant in the P24.D1 sample, thus confirming the DIGE results.

3.4. Isobaric tags for relative and absolute quantification (iTRAQ) analysis

iTRAQ technology was used for relative quantification of plasma proteins. Variation in protein abundance between samples would, if significant, suggest proteolytic degradation or the presence of undesired proteins. Results are presented in Supplementary data 1. The concentrations of several typical serum proteins detected were relatively modulated among the different samples, as presented in the quantification summary.

3.5. Selected Reaction Monitoring (SRM) using peptide standards

Because a significant number of common plasma proteins were detected as modulated by the iTRAQ analysis, we decided to use a previously described peptide standard kit developed by the Borchers group [34] to validate a large number of proteins in the original non-depleted pooled samples using SRM (Fig. 5). In a single experiment, 37 proteins were tested using a single proteotypic peptide per protein (Fig. 5A). In the four different samples tested, 33 proteins were detected and accurately quantified (Fig. 5B). The logarithmic representation of the heavy/light ratio suggests no major changes occurred in the amount of the proteins among the samples. A comparison of the individual ratios of each peptide with the average ratio of all the peptides (Supplementary data 2) revealed a group of six proteins, alpha-1-b-glycoprotein, apolipoprotein CIII, serotransferrin, vitronectin, alpha-2-microglobulin, and

A

HEMOGLOBIN B PEPTIDES	OBSERVED	CODE
EFTPPVQAAYQK	YES	PEP1
FFESFGDLSTPDAVM[Oxi]GNPK	NO	
FFESFGDLSTPDAVMGNPK	NO	
GTFATLSELHC[CAM]DK	NO	
LHVDPENFR	YES	PEP2
LLGNVLVC[CAM]VLAHHFGK	NO	
LLVVYPWTQR	YES	PEP3
M[Oxi]VHLTPEEK	NO	
MVHLTPEEK	NO	
SAVTALWGK	YES	PEP4
VLGAFSDGLAHLDNLK	YES	PEP5
VNVDEVGGEALGR	YES	PEP6
VVAGVANALAHK	NO	
LVIDEPTK	YES	EXOGENOUS

B

	AREA RATIO (CORRECTED)					
SAMPLE	pep1	pep2	pep3	pep4	pep5	pep6
P4.D1	0.6001	0.1746	5.2055	1.5347	0.1348	1.0790
P4.D2	0.5396	0.1867	3.3722	1.0780	0.1102	0.9734
P4.D3	0.7412	0.4456	4.6311	1.5477	0.1798	1.4802
P24.D1	1.1355	0.2568	9.0114	2.4042	0.2393	2.2882

C

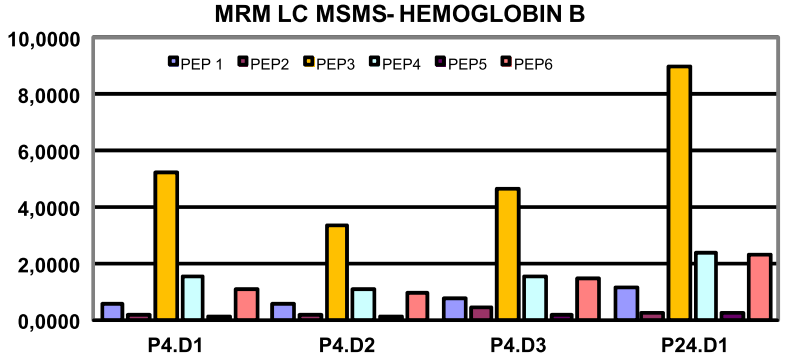


Fig. 4. Multiple reaction monitoring (MRM) analysis of hemoglobin B. A) List of theoretical proteotypic peptides generated with MRM Pilot software and the exogenous spiked peptide LVIDEPTK. B) Area ratio (corrected) expressed in arbitrary units corresponding to the six observed peptides in the four different analyzed samples. C) Numerical values corresponding to the corrected area ratio. LC = liquid chromatography; MS = mass spectrometry.

plasma retinol binding protein, whose profiles differed slightly from the average profile.

3.6. Analysis of low molecular weight proteins

If the integrity of plasma samples would be compromised, we would expect the appearance of protein fragments with lower molecular weight than the whole protein. Thus, fractionation of plasma proteins by SDS/PAGE should reveal a mobility shift of proteins due to the presence of fragments. In accordance, the number of identified proteins with higher molecular weight than expected in a gel slice should increase if plasma quality decreases. We fractionated complete and depleted plasma samples by SDS/PAGE and analyzed the low molecular weight (<20 kDa) region of the gel. The identified proteins were arranged by their predicted molecular weight according to their sequence and grouped by quartiles (Supplementary data 3). A slight but not significant increase in the number of identified proteins was observed in samples P4.D3 and P24.D1 of depleted plasma. However, there was no difference among the protein size groups (Fig. 6A and B), suggesting that the number of fragments from larger proteins is not increased by any of the treatments. It could be that the number of identified proteins would remain unchanged but with more peptides assigned to them, which would lead to an increase in their protein coverage. To assess this possibility, we determined the protein coverage of each protein size group (Fig. 6C and D). The data did not show any significant change in protein coverage among samples in any protein size group. Overall, the results indicated that the treatments did not affect significantly the integrity of plasma proteins. Although global changes were not observed, some individual proteins, particularly apolipoprotein B100 (ApoB100), appeared to increase their coverage in depleted plasma

A

Protein	Peptide	Protein	Peptide
Afamin	DADPDTFFAK	*Complement C4 gamma chain*	ITQVLHFTK
Albumin_serum	LVNEVTEFAK	*Complement factor B*	EELLPAQDIK
Alpha-1-antichymotrypsin	EIGELYLPK	*Fibrinogen alpha chain*	GSESGIFTNTK
Alpha-1B-glycoprotein	LETPDFQLFK	*Fibrinogen beta chain*	QGFGNVATNTDGK
Alpha-2-antiplasmin	LGNQEPGGQTALK	*Gelsolin*	TGAQELLR
alpha-2-macroglobulin	LLIYAVLPTGDVIGDSAK	*Haptoglobin*	VGYVSGWGR
Angiotensinogen	ALQDQLVLVAAK	*Hemopexin*	NFPSPVDAAFR
Antithrombin-III	DDLYVSDAFHK	*Heparin cofactor II*	TLEAQLTPR
Apolipoprotein A-I	ATEHLSTLSEK	*Inter-alpha-trypsin inhibitor*	AAISGENAGLVR
Apolipoprotein A-IV	SLAPYAQDTQEK	*Kininogen-1*	TVGSDTFYSFK
Apolipoprotein B-100	FPEVDVLTK	*L-selectin*	AEIEYLEK
Apolipoprotein C-I	TPDVSSALDK	*Plasma retinol-binding protein*	YWGVASFLQK
Apolipoprotein-C-III	GWVTDGFSSLK	*Plasminogen*	LFLEPTR
Apolipoprotein E	LGPLVEQGR	*Prothrombin*	ETAASLLQAGYK
Beta-2-glycoprotein I	ATVVYQGER	*Serotransferrin*	EDPQTFYYAVAVVK
Ceruloplasmin	EYTDASFTNR	*Transthyretin*	AADDTWEPFASGK
Clusterin	ELDESLQVAER	*Vitamin D-binding protein*	THLPEVFLSK
Coagulation factor XII	VVGGLVALR	*Vitronectin*	FEDGVLDPDYPR
Complement C3	TGLQEVEVK		

B

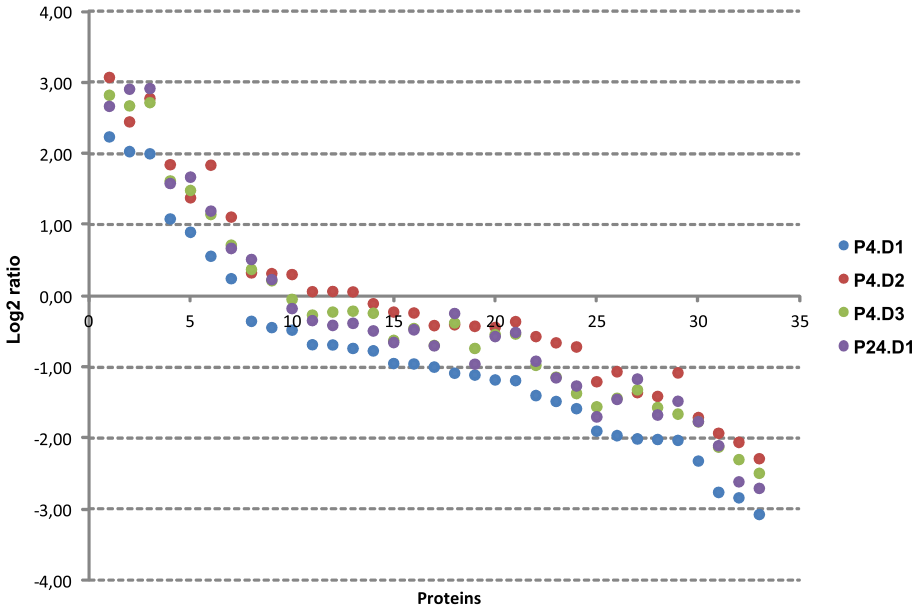


Fig. 5. Multiple reaction monitoring (MRM) analysis using peptide standards of selected plasma proteins. A) Table showing the proteotypic peptides for the thirty-seven selected proteins tested in parallel. B) Graphical logarithmic representation of the heavy/light ratio for the thirty-three observed proteins (X axis) in the four different analyzed samples.

samples P4.D3 and P24.D1. We investigated the abundance of ApoB100 by Western blot and SRM analysis but the results did not confirm any significant difference (not shown).

3.7. LC-MALDI-TOF/TOF and Western-blot validation of selected proteins

After protein identification using Mascot, the emPAI score [27] was used to estimate the absolute amount of each protein in a particular sample (Supplementary data 4). Changes in the emPAI score would have revealed protein degradation or the presence of truncated isoforms. Fig. 7 represent the average emPAI score (Y axis) of all the proteins detected (X axis) fast ($t \leq 4$ h) vs. delayed ($t = 24$ h) processing (Fig. 7A) or when comparing freeze/thaw cycles (Fig. 7B).

The average emPAI score did not vary for most proteins when the samples were compared. However, among the few exceptions detected, suggesting that the amount of these proteins might vary among samples, three were selected (apolipoprotein A1, vitronectin and kininogen 1) for Western blot validation in crude pooled samples. The results of this analysis did not reveal accumulation or degradation of the proteins (Fig. 7C and D).

4. Discussion

An increasing number of clinical biobank sample collections from large-scale population-based studies are currently available for research applications. Many proteomic studies for biomarker discovery have been developed based on access to these collections [35]. However, standardization of sample quality is a critically important requirement for valid use of the full potential of the collected samples [36]. The need for improved biomarker research has focused attention on the effects of pre-analytical variable on collected samples as key parameters determining the obtained results [37]. Although this is particularly true for plasma profiling studies, the stability of most plasma proteins to pre-analytical variables remains unexplored.

Biobanking faces standardization of a wide range of pre-analytical variables involved in sampling procedures of blood and plasma. Among these, acquisition, handling and storage may produce alterations of the proteome. In this work, we analyzed the influence of time from blood collection to plasma isolation (<4 h at RT or after a delay of 24 h at 4 °C) and the number of freeze/thaw cycles (1, 2 or 3) performed on plasma samples using several standardized proteomic techniques: (SPE MALDI-TOF, DIGE, iTRAQ, LC-MS/MS or LC-MALDI-TOF/TOF) and targeted proteomic analysis (SMR) platforms. We systematically evaluated the effects in prospectively collected human platelet-poor plasma from a representative population of 100 healthy donors (as defined by pre-established criteria) divided by gender (50%) and by age group (20 to 40, 40 to 60, and 60 to 80 years). The proteomic comparisons were based on differences in mean intensity values of peptides/protein using plasma processed under the different conditions. In general, following a thorough analysis of the results from the different analytic techniques, no detectable significant changes were observed as a result of delay in blood processing or number of freeze/thaw cycles.

The Biospecimen Reporting for the Improved Study Quality (BRISQ) has released recommendations on collection and management of human biospecimens [38]. According to these guidelines, all samples included in our study were handled identically by the biobank participants to exclude any pre-variable besides those we wanted to analyze. We followed the recommendation and used platelet-poor plasma to prevent the effects of activation of platelets [12].

Samples collected for clinical analysis are either used immediately or stored in biobanks for future research use. Unfortunately, biobanks where samples are processed and stored are often not close to the clinic where samples are collected. Thus, geography can delay the processing of blood samples for hours or even days; this interval could produce alterations in the proteome due to cell metabolism and activation [39]. To determine the extent of these alterations, we examined the effects of elapsed time to processing from venipuncture and the temperature at which samples are maintained prior to first centrifugation. It is deemed essential that the time elapsed between blood acquisition and processing in the biobank be as short as possible (<4 h, when possible), but sometimes blood samples cannot be processed until the day following extraction; in this case it is recommended that samples be maintained at low temperature to minimize proteolytic activity. However, the extent to which time and temperature can affect blood samples remains unclear. Our results indicate that few non-significant changes occurred

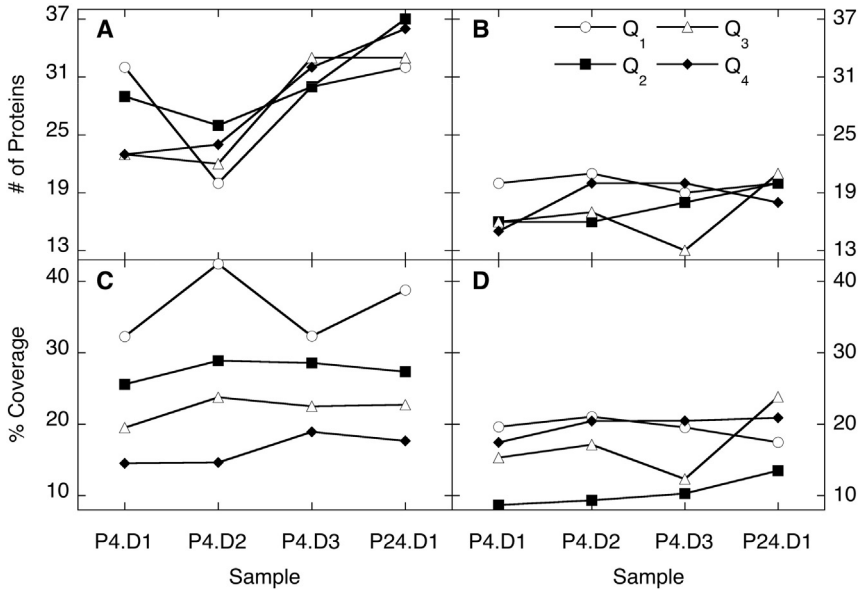


Fig. 6. Analysis of plasma proteins integrity. A, B) The number of identified proteins in the low molecular weight region of a SDS/PAGE of (A) depleted and (B) complete plasma. C, D) The protein coverage of the identified proteins in the low molecular weight region of a SDS/PAGE of (C) depleted and (D) complete plasma, respectively. Median molecular weight of quartiles 1–4: 14, 29, 53, and 93 kDa for depleted plasma; and 11, 34, 53, and 82 kDa for complete plasma.

in peptide and protein identifications in plasma from blood collected in EDTA-plasma tubes and processed within the first 4 h following collection or after storage for up to 24 h at 4 °C prior to plasma isolation. The distribution of proteins in all samples was very similar, and the only clear difference observed by DIGE was in the concentration of several hemoglobin isoforms when there was a delay in blood centrifugation. This finding has been further confirmed by targeted MRM analysis, and is in accordance with other studies demonstrating by mass spectrometry that the undesired presence of high levels of hemoglobin chains in biological fluids could be used as marker for blood contamination [40]. Also, in a previous study specifically examining serum handling conditions over several hours, both transport time and temperature were found to affect SELDI profiles [41]. In that study, the effects of time delay were minimized in samples transported on ice, although, as demonstrated by our results, hemoglobin also displayed significant variability. Comparable results were obtained after time course studies on plasma from blood collected in EDTA-plasma tubes and stored for up to a week either at 4 °C or room temperature prior to plasma isolation. These proteomic studies revealed few significant changes in peptide and protein identifications, semitryptic peptides and methionine-oxidized peptides [42]. Results obtained by Hassis et al. [43], utilizing iTRAQ, are in agreement that minimal impact of time

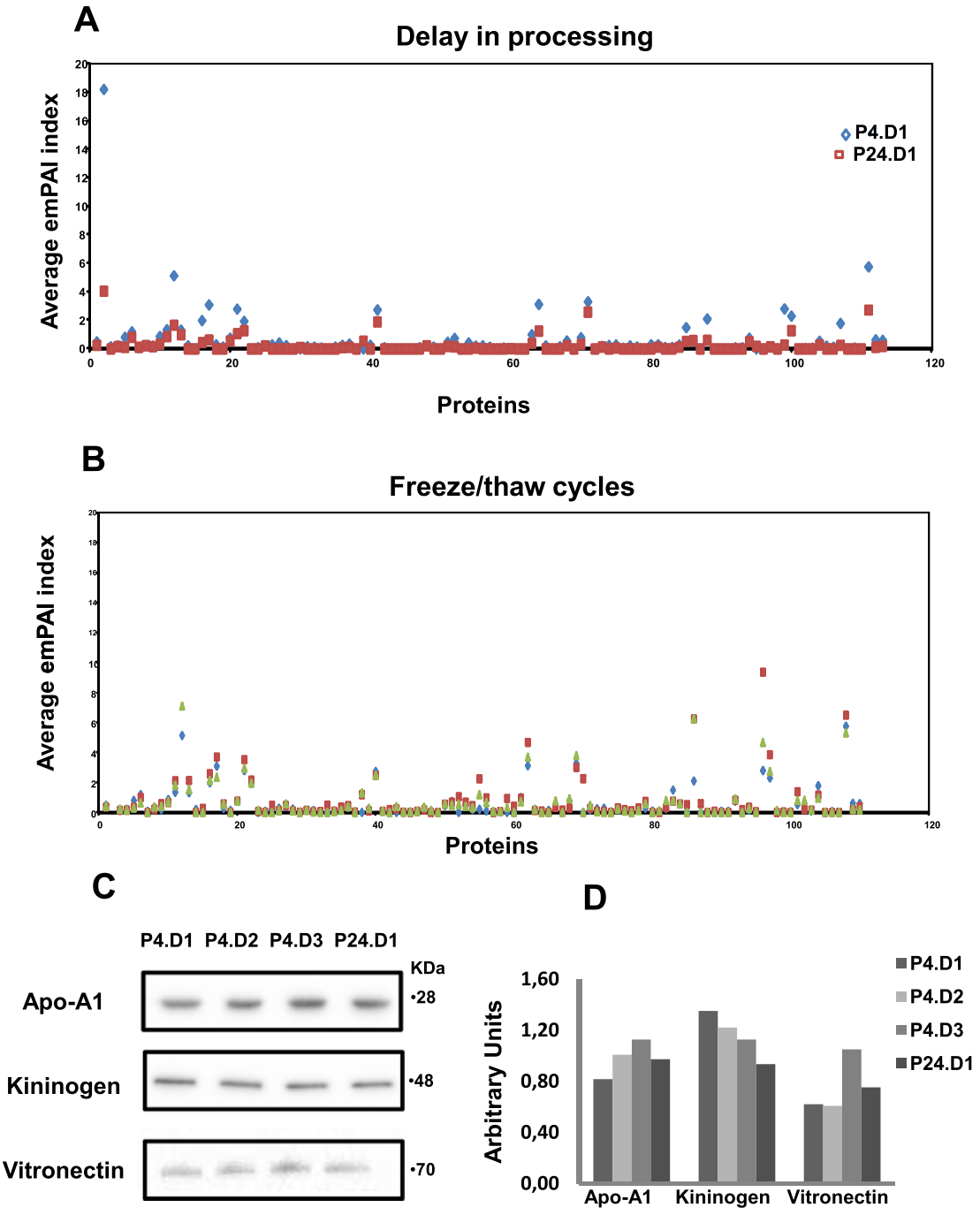


Fig. 7. LC-MALDI-TOF/TOF and Western-blot validation of selected proteins. A) Average emPAI index (Y axis) of all the proteins detected (X axis) when comparing fast processing (P4) vs. delayed processing (P24). B) The average emPAI (Y axis) of all the proteins detected (X axis) when comparing freeze/thaw cycles. C) Western blot validation of the putative alteration of the amount of selected proteins, apolipoprotein A1, vitronectin and kininogen. D) Densitometry analysis of each protein normalized to the corresponding densitometry of the Ponceau staining (not shown) of the entire lane.

and temperature on plasma occurred after specimen collection. In serum, other previous studies have demonstrated that a blood pre-centrifugation delay of 24 h at room temperature influenced the proton NMR spectroscopic profile, but, if during this delay the temperature is held at 4 °C, there was no influence on the proton NMR spectroscopic profile [44]. However, Findeisen et al. [45] proposed a "proteomics degradation clock" based in the monitoring of synthetic spiked reporter-peptides, since their concentration in both serum and plasma samples decay in a time-dependent manner along a 2 to 24 h period.

Another pre-analytical variable possibly affecting stability of plasma is the number of freeze/thaw cycles. There is no consensus on the effects of freezing and thawing on serum and plasma. The marked effects and dependence on the number of freeze-thaw cycles on serum profiles generated using magnetic bead-based SPE followed by MALDI-TOF has been shown [46], and repeated freeze-thaw cycles resulted in a trend towards increasing changes in peak intensity in MALDI-TOF studies [47]. However, other studies of serum samples reported minimal effect on detected peaks of repeated freezing and thawing of up to ten cycles, as only minor effects were seen on the proteome, based upon the analysis of about 100 peaks by MALDI-TOF MS [48] and similar results were obtained in analyses of plasma subjected to up to 25 freeze/thaw cycles [42]. In agreement with the latter report, our data indicate that up to three freeze/thaw cycles have no significant effect on the stability of proteins in the plasma samples.

Overall, our results indicate that the integrity of proteins and peptides was resistant to alteration by the pre-analytical variables considered in this study. Although there are other studies related to protein stability of clinical samples with similar results, what is remarkable about our work is the large number of plasma samples examined and that our analyses assessed protein integrity by combining a wide set of complementary proteomic approaches performed at different proteomic platform participating laboratories that all yielded similar results. Thus, we were able to examine the stability at both the peptide and protein level of a far broader range of plasma proteins than previously possible using individual techniques and fewer specimens. The peptide-level analyses performed in our work provide a robust method for accurate protein quantization in plasma samples and a better understanding of the changes in proteins induced by delayed processing or plasma freeze/thaw cycles. We believe our study is the most comprehensive performed to date to determine the changes in proteins induced by delayed sample processing and plasma freeze/thaw cycles.

This work indicates that the reevaluation of plasma collection protocols in clinical proteomics studies used to analyze biomarkers should be considered. Our data suggest that stability of plasma proteins under expanded clinical laboratory storage conditions (4 °C for up to 24 h prior to sample processing) could be considered as an acceptable time frame for plasma collection. Thus, this sampling-preparation time frame could expand the current typical standard operating procedure (processing within 4 h of collection). In addition, the utilization of plasma samples subjected to up to three freeze/thaw cycles, at least in the case of controlled thawing times of no more than 1 h, could be considered potentially acceptable for clinical proteomics studies.

5. Conclusion

In summary, although it is evident that pre-analytical variables may influence clinical proteomic studies using plasma, and these must be taken into account, the results of the present work indicate that blood proteins in plasma are broadly insensitive to such pre-analytical variables as delayed processing or freeze/thaw cycles when analyzed at the peptide level. These findings do not mean that these pre-analytical variables do not affect other analyses, for instance, immunoassays directed at intact proteins, where collection protocols may have different requirements.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2016.09.003.

Conflict of interest

I declare that there are no financial or other relations that could lead to a conflict of interest.

Sources of funding

This work was supported by the grant numbers PI12/02670 and PT13/0001/0034, integrated in the National Plan for Scientific Research, Development and Technological Innovation 2008–2011 and 2013–2016 and funded by the ISCIII-General Subdirection of Assesment and Promotion of the Research-European Regional Development Fund (FEDER) "A way of making Europe".

Disclosures

None.

Acknowledgements

This work is dedicated to the memory of our colleague and friend Juan Pablo Albar.

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