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Evaluating the effects of preanalytical variables on the stability of the human plasma proteome

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ABSTRACT

High quality clinical biospecimens are vital for biomarker discovery, verification, and validation. Variations in blood processing and handling can affect protein abundances and assay reliability. Using an untargeted LC-MS approach, we systematically measured the impact of preanalytical variables on the plasma proteome. Time prior to processing was the only variable that affected the plasma protein levels. LC-MS quantification showed that preprocessing times <6 h had minimal effects on the immunodepleted plasma proteome, but by 4 days significant changes were apparent. Elevated levels of many proteins were observed, suggesting that in addition to proteolytic degradation during the preanalytical phase, changes in protein structure are also important considerations for protocols using antibody depletion. As to processing variables, a comparison of single- vs double-spun plasma showed minimal differences. After processing, the impact ⩽3 freeze–thaw cycles was negligible regardless of whether freshly collected samples were processed in short succession or the cycles occurred during 14–17 years of frozen storage (−80 °C). Thus, clinical workflows that necessitate modest delays in blood processing times or employ different centrifugation steps can yield valuable samples for biomarker discovery and verification studies.

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Blood is an accessible and promising source for discovering biomarkers for disease screening and diagnosis as well as monitoring progression and/or therapeutic response. Changes in the protein repertoire of cells, tissues, and organs, which reach the bloodstream by active or passive means, are important clinical tools. The challenges associated with plasma proteomics include the large dynamic range of protein concentrations [1,2], variability in preanalytic and analytic processes, and inherent biological variability. Detection of low abundance proteins is facilitated by depletion or enrichment of peptides or proteins prior to mass spectrometry (MS) analyses [3]. The use of sound study designs and system suitability protocols improves analytical reproducibility and statistical power [4–7]. Ultimately, the quality of specimens affects the validity of the data. Preanalytical variables associated with collection, processing, and storage can also be confounding factors. The National Cancer Institute Biospecimen Reporting for Improved Study Quality (BRISQ) workgroup proposed guidelines for reporting specific preanalytical conditions, including factors that might influence the integrity, quality, or composition of samples [8]. However, outstanding questions remain about the most important preanalytical variables in terms of major effects on the validity of biomarker studies.

Protein and peptide integrity in plasma samples can be compromised in multiple ways, including proteolysis, oxidation, loss of posttranslational modifications, and changes in solubility [9]. Biomolecules degrade at different rates under a variety of circumstances. The rate and extent of degradation depend on the time/temperature at which blood is held prior to processing, centrifugation speed, the time/temperature prior to freezing, the number of freeze–thaw cycles, and analyte stability [10]. It is not always feasible to process clinical samples immediately after collection because the clinic and the blood processing facilities are often geographically separate. Prior to and during processing,

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proteolytic activity or cellular metabolism may alter protein content, which can also be affected by blood cell lysis at higher centrifugation speeds. Postprocessing delays prior to frozen storage or after thawing could afford time for additional *ex vivo* proteolysis. The use of broad spectrum protease inhibitors during sample collection and processing, which is costly in terms of dollars and effort, is unlikely to occur routinely in the clinical setting. Perhaps more importantly, we lack a global understanding of factors that impact protein stability in blood. Detailed protocols for serum/plasma collection and processing have been published [9,11]. In particular, there are two widely used plasma protocols that differ mainly in the centrifugation step. The Early Detection Research Network (EDRN)[1] standard operating procedure (SOP) uses a single centrifugation step no longer than 4 h post collection [11]. The Clinical Proteomic Technologies Assessment for Cancer (CPTAC) SOP entails a second, higher speed centrifugation step to obtain platelet poor plasma [12].

The effects of preanalytical variables on plasma/serum peptide and protein stability over time, assayed by using MS methods, have been reported. Analysis of the low molecular weight (LMW) plasma proteome revealed postprocessing, time-dependent changes in the MALDI TOF profiles over 48 h [13]. At the protein level, changes in a relatively small number of abundant plasma proteins including albumin, hemoglobin, serotransferrin, inter-alpha trypsin inhibitor, and fibrinogen were observed in plasma samples held at room temperature for 1 week before processing [14]. The use of plasma collection tubes containing protease inhibitors to minimize degradation as compared with EDTA has not been shown to have a significant impact on the levels of peptides or proteins [15,16]. Multiple studies have shown changes in the abundance of complement C3, at protein and peptide levels, due to preanalytical variables [13,14,17,18]. However, since the dynamic range in plasma protein concentrations exceeds that of mass spectrometers by at least 5 orders of magnitude, immunoaffinity depletion of abundant proteins is routinely employed in plasma biomarker discovery and verification studies [19]. Preanalytical variables could affect depletion, either directly via antibody binding or indirectly via nonspecific protein interactions with depletion targets. The objective of this study was to analyze the effects of well-defined preanalytical variables on the protein integrity of immunodepleted plasma and serum samples and to compare two widely used methods of plasma preparation.

Materials and methods

Blood collection

Biospecimen Research Network specimens

Donors gave consent for blood collection for research purposes as part of a UCSF Institutional Review Board-approved protocol. Male and female donors (aged 20–40, median 27, Supplemental Table 1) were requested to fast for a minimum of 12 h prior to blood collection. Donors were seated at least 5 min before the draw and the arm was positioned on a slanting armrest in a straight line from the shoulder to the wrist. A tourniquet was applied approximately 2 inches above the antecubital fossa or above area to be drawn with enough pressure to provide adequate vein visibility and the patient was asked to form a fist. The forearm was cleaned with an antiseptic wipe and allowed to dry. Then a butterfly needle was inserted the evacuated tube pushed onto the Luer adapter. The tourniquet was released once blood flow was established, within 1 min. Blood collection was performed by registered nurses at UCSF Moffitt Hospital who were provided with the protocol and briefed on the project, stressing the importance of adherence to the blood collection standard operating protocol (SOP). Two members of the research team were present during blood draws and any deviations from protocol were logged.

Magee–Womens Biorepository specimens

Plasma samples were obtained from an ongoing investigation of preeclampsia (Prenatal Exposures and Preeclampsia Prevention [PEPP]) at the Magee–Womens Research Institute and Hospital (University of Pittsburgh, Pittsburgh, PA, USA). PEPP was approved by the University of Pittsburgh Institutional Review Board, and informed consent was obtained from all participants. The PEPP committee approved the use of these previously frozen samples and deidentified clinical data.

Blood processing

Biospecimen Research Network specimens

Two published SOPS were used to process blood, a double spin protocol [12,20] and a single spin method [11]. Blood was collected in spray-coated K_2EDTA Vacutainers (BD, Franklin Lakes, NJ). After blood collection, the tubes were inverted 8 times. For double-spun plasma, one tube was centrifuged within 30 min, in a horizontal rotor, for 15 min at 1500*g*, 4 °C. The plasma was transferred to a sterile 15 ml conical tube without disturbing the buffy coat and subjected to a second centrifugation step, in a horizontal rotor, at for 15 min at 2000*g*, 4 °C. The platelet-poor plasma was aliquoted into sterile cryovials (0.75–0.2 ml/tube), immediately transferred to dry ice, and transported within 30 min to a −80 °C freezer. For single-spun plasma, tubes were centrifuged, in a horizontal rotor, for 20 min at 1200g, 20 °C, and then transferred to a sterile 15 ml conical tube without disturbing the buffy coat. Plasma was aliquoted into sterile cryovials (0.75–0.2 ml/tube) and frozen as described for double-spun plasma. Additional biospecimens were prepared by systematically altering several preanalytical variables. (1) Preprocessing holding time was increased to 6 h. (2) Preprocessing holding time was increased to 96 h and the temperature was raised to 37 °C. (3) One to three freeze–thaw cycles were performed: the first thaw was designated "0." (4) Thawed samples were held for 24 h at room temperature prior to immunodepletion. Exclusion criteria were hemolysis, insufficient sample volume, or SOP deviations.

Magee–Womens Biorepository specimens

For investigating the effects of long-term frozen storage (−80 °C), samples banked at the Magee Women's Research Institute (MWRI, Pittsburgh, PA) for 14–17 years (median = 15.3) were employed (Supplemental Table 1). Records documented the number of times that the samples were frozen and thawed (median = 3). Paired plasma and serum samples were collected from the same pregnant woman (n = 20), median gestational age [GA] = 40 weeks). The subjects were controls, women with uncomplicated pregnancies, for studies of pregnancy complications. Serum was collected into silicon-coated tubes without additives (BD) and plasma into spray-coated K_2EDTA tubes (BD). Tubes that contained serum were maintained upright; Tubes that contained plasma were inverted several times immediately after collection before they were also placed in an upright position. After 1–1.5 h at room temperature, samples were centrifuged at 2000*g* for 20 min at 25 °C. Sterile transfer pipets were used to aliquot (0.1–0.25 mL/tube) the plasma or serum into sterile cryovials prior to storage at −80 °C. An additional 10 paired control samples (median GA = 35.9 weeks) were collected and processed at MWRI under the same protocols and stored at −80 °C for <3 months prior to analyses.

[1] *Abbreviations used:* CPTAC, Clinical Proteomic Technologies Assessment for Cancer; EDRN, Early Detection Research Network; SOP, standard operating procedure.

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