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Stability of the Human Plasma Proteome to Pre-analytical Variability as Assessed by an Aptamer-Based Approach

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Abstract

Variable processing and storage of whole blood and/or plasma are potential confounders in biomarker development and clinical assays. The goal of the study was to investigate how pre-analytical variables impact the human plasma proteome. Whole blood obtained from 16 apparently healthy individuals was collected in six EDTA tubes and processed randomly under six pre-analytical variable conditions including blood storage at 0 °C or RT for 6 h (B6h0C or B6hRT) before processing to plasma, plasma storage at 4 °C or RT for 24 h (P24h4C or P24hRT), low centrifugal force at $1300 \times g$ (Low $\times g$), and immediate processing to plasma under $2500 \times g$ (control) followed by plasma storage at -80 °C. An aptamer-based proteomic assay was performed to identify significantly changed proteins (fold change ≥ 1.2 , $P < 0.05$, and false discovery rate < 0.05) relative to the control from a total of 1305 proteins assayed. Pre-analytical conditions Low $\times g$ and B6h0C resulted in the most plasma proteome changes with 200 and 148 proteins significantly changed, respectively. Only 36 proteins were changed under B6hRT. Conditions P24h4C and P24hRT yielded changes of 28 and 75 proteins, respectively. The complement system was activated in vitro under the conditions B6hRT, P24h4C, and P24hRT. The results suggest that particular pre-analytical variables should be controlled for clinical measurement of specific biomarkers.

Graphical Abstract

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Author Contributions

All authors confirmed that they have contributed to the intellectual content of this paper and have met the following three requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

The authors declare no competing financial interest.

Supporting Information

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Keywords

aptamer technology; biomarkers; human blood; plasma; pre-analytical variability; proteomics; quality assessment

1. INTRODUCTION

The quality of clinical and biobank samples is of the utmost importance when conducting biomedical research using OMICS-based approaches. Authors Lee and Kim coined the term “precision biobanking”¹ as a way of emphasizing the importance of considering pre-analytical variability. Although standard operating procedures may be in place for sample analysis, the non-standardized variability introduced during sample processing is a potential confounder in the results of clinical assays.^{2,3} Consortia like the Human Proteomics Organization (HUPO) Human Plasma Proteome Project (PPP)⁴ and the Standardisation and improvement of generic pre-analytical tools and procedures for in-vitro diagnostics (SPIDIA project; <http://www.spidia.eu/>) sought to address the problem of this phase of research in the in vitro diagnostics arena. Recently, proteomics platforms were used to evaluate the impact of different pre-analytical parameters on sample quality.^{2,5-12} However, many areas in pre-analytical sample variability remain to be investigated.

A newly released guidance of bioanalytical method validation from the United States regulatory agency, the Food & Drug Administration (FDA), recommended determination of pre-analytical effects of sample collection, handling, and storage of analytes including benchtop and freeze–thaw stability.¹³ Although the guidance does not provide further recommendations about specific sample storage temperatures, centrifugation speeds for processing whole blood to plasma, or delays in the pre-analytical process, investigation of these areas would fill the knowledge gap and facilitate bioanalytical assay development and biomarker qualification.

The timing of pre-analytical steps after blood collection or alterations in processing methods can affect sample quality and ultimately study results. The variables evaluated in this study

are closely related to the time frames of clinical practices for blood processing and storage, and the results will help fill the gap in the FDA's biomarker qualification program related to pre-analytical variability. Biomarker discovery in plasma is a natural application of proteomics, yet the impact of alterations due to different pre-analytical sample processing steps remains a question. The goal of this study was to examine the impact of plasma sample processing variations such as (1) delayed processing of blood to plasma, (2) storage temperature of whole blood or plasma, and (3) the effect of centrifugal force applied on the plasma proteome. To achieve this, an aptamer-based proteomics approach known as the SOMAscan assay^{14,15} was utilized to measure the relative abundance of 1305 proteins derived from human plasma. As demonstrated from the impact of these pre-analytical variables on metabolites, peptides, and inflammation biomarkers,¹⁶ the results from this study further suggest that pre-analytical steps should be taken into consideration when evaluating clinical protein biomarker and proteomics results.

2. MATERIALS & METHODS

2.1. Subjects

This study was approved by the FDA's Institutional Review Board, the Research Involving Human Subjects Committee, and informed consent was obtained from all participants. Twenty self-reported healthy males and females were recruited by the FDA's National Center for Toxicological Research (NCTR). Volunteers were between the ages of 18 and 65, had BMIs between 18 and 30 kg/m², and attested to not taking heparin and steroidal or non-steroidal anti-inflammatory medication within the previous 10 days. Subjects were asked to fast overnight prior to blood draw. Exclusion criteria were acute or chronic diseases, anemia, pregnancy (2nd and 3rd trimester), and use of antihistamines or selective serotonin re-uptake inhibitors (within the last 4 weeks). Although 20 subjects were enrolled, data from four subjects were not obtained and treated as missing completely at random. Proteomic data was analyzed from the blood of 16 individuals, which was subjected to six predefined processing conditions, thereby introducing pre-analytical variability.

2.2. Blood Collection and Processing

After fasting an average of 12 h, approximately 50 mL of blood was collected via venipuncture from a seated position using a 21-gauge BD Safety-Lok blood collection system (Fisher Scientific, BD part # 368652) into six 10 mL capacity conventional BD Vacutainer K₂ EDTA tubes (Fisher, BD part # 368589). De-identified blood samples were transferred for processing. To assure that the proteome gradient, which might be associated with the order of collection into the tubes, did not introduce a systematic error in the results, William's design based on replicates of a standard 6 × 6 Latin square was used to generate random sequences of treatment-by-collection tubes for reducing variability between the collection tubes within the subject. Each of the six tubes of whole blood was stratified randomly into the following variable categories: (1) stored for 6 h on wet ice (i.e., 0 °C) before processing to plasma (B6h0C), (2) stored for 6 h at room temperature before processing to plasma (B6hRT), (3) immediately centrifuged (control), (4) immediately centrifuged at 1300 × *g* (Low×*g*), (5) immediately centrifuged, and the plasma was stored for 24 h at RT (P24hRT), and (6) immediately centrifuged, and the plasma was stored for

24 h at 4 °C (P24h4C). For each condition, blood was processed by centrifugation at $2500 \times g$ or $1300 \times g$ (Low $\times g$ condition) for 15 min at RT, between 19 and 22 °C, and the plasma supernatant was carefully removed to a new tube where it was briefly mixed. The processed plasma under each condition was aliquoted into Eppendorf tubes, snap-frozen in liquid nitrogen, and then stored at -80 °C until analysis (Figure 1).

2.3. Proteomic Analysis

The SOMAscan assay (version 1.3k; SomaLogic) utilizes single-stranded DNA slow off-rate modified aptamers (SOMAmers) to quantify 1305 low-, middle-, and high-abundance proteins in a small volume of biological fluid and has been described previously.^{14,15} Briefly, human plasma samples (50 μ L) were incubated with SOMAmers pre-immobilized with streptavidin-coated beads. After a series of catches and washes, the SOMAmers that were specifically bound to their cognate proteins were quantified by hybridization of SOMAmers to custom DNA microarrays and scanned using an Agilent C scanner (G2505C). The raw outcome for each protein identified in the SOMAscan assay was relative fluorescence units (RFUs). The raw data underwent hybridization normalization to remove variation introduced during the hybridization and scanning processes, followed by median normalization to eliminate intra-run bias, and finally calibrated to account for inter-run differences.¹⁴

2.4. Statistical Analysis

A repeated measure ANOVA profile model was used to evaluate changes in blood sample quality based on the observed effect of each treatment condition on each protein. The ANOVA model is robust in analyzing minor departures from normality given the large number of proteins with variable range/scales. Two sided statistical tests were adjusted for multiplicity testing using Dunnett's method to control the Type I error rate. To control for the false discovery rate (FDR), the Benjamini–Hochberg method was chosen. The fold change for each treatment condition relative to the control condition was computed for each protein. Evaluation of reproducibility for the SOMAscan assay demonstrated that the assay had a median intra-run CV of 3–5%^{14,17} and inter-run CV of 5–6%.¹⁴ Our previous evaluation of the assay revealed that a combination of 1.2-fold change with $p < 0.05$ could achieve highly reliable quantitative results with a low false discovery rate (FDR).¹⁸ Based on these data, a fold change cutoff of 1.2, $p < 0.05$, and $FDR < 0.05$ were used as stringent criteria to identify significantly changed proteins. The SAS System for Windows (v9.3) was used for statistical analysis. Gene ontology analysis was performed using PANTHER (v13.1, <http://www.pantherdb.org>) for significantly changed proteins under each pre-analytical condition and for all the 1305 proteins assayed by SOMAscan as a reference. Unsupervised multilevel principal component analysis (PCA) was used to visualize the data. The software R 3.3.1 (package ggplot2, multigroup, and mixOmics) was also used for multivariate data analysis and visualization.¹⁹⁻²¹

3. RESULTS

3.1. Protein Abundance Changes Resulting from Each Pre-analytical Condition

SOMAscan analysis of plasma samples under variable pre-analytical conditions revealed that a total of 343 (~25%) proteins underwent statistically significant changes in abundance (> 1.2 -fold, $p < 0.05$, and $FDR < 0.05$) compared to the control (Table S1). Of the five defined pre-analytical factors, plasma that was processed immediately using lower centrifugation speed and stored in -80°C without delay ($\text{Low} \times g$) resulted in 200 (15%) proteins changed, with 4 (0.3%) decreased and 196 (15%) increased in abundance compared to the control as shown in the volcano plot (Figure 2A). For two delayed plasma processing conditions, blood samples stored at 0°C (B6h0C) prior to processing to plasma resulted in changes of 148 (11%) proteins with 117 (9%) decreased and 31 (2.4%) increased (Figure 2B), while blood stored at room temperature for 6 h (B6hRT) altered the abundance of 36 (2.8%) proteins with 17 (1.3%) decreased and 19 (1.5%) increased (Figure 2C). For the remaining two conditions, storing immediately processed plasma at 4°C for 24 h (P24h4C) resulted in changes of 28 (2.1%) proteins with 11 (0.8%) decreased and 17 (1.3%) increased (Figure 2D), while storing immediately processed plasma at room temperature for 24 h (P24hRT) altered 75 (6%) proteins with 35 (2.7%) decreased and 40 (3.1%) increased (Figure 2E).

3.2. Unique and Common Protein Changes between Pre-analytical Conditions

When significant protein changes were compared between pre-analytical conditions, there was not an overlap of proteins across all conditions; rather, each condition had its own specific signature. $\text{Low} \times g$ had 111, P24hRT had 39, P24h4C had 8, B6hRT had 10, and B6h0C had 51 proteins unique to that particular condition (Figure S1A). When pre-analytical conditions were compared, it was found that the most significant overlaps of the changed proteins were between $\text{Low} \times g$ and B6h0C (Table S2) and between B6hRT, P24h4C, and P24hRT (Table 1). There were 83 proteins commonly changed between $\text{Low} \times g$ and B6h0C, 22 proteins between B6hRT and P24hRT, and 17 proteins between P24h4C and P24hRT. In addition, nine proteins were commonly changed among B6hRT, P24h4C, and P24hRT. It is intriguing that the common proteins changed between B6hRT, P24h4C, and P24hRT were all in the same direction (increased or decreased) (Table 1). These results indicate the distinct and similar protein abundance changes between these pre-analytical conditions.

3.3. Gene Ontology Analysis of Changed Proteins

Gene ontology analysis of molecular functions for the significantly changed proteins indicated that proteins with catalytic activities were enriched in the changed proteins in each pre-analytical condition (Figure 3A) as compared to all the proteins in the SOMAscan assay. Further analysis of cellular components revealed that significantly changed proteins were enriched in cell parts and organelles in $\text{Low} \times g$ (70% of the changed proteins), B6h0C (58%), and P24h4C (60%) as compared to the control (40%) (Figure 3B). Changes in extracellular matrix proteins were enriched in B6hRT (31%), P24h4C (13%), and P24hRT (11%) as compared to the total SOMAscan proteins (5%). In addition, changes of proteins from extracellular regions were enriched in B6hRT (31%). These data indicate that blood

processing with low centrifugal force and blood storage at low temperature likely affects compartmentalized proteins with an additional impact on extracellular proteins for extended storage of blood at RT and plasma for 24 h.

3.4. Protein Changes in the Complement System

There were seven complement components that changed in at least one of the pre-analytical conditions. In Low×g and B6h0C, only complement component C3a was changed with 1.41- and 1.39-fold decreases in abundance, respectively. In contrast, complement components C3b and C4b were increased in B6hRT (1.8- and 3.4-fold), P24h4C (1.4- and 2.7-fold), and P24hRT (4.2- and 7.4-fold) although the total protein level of C4 was decreased (1.4-fold) in P24hRT (Table 2). In addition, a slight increase of the C1q binding protein and a decrease of C1r were observed in P24hRT. Taken together, the results suggest that the complement system was activated in vitro in the prolonged processing of blood or storage of plasma above 0 °C.

3.5. PCA Analysis

An unsupervised multilevel PCA was applied to the 343 significantly changed proteins from the six sample processing methods. Without informing the data, subjects in both the B6h0C and P24hRT conditions were clustered well within each group and were well separated from other conditions in the PCA plots (Figure 4). Although partially clustered with the controls, subjects in Low×g were separated with other groups. Subjects in B6hRT and P24h4C were clustered, suggesting their similarity in protein changes. It is acknowledged that data from subject 8 lends itself to clear separation from the rest of the subjects, which appears to be an artifact of sensitivity to prolonged whole blood storage.

4. DISCUSSION

Interest in pre-analytical conditions on protein quality has risen in protein biomarker studies. One report suggests processing whole blood to plasma within 4 h for proteomic analysis.²² We agree with their suggested procedures that a temperature of –80 °C be used for long-term storage of processed plasma samples for proteomic analysis and along with others that blood samples be collected in EDTA tubes as a means to preserve biomolecules²³⁻²⁵ although Ilies et al. suggested that heparinized plasma could be just as valuable as EDTA and citrate for protein profiling.¹² Minimizing the time between collection, processing, and freezing and/or analysis is an overarching theme in many investigations.^{2,23-26} The SOMAscan proteomic platform applied is novel to this field of study and has been evaluated to be stable and reproducible in both other and our own studies.^{14,17,18,27} It is a means to investigate intact protein versus digestion into peptides as is the case in certain mass spectrometry (MS)-based plasma pre-analytic proteomic evaluations where abundant proteins were depleted and samples were evaluated as a pool.⁶⁻⁹ Proteins and peptides largely remained robust in these MS-based studies until delays in processing blood reached 4 days⁷ and beyond.^{8,9} Pasella et al. found that some proteins are more labile at lower temperatures than when stored at RT,⁹ which agrees with our findings. Whereas the study conducted by Mateos et al. yielded no changes with delayed processing at differing temperatures (blood held for <4 h at RT or 24 h at 4 °C) by using multiple MS-based

techniques,⁶ our study revealed significant changes under similar conditions (B6hRT and B6h0C). Not only did most MS-based studies reveal negligible differences among variables tested, Qundos et al. investigated post-processing to plasma delays from 1 to 36 h before analysis using antibody suspension bead arrays and found only 1 out of 373 antibodies that showed time-dependent sensitivity to the delays.¹⁰ The SOMAscan assay covers both low- and high-abundance serum/plasma proteins, and the assay measures intact proteins, different from most of the MS-based studies in which proteins were digested and measurement was at the peptide level. Under the pre-analytical conditions investigated in this study, each condition affected a distinct set of proteins, while some common changes between two or more conditions were observed, suggesting different or similar molecular/biochemical or physical properties could contribute to these changes.

4.1. Effects of Cellular Compartments on Plasma Proteome Quality

In this study, Low $\times$ g and B6h0C resulted in the most changes in the human plasma proteome. Gene ontology analysis revealed proteins from a variety of cellular parts, and organelles were enriched in these changes. Apart from the proteins “freely” solubilized in the plasma as single molecules or protein complexes, many proteins are also compartmentalized by membranes such as those in organelles and extracellular vesicles including exosomes, microvesicles/microparticles, and apoptotic bodies.^{28,29} Proteins can also bind to these vesicles. The sizes of exosomes, microvesicles, and apoptotic bodies are 30–100 nm, 100–1000 nm, and 1–5 μ m, respectively,³⁰ which are in the range of the median size of platelets (5.4 μ m³, equivalent to a diameter of 2.2 μ m in spheres).³¹ Centrifugation of blood samples at 250–2000 $\times$ g for 10 min has been used for spinning down and enriching platelets for the preparation of platelet-rich plasma.³² Variable centrifugation speed (1300 $\times$ g vs 2500 $\times$ g) would likely result in differential separation of large vesicles/microvesicles, apoptotic bodies, and platelets due to heterogeneous diameter and buoyant density of these components. As observed in this study, 98% of the 200 changed proteins were augmented in abundance in the plasma prepared at Low $\times$ g. There is also a possibility that slower sedimentation of blood cells in Low $\times$ g could cause partial lysis or shaving of surface proteins, leading to higher amounts of cellular proteins in prepared plasma. Cold temperatures induce platelet aggregation,³³ and possible aggregation of vesicles and proteins in whole blood may decrease the amount of protein in plasma when the aggregates are sedimented by centrifugation. It was observed that 79% of the 148 changed proteins were decreased in abundance in B6h0C. Among the 83 proteins that changed commonly between Low $\times$ g and B6h0C, 79 proteins were changed in opposite directions (increased in Low $\times$ g, decreased in B6h0C; Table S2). Thus, the majority of these protein changes could result from the separation of these vesicles or aggregates based on their physical properties rather than by the biological processes of up- or down-regulation in transcription and translation. Also noted, freezing at <0 °C induces hemolysis, which leads to an increase in protein measurement.³⁴ Although care was taken not to freeze the blood samples held on wet ice prior to processing, a low degree of hemolysis may explain the increase in abundance of 31 proteins in the pre-analytical condition of B6h0C as hemoglobin was increased (2.2-fold) in the plasma processed under this condition but not under any other conditions (Table S1).

4.2. Effects of Enzymes on Plasma Proteome Quality

Gene ontology analysis indicated that proteins with catalytic activities were enriched in the changed proteins in each pre-analytical variable as compared to the control. Enzyme activity renders the blood/plasma samples sensitive to storage time and temperature. Among the top changed proteins, phosphoglycerate mutase 1, an enzyme involved in glycolysis,³⁵ was increased in plasma processed under all pre-analytical conditions except B6h0C. Neutrophil collagenase (or MMP-8) and stromelysin-1 (or MMP-3) are metalloproteinases that can cleave collagens.^{36,37} Their levels were decreased in the B6hRT, P24h4C, and P24hRT processing conditions. Interestingly, the level of sonic hedgehog protein (SHH) was also reduced in plasma processed under these three conditions. The C-terminal domain of the SHH precursor has autoprolysis activity,³⁸ which could contribute to the degradation of this protein. Changes of enzymes due to variable storage time and temperature not only affect the validity of these proteins as biomarkers but also affect peptide and metabolite biomarkers.

4.3. Activation of the Complement System

The complement system plays critical roles in innate immune response, and it is activated in many diseases.³⁹ Moreover, complement component peptides are often identified as disease biomarkers. Component C3 plays a central role in the activation of the complement system. Plasma levels of complement component C3a were decreased in Low×g and B6h0C, and no complement components were increased in these two conditions. However, complement components C3b and C4b were increased in B6hRT, P24h4C, and P24hRT, while C4 was decreased in P24hRT. Our previous data suggest that the total protein content of C3 was not affected by pre-analytical sample processing variations; however, peptides from C3 were increased in P24hRT and/or P24h4C.¹⁶ Significant increases of peptides from C4b were also observed in B6hRT, P24h4C, and P24hRT from our previous study.¹⁶ The data from this study suggests that C3 and C4 were activated in vitro and C3 convertase was also possibly activated since C3b and C4b are the respective subunits of two forms of C3 convertases. The increased level of C3d also indicates that enzymatic activity of factor I could persist in these plasma samples. It is possible that C3 could be activated through extrinsic proteases such as thrombin,⁴⁰ which was increased under the condition P24h4C. Up-stream activation of C1 complex was not observed since C1 subcomponents were not increased in B6hRT and P24h4C. In contrast, a slight increase of the C1q binding protein and decrease of C1r in P24hRT were observed (Table 2). Kaisar et al. took an interesting look at the human plasma degradome and noted an increase in proteolytic fragmentation of the complement members C1r, C3, C4B, and C5 over time (30 min to 2 days at RT) before processing to plasma.¹¹ Taken together, these results suggest special blood/plasma sample quality control should be employed to avoid artificial complement system activation in vitro for clinic assays of the complement system.

4.4. Value of Stable Proteins

This study found proteins changed (as defined in the Materials & Methods section) in human plasma levels under variable pre-analytical conditions. Considerable attention should be paid to sample quality in identifying these changed proteins as biomarkers. Important

also are the proteins that were relatively stable to the pre-analytical variables applied in this study. The remaining 962 proteins (approximately 75%) did not experience a significant alteration in abundance under any condition examined (Table S3). Using the same version of the SOMAscan assay, Kim et al. observed a similar proportion (70%) of proteins with a Spearman $r = 0.75$ from whole blood samples collected in EDTA and processed to plasma either immediately or after a 24 h delay.²⁷ The commonalities of the stable proteins observed between the conditions are presented in Figure S1B (Table S4). Besides the 962 proteins that were commonly stable among all the pre-analytical variables investigated, other proteins were changed under one or more conditions but stable under the other conditions. For example, only two and one protein were stable solely in the B6h0C and Low×g conditions, respectively. These stable proteins are valuable for biomarker development, especially those proteins that were stable under all the pre-analytical conditions investigated in this study. When biomarkers are among the changed proteins associated with pre-analytical conditions, special quality controls are required and validated for plasma sample processing.

Results from this study highlight protein abundance sensitivities associated with the specific pre-analytical conditions investigated. It should be noted that this study design is far from being inclusive of every possible source of variation in sample storage and processing. For pre-analytical conditions other than those investigated in this study, different sets of proteins could be changed, and protein stability should be examined for each condition.

4.5. Apparent Protein Quantification

SOMAscan assays as well as other multiplex immunoassays are affinity-based assays in which the reagent targets a certain region or epitope of a protein for molecular recognition. Therefore, these assays measure apparent quantitative changes, at least until the mechanism of decrease or increase in concentration is established. For example, near-complete proteolysis near the C-terminus of a target protein may result in a major quantitative change as observed by an assay employing a monoclonal antibody that targets the C-terminal region but for the exact same sample may result in no observed change for an assay based on an aptamer that binds to the mid-region of the targeted protein.

Recently, our lab measured the Bio-Plex panel of 37 inflammation biomarkers for their stability to the same pre-analytical variables.¹⁶ Among detectable inflammation biomarkers, 18 inflammation biomarkers were commonly quantitated by both the SOMAscan and Bio-Plex multiplex immunoassays (Table S5). Both assays revealed that seven proteins did not show significant changes under any pre-analytical variables. Among the remaining 11 proteins, consistent results were achieved for most of the pre-analytical conditions in which proteins were relatively stable; however, there were discrepancies between the two assays for significant abundance changes under individual pre-analytical conditions, especially for P24RT in which seven proteins apparently increased in abundance as quantitated by the Bio-Plex assay but remained stable as measured by the SOMAscan assay. While samples from 20 human subjects were analyzed by the Bio-Plex assay, results from the SOMAscan assay were obtained from only 16 subjects. For some proteins such as sCD30/TNFRSF8, sIL-6Ra, gp130/sIL-6R β , sTNF-R1, and sTNF-R2, apparently, the Bio-Plex assay measured the soluble forms of these proteins as increased upon P24RT but not necessary for the

SOMAscan aptamers to recognize the same region of each of these proteins. All these factors could contribute to the discrepancies in protein quantitation. From this aspect, it may be beneficial to conduct biospecimen stability studies using the same assay that is to be employed for biomarker discovery or validation. Validation of different assays for the same proteins or elucidation of the mechanisms of biomarker changes is necessary prior to biomarker validation using different assays.

5. CONCLUSIONS

Variability in processing blood to plasma and storage resulted in distinct protein abundance changes in each condition and certain common changes between conditions. Low centrifugal force in blood processing resulted in increased abundance of affected proteins in plasma, while storage of blood at 0 °C for prolonged time before processing to plasma decreased the plasma levels of most affected proteins. Plasma proteins were most sensitive to these two investigated conditions, resulting in the most protein changes in plasma. Fewer proteins were changed when blood was stored at RT for 6 h prior to processing to plasma or when plasma was stored at low temperature or RT for 24 h. However, residual enzymatic activities in blood/plasma, which are sensitive to storage time and temperature, could commonly affect the quality of plasma samples as demonstrated in the in vitro activation of the complement system. Undoubtedly, most proteins were robust and presented no significant changes despite the variances in pre-analytical processing. These resilient proteins could be useful as reference proteins to standardize abundance comparisons if they do not serve as diagnostic or prognostic biomarkers in clinical measurement. Careful consideration should be taken if proteins of interest lie within the category of significantly changed proteins identified in this study to avoid analytical inaccuracy of biomarker measurement using clinical assays. Nonetheless, it is recommended that blood samples be processed and frozen immediately under more stringent and consistent standard operating procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Diagram showing blood sample collection and processing under six pre-analytical conditions. Applied centrifugal force was $2500 \times g$ for all processing conditions except Low $\times g$ at $1300 \times g$.

Figure 2.

Volcano plots showing protein changes in variable pre-analytical conditions compared to the control. The vertical lines correspond to a 1.2 fold change in increased and decreased abundance, while the horizontal line represents a false discovery rate (FDR) of 0.05. Proteins that did not experience a statistically significant change (FDR > 0.05) are shown as black dots and fall below the horizontal line. The red dots represent proteins with increased abundance (fold change ≥ 1.2 and FDR < 0.05), and green dots represent proteins with decreased abundance (fold change ≤ -1.2 and FDR < 0.05) as compared to the control. Yellow dots symbolize proteins that statistically differed from the control, but their fold changes were <1.2.

Figure 3.

Gene ontology classification of (A) molecular functions and (B) cellular components for significantly changed proteins under each pre-analytical condition. The numbers within the pie charts represent the percentages of proteins classified in the GO categories. All proteins, a total number of 1305 proteins in the SOMAscan assay; Low $\times$ g, whole blood was immediately centrifuged at $1300 \times g$, and then plasma was directly frozen and stored at -80°C ; B6h0C, whole blood was stored for 6 h on wet ice (0°C) before processing to plasma at $2500 \times g$; B6hRT, whole blood was stored for 6 h at room temperature before processing to plasma at $2500 \times g$; P24h4C, whole blood was immediately centrifuged at $2500 \times g$, and the plasma was stored for 24 h at 4°C ; P24hRT, whole blood was immediately centrifuged at $2500 \times g$, and the plasma was stored for 24 h at room temperature.

Figure 4.
Multilevel principal component analysis of significantly changed proteins under variable pre-analytical conditions.

Table 1.

Proteins Changed Commonly between Pre-analytical Conditions B6hRT, P24h4C, and P24hRT^a

UniProt	protein	B6hRT		P24h4C		P24hRT	
		FC	FDR	FC	FDR	FC	FDR
P18669	phosphoglycerate mutase 1	11.08	5.3×10^{-10}	2.81	9.1×10^{-06}	50.35	1.8×10^{-10}
P0C0L5	complement C4b	3.41	6.5×10^{-10}	2.69	9.1×10^{-04}	7.35	1.8×10^{-10}
P08254	stromelysin-1	-2.85	7.4×10^{-07}	-2.22	5.5×10^{-05}	-2.78	1.6×10^{-07}
Q15465	sonic hedgehog protein	-2.12	5.3×10^{-10}	-1.36	5.0×10^{-06}	-4.76	1.8×10^{-10}
P01024	complement C3b	1.83	5.1×10^{-07}	1.37	8.4×10^{-04}	4.20	1.8×10^{-10}
P12277 P06732	creatine kinase M-type:creatine kinase B-type heterodimer	-1.71	1.3×10^{-05}	-1.53	3.4×10^{-05}	-1.54	1.5×10^{-05}
P22894	neutrophil collagenase	-1.66	6.4×10^{-04}	-2.03	1.9×10^{-09}	-2.02	7.6×10^{-10}
Q13443	disintegrin and metalloproteinase domain-containing protein 9	-1.28	3.9×10^{-08}	-1.36	4.4×10^{-10}	-1.75	1.8×10^{-10}
P55010	eukaryotic translation initiation factor 5	1.22	7.3×10^{-05}	1.26	1.4×10^{-06}	1.42	6.3×10^{-11}
P58417	neurexophilin-1	2.65	5.3×10^{-10}	1.22	6.3×10^{-01}	2.62	8.8×10^{-11}
P04155	trefoil factor 1	-2.28	5.3×10^{-10}	-1.06	1.0×10^{00}	-2.45	1.8×10^{-10}
P02144	myoglobin	1.48	3.2×10^{-08}	1.03	1.0×10^{00}	1.61	5.7×10^{-10}
P40225	thrombopoietin	-1.38	3.6×10^{-10}	-1.05	1.0×10^{00}	-1.27	6.5×10^{-05}
Q9UHA7	interleukin-36 alpha	1.36	1.2×10^{-02}	1.24	1.0×10^{00}	1.79	4.8×10^{-09}
P00749	urokinase-type plasminogen activator	-1.32	2.6×10^{-09}	-1.16	1.4×10^{-03}	-1.24	2.2×10^{-03}
O15123	angiotensinogen-2	-1.30	7.6×10^{-03}	-1.20	1.0×10^{00}	-1.33	1.5×10^{-02}
Q04609	glutamate carboxypeptidase 2	-1.28	3.2×10^{-05}	-1.12	2.6×10^{-02}	-1.47	3.4×10^{-07}
P10768	S-formylglutathione hydrolase	1.27	6.9×10^{-05}	1.08	5.1×10^{-01}	1.45	6.6×10^{-11}
P09237	matrilysin	-1.27	2.1×10^{-09}	-1.05	1.0×10^{00}	-2.65	1.8×10^{-10}
P13497	bone morphogenetic protein 1	-1.26	9.2×10^{-09}	-1.15	1.2×10^{-02}	-1.37	1.1×10^{-10}
O75556	mammaglobin-B	1.26	3.7×10^{-06}	1.11	8.5×10^{-03}	1.27	1.7×10^{-09}
P36507	dual specificity mitogen-activated protein kinase kinase 2	1.25	3.3×10^{-05}	1.03	1.0×10^{00}	1.75	1.8×10^{-10}
P30566	adenylosuccinate lyase	1.18	1.0×10^{00}	3.34	8.8×10^{-10}	1.54	1.5×10^{-05}
O95954	formimidoyltransferase-cyclodeaminase	-1.18	1.6×10^{-03}	-1.62	2.5×10^{-10}	-2.17	1.8×10^{-10}
P21695	glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic	1.14	1.0×10^{-02}	1.25	9.6×10^{-06}	1.28	8.3×10^{-07}
P17174	aspartate aminotransferase, cytoplasmic	1.14	1.0×10^{00}	1.65	3.3×10^{-10}	1.53	4.8×10^{-11}

UniProt	protein	B6hRT		P24h4C		P24hRT	
		FC	FDR	FC	FDR	FC	FDR
P62826	GTP-binding nuclear protein Ran	-1.12	7.6×10^{-01}	-1.68	2.4×10^{-03}	-2.03	5.2×10^{-06}
Q12907	vesicular integral-membrane protein VIP36	1.12	9.6×10^{-03}	1.21	3.7×10^{-07}	1.26	4.0×10^{-10}
P06703	protein S100-A6	-1.05	1.0×10^{00}	-1.42	4.4×10^{-04}	-1.40	3.2×10^{-04}
Q8WWK9	cytoskeleton-associated protein 2	1.05	1.0×10^{00}	1.31	1.1×10^{-05}	1.26	1.2×10^{-05}

^aFC, fold change as compared to the control condition, whole blood was immediately centrifuged at $2500 \times g$, and then plasma was directly frozen and stored at $-80\text{ }^{\circ}\text{C}$; FDR, false discovery rate calculated based on *P*-values, and the multiplex measurement was adjusted using the Benjamini and Hochberg method; B6hRT, whole blood was stored for 6 h at room temperature before processing to plasma at $2500 \times g$; P24h4C, whole blood was immediately centrifuged at $2500 \times g$, and the plasma was stored for 24 h at $4\text{ }^{\circ}\text{C}$; P24hRT, whole blood was immediately centrifuged at $2500 \times g$, and the plasma was stored for 24 h at room temperature.

Table 2.

Complement Components Changed under Pre-analytical Conditions^a

UniProt	protein	B6h0C		B6hRT		Low×g		P24h4C		P24hRT	
		FC	FDR	FC	FDR	FC	FDR	FC	FDR	FC	FDR
Q07021	C1 Q binding protein	1.05	8.9×10^{-01}	1.12	9.5×10^{-03}	1.10	8.1×10^{-03}	1.13	1.6×10^{-04}	1.24	1.4×10^{-10}
P00736	C1r	-1.15	1.1×10^{-02}	-1.13	1.1×10^{-01}	-1.01	1.0×10^{00}	-1.07	1.0×10^{00}	-1.32	1.6×10^{-08}
P01024	C3a	-1.39	4.5×10^{-02}	-1.42	9.9×10^{-02}	-1.41	3.2×10^{-02}	-1.31	5.2×10^{-01}	-1.22	1.0×10^{00}
P01024	C3b	1.28	7.4×10^{-02}	1.83	5.1×10^{-07}	1.04	1.0×10^{00}	1.37	8.4×10^{-04}	4.20	1.8×10^{-10}
P01024	C3d	1.04	1.0×10^{00}	-1.06	1.0×10^{00}	-1.03	6.0×10^{-01}	1.02	1.0×10^{00}	1.22	2.2×10^{-03}
P0C0L4, P0C0L5	C4	-1.02	1.0×10^{00}	-1.07	6.9×10^{-02}	-1.04	2.5×10^{-01}	-1.04	1.1×10^{-01}	-1.35	4.9×10^{-11}
P0C0L5	C4b	-1.09	1.0×10^{00}	3.41	6.5×10^{-10}	1.17	1.0×10^{00}	2.69	9.1×10^{-04}	7.35	1.8×10^{-10}

^aFC, fold change as compared to the control condition, whole blood was immediately centrifuged at $2500 \times g$ and then plasma was directly frozen and stored at -80°C ; FDR, false discovery rate calculated based on P -values, and the multiplex measurement was adjusted using the Benjamini and Hochberg method; B6h0C, whole blood was stored for 6 h on wet ice (0°C) before processing to plasma at $2500 \times g$; B6hRT, whole blood was stored for 6 h at room temperature before processing to plasma at $2500 \times g$; Low×g, whole blood was immediately centrifuged at $1300 \times g$ and then plasma was directly frozen and stored at -80°C ; P24h4C, whole blood was immediately centrifuged at $2500 \times g$ and the plasma was stored for 24 h at 4°C ; P24hRT, whole blood was immediately centrifuged at $2500 \times g$ and the plasma was stored for 24 h at room temperature.