Global Stability of Plasma Proteomes for Mass Spectrometry-Based Analyses*[S]

Lisa J. Zimmerman‡§, Ming Li¶‖, Wendell G. Yarbrough‖, Robbert J. C. Slebos‡‖, and Daniel C. Liebler‡§‡‡**

Peptide-based mass spectrometry approaches, such as multiple reaction monitoring, provide a powerful means to measure candidate protein biomarkers in plasma. A potential confounding problem is the effect of preanalytical variables, which may affect the integrity of proteins and peptides. Although some blood proteins undergo rapid physiological proteolysis *ex vivo*, the stability of most plasma proteins to preanalytical variables remains largely unexplored. We applied liquid chromatography-tandem mass spectrometry shotgun proteomics and multiple reaction monitoring analyses to characterize the stability of proteins at the peptide level in plasma. We systematically evaluated the effects of delay in plasma preparation at different temperatures, multiple freeze-thaw cycles and erythocyte hemolysis on peptide and protein inventories in prospectively collected human plasma. Time course studies indicated few significant changes in peptide and protein identifications, semitryptic peptides and methionine-oxidized peptides in plasma from blood collected in EDTA plasma tubes and stored for up to a week at 4 °C or room temperature prior to plasma isolation. Similarly, few significant changes were observed in similar analyses of plasma subjected to up to 25 freeze-thaw cycles. Hemolyzed samples produced no significant differences beyond the presence of hemoglobin proteins. Finally, paired comparisons of plasma and serum samples prepared from the same patients also yielded few significant differences, except for the depletion of fibrinogen in serum. Blood proteins thus are broadly stable to preanalytical variables when analyzed at the peptide level. Collection protocols to generate plasma for multiple reaction monitoring-based analyses may have different requirements than for other analyses directed at intact proteins. ***Molecular & Cellular Proteomics 11: 10.1074/mcp.M111.014340, 1–12, 2012.***

The human plasma proteome contains proteins from all tissues and its composition is hypothesized to reflect dynamic states of human health (1). Although immunoassays are the most widely used laboratory diagnostics, the challenge of producing high quality antibodies poses a barrier to the systematic evaluation of many new plasma protein biomarker candidates (2). The analysis of proteins by mass spectrometry (MS) provides a powerful alternative approach to systematically configure assays for potential disease biomarkers (2, 3). MS-based analyses of proteins, including targeted analyses by multiple reaction monitoring (MRM)[1] measure peptides produced by proteolytic digestion of plasma proteomes (2). Standardized MRM analyses display performance characteristics and interlaboratory reproducibility consistent with the eventual development of MRM-based clinical assay platforms (4–6). Detection of plasma proteins at low concentrations can be achieved with immunoaffinity enrichment of peptides or intact proteins prior to MRM analysis (5, 7, 8). MS analyses targeted to peptides thus appear likely to emerge in clinical laboratory practice in the near future (3).

Preanalytical variables, including conditions of plasma collection, preparation, and storage may affect the stability of plasma proteins. Processing and storage conditions affect the performance of many immunoassays (see (9–11) for recent examples), but relatively little is known about the impact of these variables on MS-based measurements made at the peptide level. Both the National Cancer Institute Early Detection Research Network (EDRN) and the Human Proteome Organization (HUPO) have made recommendations for specimen acquisition and handling practices associated with biomarker discovery and validation studies (12–14). However, these recommendations were based primarily on data from matrix-assisted laser desorption/ionization (MALDI) and surface-enhanced laser desorption/ionization (SELDI) analyses, which detect a relatively small number of abundant, low molecular weight proteins and peptides in undigested plasma or serum. Moreover, the changes observed with these platforms typically reflect physiologic proteolysis *ex vivo* of a small number of proteins involved in the coagulation and complement pathways (15–17). Evidence-based guidelines for blood collection and processing for targeted MS analyses require

From the ‡Jim Ayers Institute for Precancer Detection and Diagnosis and Departments of §Biochemistry, ¶Biostatistics, ‖Cancer Biology and **Otolaryngology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

Received September 13, 2011, and in revised form, January 23, 2012

⌘ *Author's Choice*—Final version full access.

Published, MCP Papers in Press, February 2, 2012, DOI 10.1074/mcp.M111.014340

[1] The abbreviations used are: MRM, multiple reaction monitoring; HUPO, Human Proteome Organization; MALDI, matrix-assisted laser desorption ionization; SELDI, surface-enhanced laser desorption/ionization; GLM, generalized linear mode.

new information about the stability of a broad range of plasma proteins, as indicated by measured levels of their constituent peptides.

To approach this problem, we employed a standardized shotgun proteomic analysis platform to perform global surveys of peptide content of plasma following tryptic digestion. This platform, which is similar to that characterized recently by the National Cancer Institute Clinical Proteomic Technology Assessment for Cancer (CPTAC) network (18, 19), generates ~13,000 MS/MS spectra, which identify ~1000 distinct peptides mapping to about 200 proteins in a single liquid chromatography tandem MS (LC-MS/MS) analysis of an unfractionated plasma digest.

Using shotgun proteomics and targeted analyses by MRM, we examined the stability of blood proteomes at the peptide level under variable storage and handling conditions commonly encountered in a clinic setting, where blood is routinely drawn for purposes other than proteomic analyses. Our experiments characterized changes at the peptide level in plasma proteins as a function of (1) time from blood collection to plasma isolation at 4 °C or room temperature, (2) number of freeze-thaw cycles performed on plasma samples, and (3) hemolysis prior to plasma preparation. Finally, we compared at the peptide level proteomes from serum and plasma samples prepared simultaneously from the same individuals. The data indicate that peptide inventories are surprisingly resistant to alteration by preanalytical variables and that peptide-level analyses provide a robust means of protein quantitation in blood samples subject to variable processing and storage conditions.

EXPERIMENTAL PROCEDURES

Materials and Reagents—Iodoacetamide and ammonium bicarbonate (>99.0% purity) were from Sigma (St. Louis, MO); *d,l*-1,4 dithiotheitol (DTT) was from Thermo Scientific (Rockford, IL); 2,2,2-trifluoroethanol (TFE) was from Acros (New Jersey). The *Escherichia coli* alkaline phosphatase peptide U-$^{13}C_6$, U-$^{15}N_4$-Arg-AAQGDITAPGGAR (>99% isotopic purity) was supplied by New England Peptide, LLC (Gardner, MA) at over 95% chemical purity. MS grade trypsin (Trypsin Gold, cat#TB309) was purchased from Promega (Madison, WI). HPLC grade water and acetonitrile were from Mallinckrodt Baker (Phillipsburg, NJ, USA).

Sample Collection and Preparation—Blood specimens from 10 subjects were collected during the Yul Brynner Head and Neck Cancer screening day on April 20, 2007 at Vanderbilt University and the Veterans Administration Medical Center in Nashville, TN. Demographic information for the participants is provided in supplemental Table S1. All protocols for this study were approved by the Vanderbilt Institutional Review Board (IRB) and all patients signed informed consent to donate their biological specimens for research. Blood was collected by venipuncture in 7 ml Vacutainer lavender tubes (with K_2EDTA (spray-dried) - product number 366643) (BD Diagnostics, Franklin Lakes, NJ) and immediately placed on ice. The tubes were transported to a central location within 30 min where a 1.5 ml portion of the blood was pipeted into a 1.5 ml Eppendorf tube. From this tube, 100 μl aliquots of whole blood were transferred into eight 96-well plates using one plate for each of the eight conditions: 4, 24, 72, or 168 h at 4 °C or 23 °C (room temperature). A separate 100 μl aliquot was also taken from each sample as the "0 h " time point. These were collected in strips of eight microcentrifuge tubes, which were frozen on dry ice at regular intervals. All processing was performed in a cold room at 4 °C and the maximum time between blood collection and freezing of the 0 h time point was 1 h. Blood was centrifuged at the designated times for 15 min at 1500 × *g* and plasma was removed via pipette and stored at −80 °C until further analysis.

For studies of the effects of freeze/thaw cycles, hemolysis, and for plasma/serum comparisons, two additional blood sample sets were obtained at a later date from the Jim Ayers Institute Tissue and Blood Collection Repository at Vanderbilt University. Demographic information for the participants is provided in supplemental Table S1. These blood samples were collected in 10 ml BD Vacutainer lavender tubes (spray-coated with K_2EDTA, product number 366643) (BD Diagnostics, Franklin Lakes, NJ) and used for plasma isolation under a Vanderbilt IRB-approved protocol. The first additional set consisted of three blood samples, from which plasma was collected immediately after centrifugation at 1500 × *g* for 15 min at 4 °C. This plasma was aliquoted in 200 μl portions, which were taken either fresh or after 1, 2, 3, 5, 10, or 25 freeze-thaw cycles between 4 °C and −80 °C. For each freeze-thaw cycle, the samples were frozen at −80 °C for 24 h and then thawed at room temperature for 30 min. For the hemolysis study, 10 samples each of hemolyzed and nonhemolyzed plasma were selected from the Ayers Institute plasma inventory. These samples had been collected according to a standard procedure corresponding to the "t = 0" samples described above. Hemolysis was assessed by visual observation of the color of the plasma (photographs of the samples are presented in supplemental Fig. S1). All samples were stored at −80 °C until further analysis.

For plasma/serum comparisons, two blood samples were taken from each of 10 participants. One was collected into a lavender top BD Vacutainer tube and EDTA plasma was prepared as described above for t = 0 samples. The other was collected into a 10 ml red top BD Vacutainer tube (no additive, product number 366430) and allowed to sit at room temperature for one 1 h. Blood was centrifuged to remove the fibrin clot for 4 °C for 10 min at 2000 × *g* and the serum was carefully transferred using a Pasteur pipette. All samples were stored at −80 °C until further analysis.

Sample Preparation for LC-MS Analyses—For time course studies, plasma samples (4 μl each) from 10 subjects corresponding to each temperature and time point were combined to create sample pools of 40 μl. Three aliquots from each pool were then processed for digestion and analysis. For freeze-thaw, hemolysis and plasma-serum comparisons, individual samples were processed for digestion and analysis. Plasma pool samples (5 μl aliquots) from time course studies was resuspended in 100 μl of TFE/50 mM ammonium bicarbonate pH 8.0 (1:1, v/v) and reduced with 10 μl 50 mM dithiothreitol at 60 °C for 20 min, followed by alkylation with 10 μl of 100 mM iodoacetamide in the dark at ambient temperature for 20 min. The reduced and alkylated protein mixtures were diluted to 0.5 ml with ammonium bicarbonate (50 mM, pH 8.0) followed by addition of trypsin at a trypsin/protein ratio of 1:50 (w/w). The mixtures were incubated overnight at 37 °C and evaporated to dryness *in vacuo*. Plasma and serum individual samples (10 μl aliquots) from the freeze-thaw, hemolysis, and plasma-serum studies were prepared by the same procedure, except that the volumes of all components were doubled. Prior to LC-MS/MS and MRM analyses, all digests were reconstituted in 0.1% formic acid to a final concentration of 0.2 μg/μl.

Analysis by LC-MS/MS—LC-MS/MS analyses were performed in duplicate on an LTQ-XL mass spectrometer (ThermoFisher, San Jose, CA) equipped with an Eksigent 1D Plus nanoLC pump and AS-1 autosampler (Dublin, CA). Peptides were separated on a packed capillary tip (Polymicro Technologies, 100 μm × 11 cm) with Jupiter

C18 resin (5 μm, 300 Å, Phenomenex) using an in-line solid-phase extraction column (100 μm $\times$ 6 cm) packed with the same C18 resin using a frit generated with liquid silicate Kasil 1 (20) similar to that previously described (21). Mobile phase A consisted of 0.1% formic acid and mobile phase B consisted of 0.1% formic acid in acetonitrile. A 95 min gradient was performed with a 15 min washing period at a flow rate of 1.5 μl min^{-1} (100% A for the first 10 min followed by a gradient to 98% A at 15 min) to allow for solid-phase extraction and removal of any residual salts. Following the washing period, the flow rate was decreased to 700 nL min^{-1} while the gradient was increased to 25% B by 50 min, followed by an increase to 90% B by 65 min and held for 9 min before returning to the initial conditions.

Centroided MS/MS scans were acquired on the LTQ using an isolation width of 2 *m/z*, an activation time of 30 ms, an activation q of 0.250 and 30% normalized collision energy using 1 microscan with a max ion time of 100 ms for each MS/MS scan and 1 microscan with a max ion time of 100 for each full MS scan. The mass spectrometer was tuned prior to analysis using the synthetic peptide TpepK (AVAGKAGAR), so that some parameters may have varied slightly from experiment to experiment, but typically the tune parameters were as follows: spray voltage of 2 KV, a capillary temperature of 150 °C, a capillary voltage of 50 V and tube lens of 120 V. A full scan was obtained for eluting peptides in the range of *m/z* 400–2000 followed by five data-dependent MS/MS scans with a minimum threshold of 1000 set to trigger the MS/MS spectra. MS/MS spectra were recorded using dynamic exclusion of previously analyzed precursors for 60 s with a repeat duration of 1.

MS/MS spectra collected as .raw files were converted into mzData format with an in-house developed software tool called ScanSifter and searched using the MyriMatch version 1.1.2 search algorithm against the IPI human database version 3.37 containing 69,164 entries (22). The database was concatenated with the reverse sequences of all proteins in the database to allow for the determination of false discovery rates. A static modification for carbamidomethylation was defined for cysteines, whereas dynamic modifications reflecting oxidation of methionines and formation of N-terminal pyroglutamines were allowed. Any number of missed cleavages was allowed and peptides were allowed to have a single nontryptic end. A precursor error of 1.25 *m/z* was allowed, but fragment ions were required to match within 0.5 *m/z*. The search results were filtered and assembled using IDPicker version 2.0 (23). Peptide identification stringency was set at a maximum of 2.5% reversed peptide identifications (5% overall peptide FDR) and a minimum of two distinct peptides to identify a given protein within the full data set. Distinct peptides have dissimilar precursor masses or charge states, but may reflect variant peptides of the same primary protein sequence. IDPicker employs a bipartite graph analysis and efficient graph algorithms to identify protein clusters with shared peptides and to derive the minimal list of proteins. This bipartite parsimony technique simplifies protein lists by consolidating results that map to redundant database entries and also improves the accuracy of protein identification (24). Such protein groups consist almost exclusively of isoforms or identical proteins resulting from redundancy in the database.

Quantitative Comparisons of Shotgun Proteomic Data Sets—We used a Poisson-based generalized linear model (GLM) approach that employs a quasi-likelihood Poisson distribution and is implemented in a graphical user interface software package called Quasi-Tel, as we described previously (25). Normalization between different runs was achieved by using the number of confident identifications as offset in the GLM. Proteins with GLM-generated *p* values of less than 0.05 were considered significantly different.

Quasitel output generated at the peptide level guided in the selection of peptides to be monitored by LC-MRM-MS. The two most extreme conditions in the time point series and freeze thaw cycles were compared at the peptide level, respectively. Peptides were filtered by GLM-generated *p* values of less than 0.05 and sorted by spectral counts. Peptides with the highest spectral count differentials between conditions were selected for MRM analyses.

Liquid-chromatography MRM Mass Spectrometry—All MRM analyses were performed on a TSQ Vantage triple quadrupole mass spectrometer (ThermoFisher Scientific) equipped with an Eksigent Ultra nanoLC 1D pump and AS-2 autosampler (Eksigent Technologies, Dublin CA). Peptides were resolved using a PicoFrit (New Objective) column (75 μm $\times$ 11 cm, 10 μm i.d. tip) self-packed with ReproSil-Pur C18 AQ resin (3 μm particle size). Separations were performed at a flow rate of 300 nL min^{-1} using 0.1% (v/v) formic acid in water (mobile phase A) and 90% (v/v) acetonitrile with 0.1% (v/v) formic acid in water (mobile phase B). Two microliter injections of the plasma digests (0.2 μg μl^{-1}) were separated using a binary gradient of 3–20% B in 3 min, 20–60% B in 35 min, 60–90% B in 2 min, and at 90% B for 4 min.

MRM analyses were performed with an electrospray voltage of 1300 $\pm$ 100 V and capillary temperature 210 °C. A single scan event was used to monitor four MRM transitions per peptide using the following parameters: Q1 and Q3 unit resolution 0.7 Da FWHM, Q2 gas pressure of 1.5 mTorr, scan width of 0.005 *m/z* and a scan time of 8 ms. Typically 8–10 scans are acquired across each chromatographic peak.

Quantitative measurements of plasma peptides were performed with a labeled reference peptide method (26), in which a single reference peptide ^{13}C-^{15}N-Arg-AAQGDITAPGGAR was spiked into samples at a concentration of 50 fmol μg^{-1} and used as a normalization standard. Each plasma sample was analyzed in three complete process replicates. Skyline was used to process all acquired data (27). Peptide peak areas were calculated as the sum of the integrated peak areas for the monitored transitions. The peak areas for each plasma peptide and for the bacterial peptide were calculated and exported in an Excel file. Data were normalized by dividing each plasma peptide peak area by the peak area of the reference peptide ^{13}C-^{15}N-Arg-AAQGDITAPGGAR. The normalized peak areas for each peptide were used to compare levels of specific peptide forms between experimental conditions.

RESULTS

Plasma Preparation Time Course Studies—The first preanalytical variable examined was the effect of delay from time of blood collection in EDTA-containing tubes to the time of plasma isolation by centrifugation. We collected blood from 10 volunteers and processed a 200 μl aliquot of each sample immediately to plasma. This sample represented time zero. Additional 200 μl aliquots of each blood sample were placed in 96-well plates and held for 4 h, 24 h, 3 days, and 1 week at either room temperature or 4 °C prior to plasma isolation. Because these experiments generated a large number of samples, we prepared and analyzed in triplicate pools from the 10 individual samples for each time point. We then compared the LC-MS/MS data sets to detect differences in (1) the numbers of MS/MS spectra collected, (2) confident peptide identifications (numbers of MS/MS spectra matched to peptides), (3) unique peptide identifications, (4) protein group identifications (numbers of indistinguishable proteins as defined in (24)), (5) the yield of semi-tryptic peptides, and (6) the yield of methionine oxidized peptides (Fig. 1). Summaries of these parameters for all of the experiments are presented in

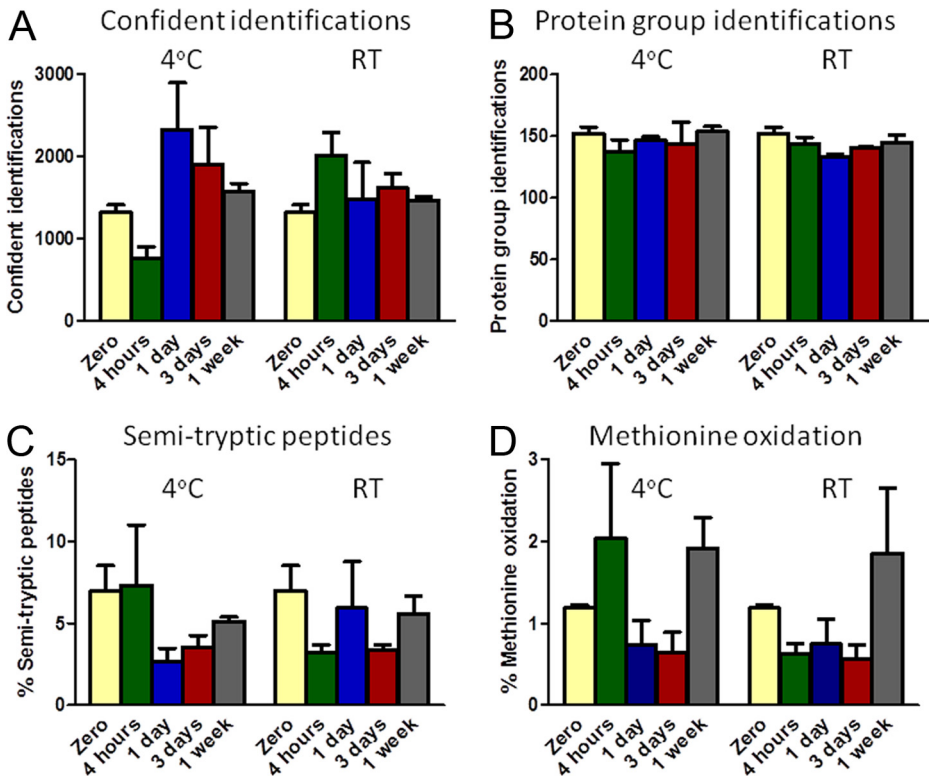

FIG. 1. **Analysis metrics from shotgun proteomic analyses of plasma from blood collected into EDTA tubes and then either processed immediately or stored for 4 h, 1 day, 3 days, or 1 week at 4 °C or room temperature prior to processing.** Metrics shown are: *A*, confident identifications (MS/MS spectra matched to database sequences at 1% FDR), *B*, protein group identifications (nonredundant protein forms), *C*, percent semi-tryptic peptides, and *D*, percent methionine oxidized peptides. All values represent the mean and standard deviation of three LC-MS/MS analyses of pooled samples as described under "Experimental Procedures."

supplemental Table S2. IDPicker reports listing all peptide and protein identifications and supporting database search results are provided as Supplemental Information.

Collection of similar numbers of MS/MS spectra indicated consistent LC-MS/MS system performance across the analyses. Although data for confident peptide identifications, peptide identifications, and protein identifications were largely consistent over time at both temperatures, statistical analysis revealed that the plasma pool corresponding to the 4 h and 4 °C time point yielded fewer peptide and protein group identifications than the other 4 °C time point plasma samples. This sample appeared to be an outlier, as there was no significant trend for decreasing peptide and protein identifications across the time course.

To detect protein degradation by endo- or exo-proteases, we assessed the yield of semitryptic peptides. No significant change in the percentage of semitryptic peptides with time was detected at either room temperature or 4 °C. To assess protein oxidation, we measured the numbers of identified peptides containing oxidized methionines. No significant differences were noted at either temperature over the time course.

We compared the spectral count data for individual proteins at different time points. Spectral counts for proteins at time zero were plotted against counts from the 24 h and 1 week time points and assessed by the Spearman correlation coefficient (Fig. 2*A*). (Comparisons of the time zero data with all of the other time points are shown in supplemental Figs. S1–S4). Only proteins observed with an average spectral count of at least one across all time point analyses were used in these comparisons. Protein degradation that decreased peptide identifications and spectral counts at later time points should decrease the correlation coefficient (r). However, r values were similar across all time point comparisons. The lowest r value (0.87) was observed in comparison of the time zero with the 4 h at 4 °C data, which, as noted above, appeared to be an outlier supplemental Fig. S2).

To identify proteins that underwent the greatest changes across the complete time course, we performed statistical comparisons of the shotgun datasets from time zero and 1 week for both temperatures. Comparison at the protein level indicated a small number of proteins with significant differences ($p < 0.05$), including albumin, serotransferrin, hemoglobin, fibrinogen, and others (supplemental Table S3). However, the observed differences between the two groups were less than two-fold and statistical significance may simply reflect the higher accuracy by which the most abundant proteins can be measured through spectral counting. To confirm

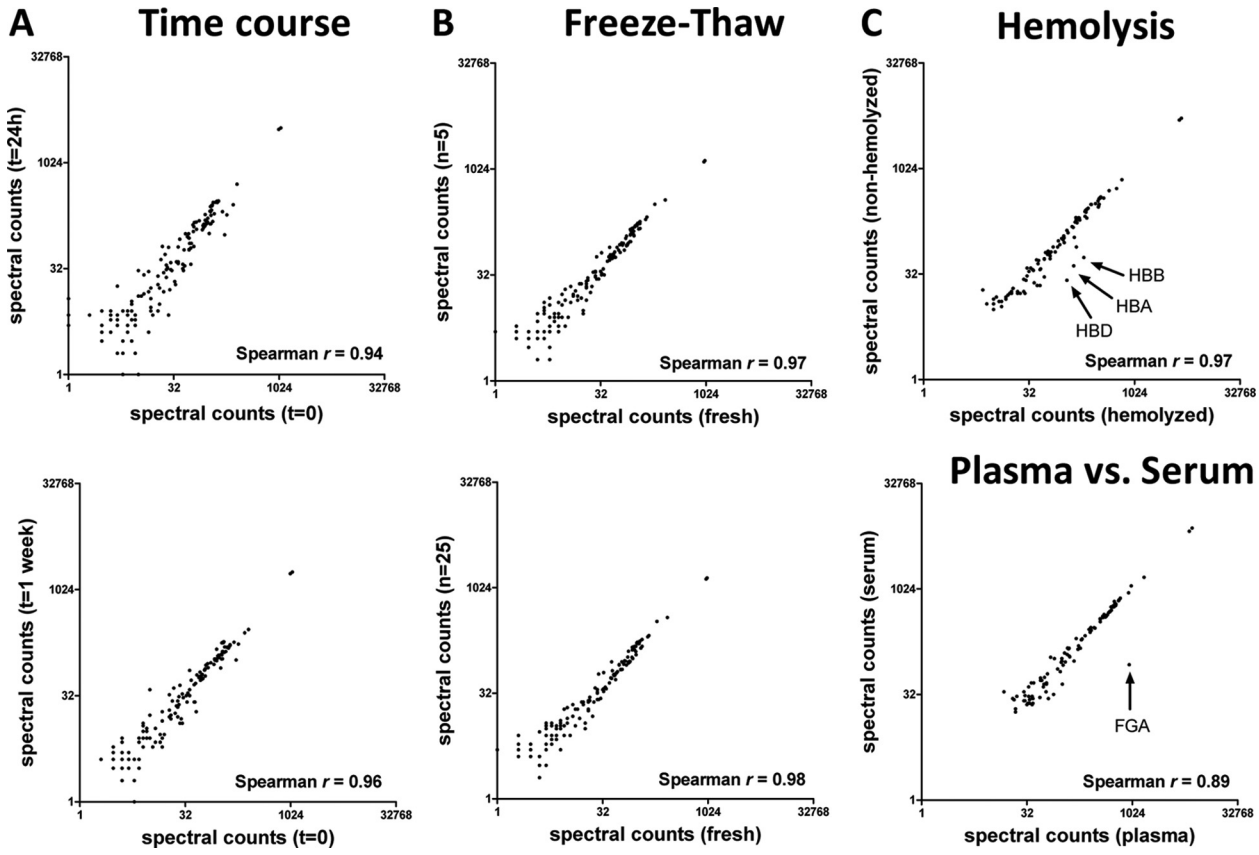

FIG. 2. ***A*, Linear correlation plots of the spectral counts for proteins identified in triplicate LC-MS/MS analyses between the zero time point plasma samples and those collected after 24 h (*upper* panel) or 1 week (*lower* panel) at 4 °C prior to processing.** The protein represented by the largest number of spectral counts within all comparisons was albumin. *B*, Linear correlation plots of the spectral counts for proteins identified in triplicate LC-MS/MS analyses between zero and five (*upper* panel) or zero and 25 (*lower* panel) freeze-thaw cycles. *C*, Linear correlation plots of the spectral counts for proteins identified in triplicate LC-MS/MS analyses of EDTA plasma from hemolyzed *versus* nonhemolyzed blood (*upper* panel) and from EDTA plasma *versus* serum (*lower* panel).

these apparent differences, we chose a panel of peptides representing these proteins based on differential spectral counts. We also examined the spectral count data for related sequences that differed by tryptic cleavages (supplemental Table S4). Most of the selected peptides were fully cleaved forms and had the highest spectral counts within each sequence family. In addition, other peptides from the same proteins, but with similar spectral counts between conditions were also selected as negative controls.

MRM assays were configured for these peptides and for other peptides from the same proteins that showed no spectral count differences between the time points. Only the blood samples from the room temperature experiment were analyzed by MRM, as time dependent effects were anticipated to be most pronounced in these samples. The peptides selected for each protein and the MRM results across the time series are shown in Fig. 3. In most cases, the spectral counts show that these differential peptides increased over time. For instance, peptides DVFLGMFLYEYAR and ALVLIAFAQYLQQCPFEDHVK from albumin and the two peptides GFTFATLSELHCDKLHVDPENFR and GFTFATLSELHCDKLHVD from hemoglobin all increased with time at room temperature. The MRM measurements all confirmed trends in the spectral count data. For albumin, peptides DVFLGMFLYEYAR and ALVLIAFAQYLQQCPFEDHVK were found to be differential by spectral counts and this was confirmed by MRM, as were changes in the ITIH4 peptide ELDRDTVFALVNYIFFK and hemoglobin peptides GFTFATLSELHCDKLHVDPENFR and GFTFATLSELHCDKLHVD. All peptides selected for MRM analysis, together with the monitored transitions and spectral count data are listed in supplemental Table S3.

We also monitored the formation of "ladder" peptides derived from fibrinogen, which have been detected as time-dependent products of degradation by exopeptidases in plasma and serum (15, 17, 28). We monitored several previously reported truncation products of the fibrinogen alpha peptide ADSGEGFLAEGGGVR in plasma collected after 0, 4h, 24 h, or 1 week at room temperature (Fig. 4*A*). Although these ladder peptides are rapidly formed *ex vivo* by degradation of fibrinogen in serum (17), the parent peptide remained the predominant form observed and there was minimal formation of the highly truncated peptides, FLAEGGGVR and LAEGGGVR.

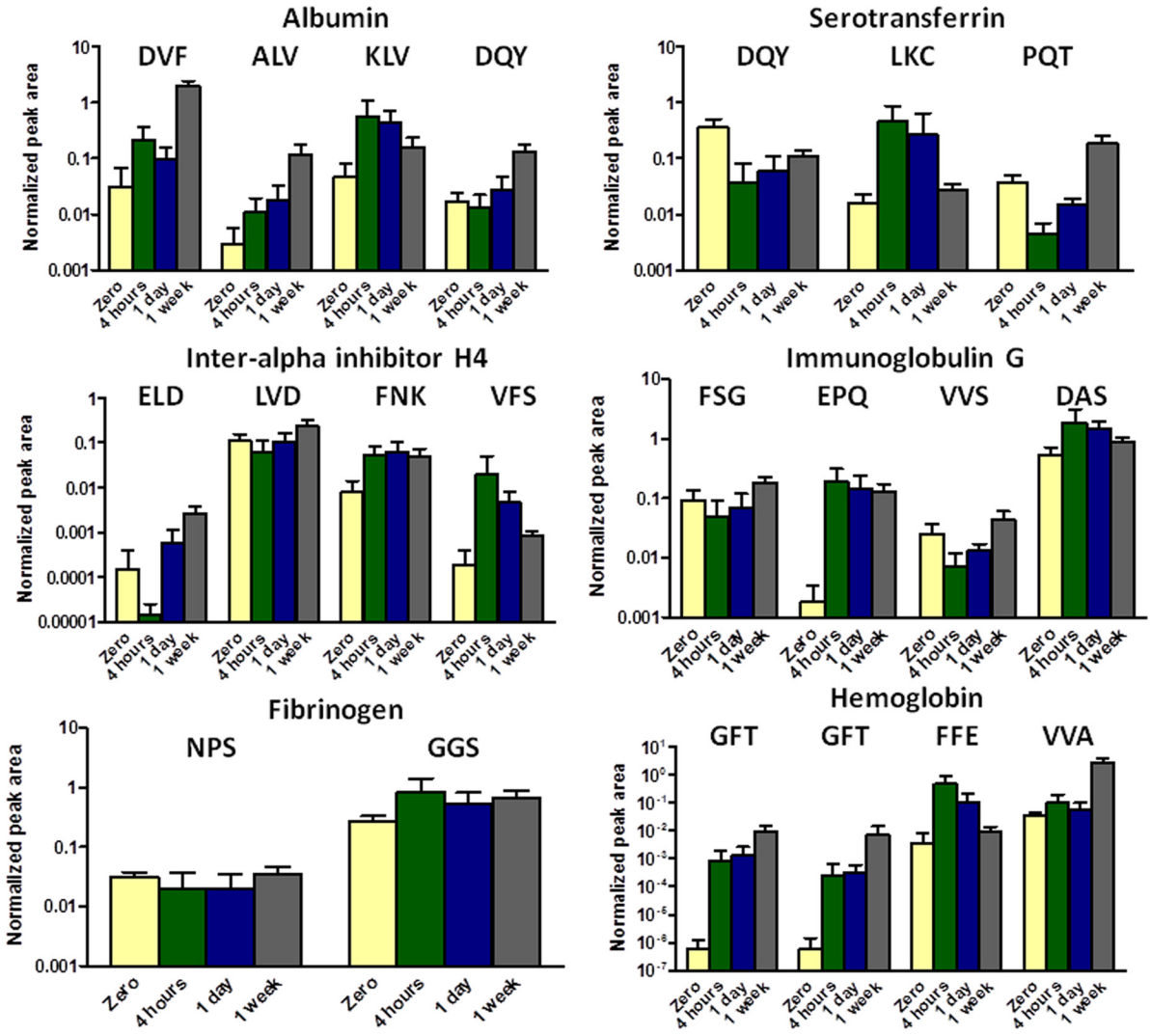

FIG. 3. **MRM analyses of selected peptides from six proteins in time course experiments.** Peptides from each protein were selected based on spectral count differences (peptides DVFLGMFLYEYAR, ALVLIAFAQYLQQCPFEDHVK (albumin); DQYELLCLDNTR (serotransferrin); GTFATLSELHCDKLHVDPENFR (GTF(1)), GFTFATLSELHCDKLHVD (GTF(2)) (hemoglobin); ELDRDTVFALVNYIFFK, LVDKFLEDVK (inter alpha trypsin inhibitor); FSGSGSGTDFTLTISR, EPQVYTLPPSRDELTK (IgG)) in shotgun analyses. Other peptides from the same proteins were selected based on spectral count similarity (KLVAASQAALGL, AQYLQQCPFEDHVK (albumin); LKCDEWSVNSVGK, PQTFYYAVAVVK (serotransferrin); FFESFGDLSTPDAVMGNPK, VVAGVANALAHK hemoglobin); FNKPFVFLMIEQNTK, VFSNGADLSGVTEEAPLKLSK (inter alpha trypsin inhibitor); VVSVLTVVHQDWLNGK, DASGVTFTWTPSSGK (IgG). Peptide peak areas were normalized by the labeled reference peptide standard method, as described under "Experimental Procedures." Plasma samples corresponding to each experimental condition were analyzed in triplicate and values shown are mean ± S.D.

Plasma Freeze-Thaw Studies—To study the effects of freeze-thaw cycles on protein stability in the plasma proteome, additional plasma samples were collected and either analyzed fresh or subjected to 1, 2, 3, 5, 10, or 25 freeze thaw cycles prior to analysis. Shotgun analyses yielded an equivalent number of MS/MS spectra for each sample and no significant changes were observed in numbers of peptides and protein group identifications regardless of the number of freeze thaw cycles. Likewise, freeze-thaw cycles appeared to have no effect on the proportion of semitryptic peptides generated or the levels of methionine-oxidized peptides (Fig. 5).

Correlation plots (Fig. 2B) compared the protein spectral counts for individual proteins in fresh plasma samples with counts for the same proteins after 5 or 25 freeze-thaw cycles. The Spearman correlation coefficients were 0.97 and 0.98, respectively, indicating no detectable changes in protein abundance based on spectral counts after multiple freeze-thaws. (Comparisons of the fresh plasma samples with plasma samples after 1, 2, 3, and 10 freeze-thaw cycles are shown in supplemental Fig. S4.)

MRM assays were configured for a few peptides that appeared to be different based upon spectral count comparisons between zero and 25 freeze thaw cycles. These peptides

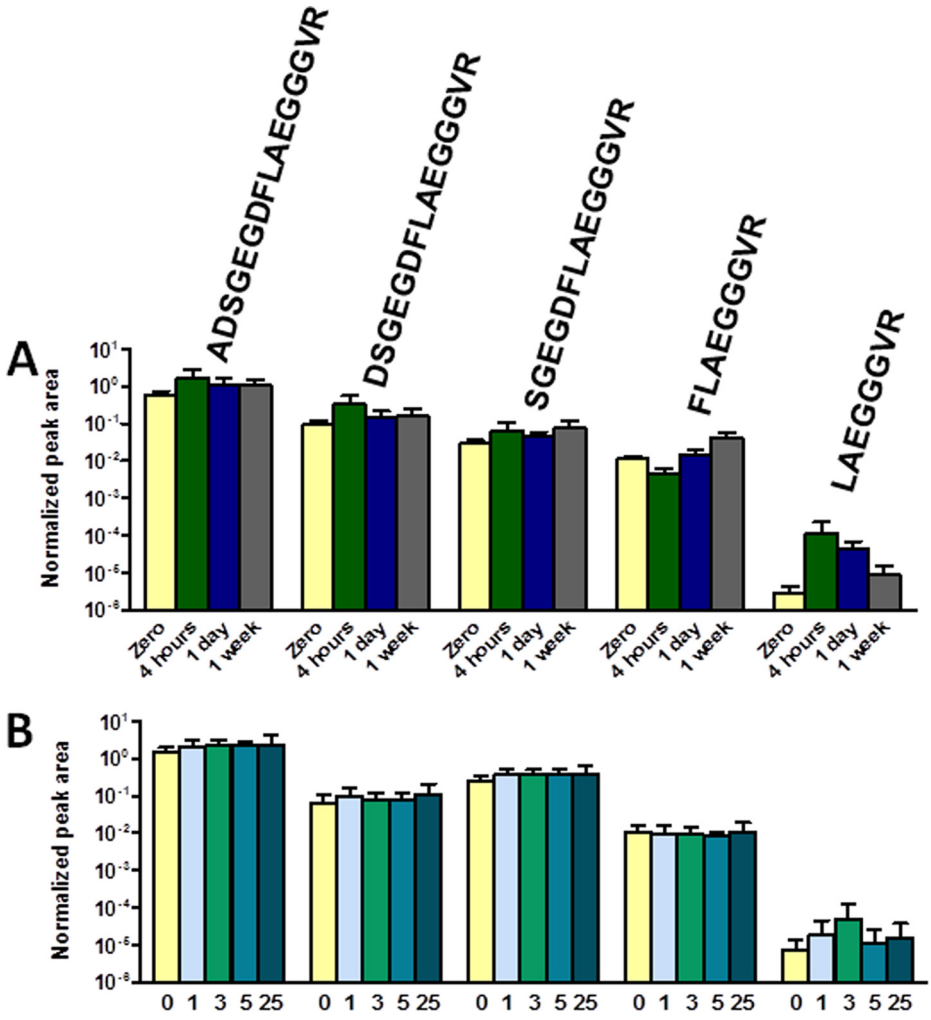

FIG. 4. ***A*, MRM analyses of ladder peptides from fibrinogen alpha in plasma collected after 4 h, 24 h, 3 days, and 1 week at 4 °C or room temperature.** The parent tryptic peptide from fibrinogen alpha, ADSGEGFLAEGGGVR, and its exopeptidase truncation products were monitored. Peptide peak areas were normalized by the labeled reference peptide standard method, as described under "Experimental Procedures." Plasma samples corresponding to each experimental condition were analyzed in triplicate and values shown are mean ± S.D. *B*, MRM analyses of ladder peptides from fibrinogen alpha in plasma collected fresh or after 1, 3, 5, 10, and 25 freeze-thaw cycles. The parent tryptic peptide from fibrinogen alpha, ADSGEGFLAEGGGVR, and its exopeptidase truncation products were monitored. Peptide peak areas were normalized by the labeled reference peptide standard method, as described under "Experimental Procedures." Plasma samples corresponding to each experimental condition were analyzed in triplicate and values shown are mean ± S.D.

were derived from the high abundant proteins, including albumin, complement C5, immunoglobulin, and fibrinogen (Fig. 6). The albumin-derived peptide EFNAETFTFHADICTLSEK had the largest difference in spectral counts between zero (16 counts) and 25 freeze thaws (198 counts). Despite modest apparent differences between some peptides between zero and 25 freeze-thaws, we observed no significant trends with a dependence on freeze-thaw cycles. We also monitored the ladder peptides derived from the fibrinogen alpha peptide ADSGEGFLAEGGGVR in a similar manner as for the time course series. No significant changes in the ladder peptides were detected with increase in freeze-thaw cycles (Fig. 4*B*).

Comparison of Plasma Proteomes from Hemolyzed and Nonhemolyzed Blood—Hemolysis is a potentially important preanalytical variable, because erythocyte proteins (particularly hemoglobin) may contaminate plasma and because hemoglobin-derived iron may catalyze oxidation and protein cleavage reactions (29). Hemolyzed plasma samples were initially identified from our collection database, which indicates evidence of hemolysis at the time of collection; we then selected samples by visual inspection for strongly reddish color. Both the hemolyzed and nonhemolyzed samples were frozen and previously unthawed prior to analysis. (A photo of the entire set is presented in supplemental Fig. S1).

We compared hemolyzed and nonhemolyzed plasma samples by shotgun proteomic analysis. The presence of hemolysis did not affect any measure of peptide or protein identifications, semitryptic peptides or methionine-oxidized peptides (Fig. 7).

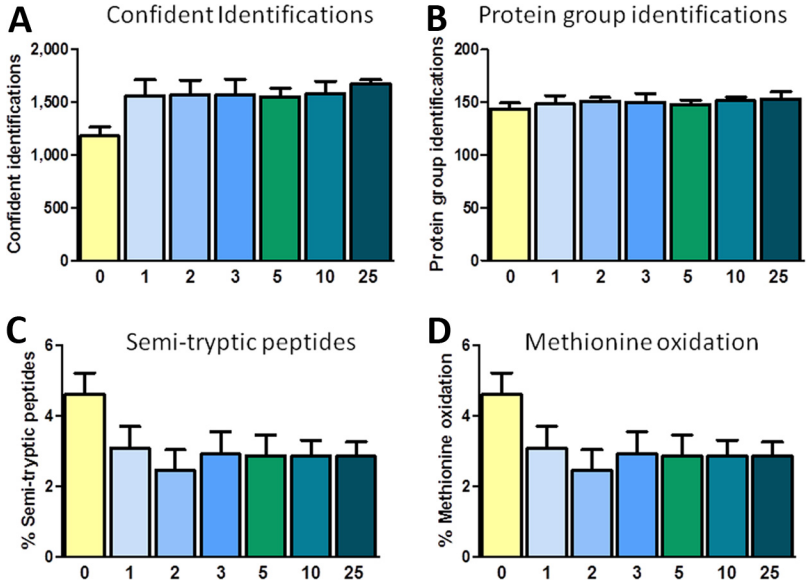

FIG. 5. **Analysis metrics from shotgun proteomic analyses of EDTA plasma either analyzed fresh or subjected to 1, 3, 5, 10, or 25 freeze-thaw cycles.** Metrics shown are: *A*, confident identifications (MS/MS spectra matched to database sequences at 5% FDR), *B*, protein group identifications (nonredundant protein forms), *C*, percent semi-tryptic peptides and *D*, percent methionine oxidized peptides. All values represent the mean and standard deviation from three LC-MS/MS analyses as described under "Experimental Procedures."

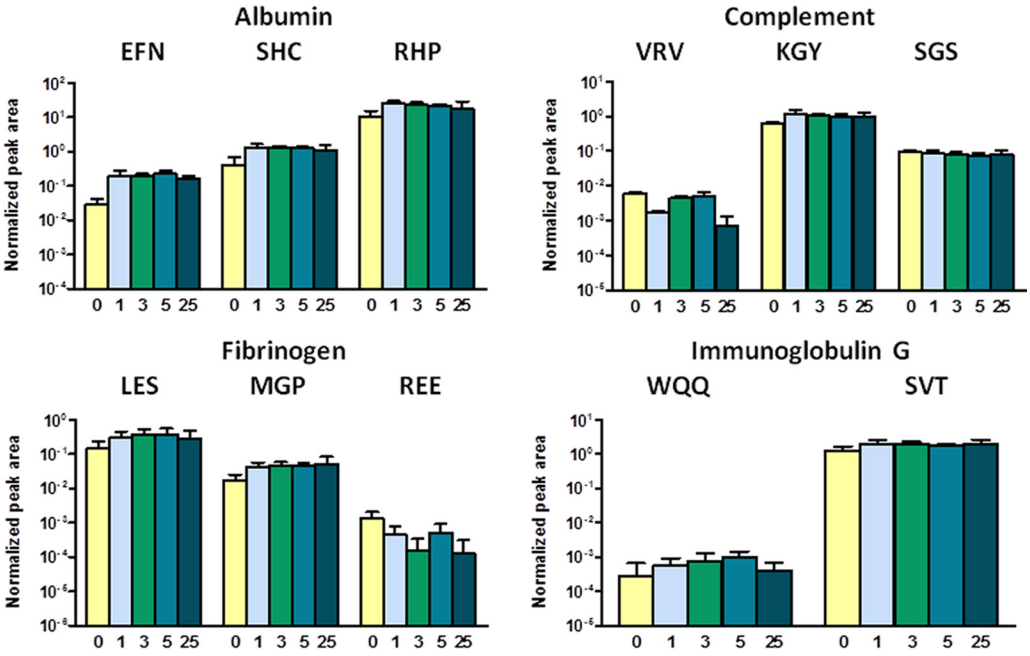

FIG. 6. **MRM analyses of selected peptides from four proteins in freeze-thaw experiments.** Peptides from each protein were selected based on spectral count differences (peptides EFNAETFTFHADICTLSEK, SHCIAEVENDEMPADLPSLAADFVESK (albumin); VRVELLHNPAFCSLATTK (complement C3); LESDVSAQMEYCR (fibrinogen beta); WQQGNVFSCSVMHEALHNHYTQK (IgG)) in shotgun analyses. Other peptides from the same proteins were selected based on spectral count similarity (peptides RHPDYSVVLLLR (albumin); KGYQQLAFR, SGSDEVQVGQQR (complement C3); MGPTELLIEMEDWKGDK, REEAPSLRPAPPPISGGGYR (fibrinogen beta); SVTCHVK (IgG)). Peptide peak areas were normalized by the labeled reference peptide standard method, as described under "Experimental Procedures." Plasma samples corresponding to each experimental condition were analyzed in triplicate and values shown are mean ±S.D.

The correlation plots indicated near identity of protein spectral counts ($r = 0.97$) between the hemolyzed and nonhemolyzed plasma samples (Fig. 2*C*). The four outliers noted in the hemolyzed plasma samples were hemoglobin proteins alpha, beta, gamma, and delta, which derive from the rupture of erythocytes.

Comparison of Plasma and Serum Proteomes—We compared proteomes of plasma and serum collected at the same time from 10 patients. Because serum preparation involves activation of proteases in the clotting factor cascade and cleavage of fibrinogen, it seems possible that other blood proteins might be subject to adventitious proteolysis. We performed shotgun proteome analyses of the plasma and serum samples. No significant differences were found in the numbers of peptide and protein identifications or in the overall

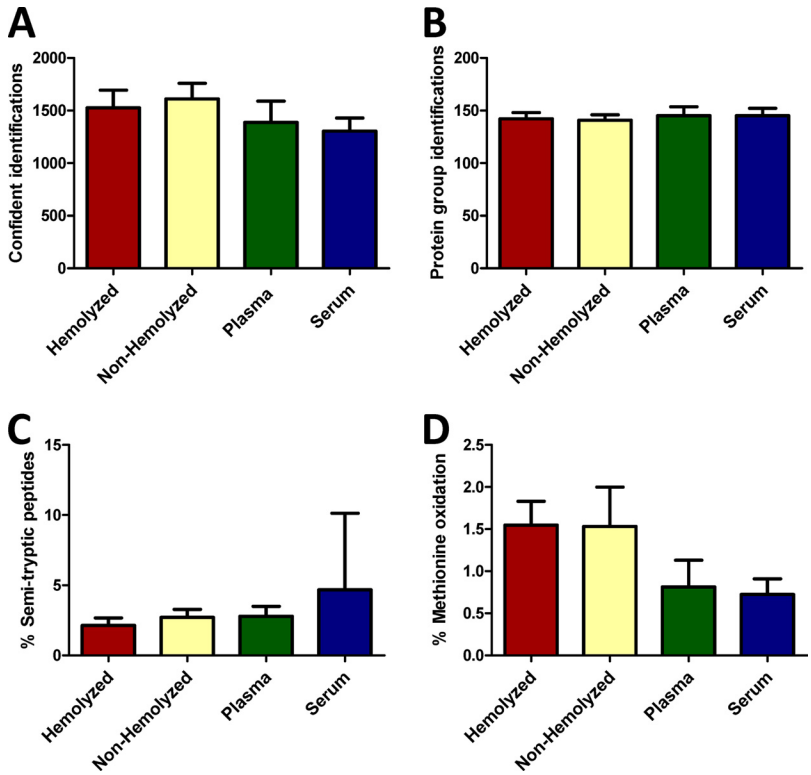

FIG. 7. **Analysis metrics from shotgun proteomic analyses of EDTA plasma from either hemolyzed or nonhemolyzed blood and from either plasma or serum.** Metrics shown are: *A,* confident identifications (MS/MS spectra matched to database sequences at 5% FDR), *B*, protein group identifications (nonredundant protein forms), *C*, percent semi-tryptic peptides and *D*, percent methionine oxidized peptides. All values represent the mean and standard deviation from three LC-MS/MS analyses as described under "Experimental Procedures."

percentages of semitryptic peptides or methionine oxidized peptides between the two sample types (Fig. 7). Although the percentages of semitryptic peptides did not differ significantly between plasma and serum, we noted higher variability of these in serum. In correlation analyses, the Spearman correlation coefficient for protein spectral counts in plasma *versus* serum was slightly lower ($r = 0.90$) than in most of the other comparisons described above (Fig. 2*C*). The primary outlier was fibrinogen alpha, which displayed 10-fold higher spectral counts in plasma than in serum. Two points not shown on the plot include the fibrinogen beta and gamma forms both of which were not identified in serum. Collectively; however, fibrinogen alpha, beta, and gamma proteins represent a 25-fold higher level in plasma than that observed in serum.

DISCUSSION

Quantitative plasma protein measurements may be affected by proteolysis or oxidation because of uncontrolled preanalytical variables. Previous MALDI- and SELDI-based studies suggested that plasma and serum proteins were highly labile, although it is now clear that the changes observed with these platforms involve a relatively small number of proteins that are subject to physiologic proteolysis (*e.g.* fibrinogens, complement) (15–17). Nevertheless, the concern is that proteolysis and concomitant oxidative damage on a broader scale might create considerable collateral damage to plasma proteins, thus potentially compromising their measurement. We asked whether systematic manipulation of common preanalytical variables could produce sufficient damage to compromise peptide measurements by LC-MS/MS. We expected that delayed processing, multiple freeze/thaws, and hemolysis would generate numerous proteolytic and oxidative changes, yet we found little evidence for accumulation of damage. We detected a few semi-tryptic peptides from abundant plasma proteins and observed modest changes in previously reported ladder peptides derived from fibrinogen. Our data suggest that plasma protein stability, represented at the peptide level, is considerably greater than would be inferred from the existing literature. These findings have important implications for the use of plasma and serum specimens for peptide-level protein quantitation.

To detect damage across the plasma proteome, we used shotgun proteome analyses, which enabled an unbiased survey of plasma peptides. We recognize that this approach has limitations. First, our single dimension LC-MS/MS analyses primarily sampled peptides derived from approximately the 200 most abundant plasma proteins, as we and others have previously demonstrated (30–32). We assumed that these proteins should be no more susceptible to proteolysis and oxidation than lower abundance proteins. Moreover, semi-

tryptic or oxidized peptides from higher abundance proteins should be easier to detect, if formed in sufficient yield. Second, spectral count comparisons in shotgun proteomics do not provide quantitative measurements as precise as those from MRM analyses. It is especially important to note that spectral count-based comparisons at the peptide level are less robust than at the protein-level, where multiple peptides are sampled. Nevertheless, MRM experiments to sample the numbers of peptides we detected in our shotgun analyses would have been prohibitively costly in both reference standards and instrument time. Despite the lower precision of spectral count data relative to MRM, our platform is capable of detecting small changes in proteome composition, as we have demonstrated previously (25). Indeed, the consistency of the shotgun datasets, in numbers of MS/MS spectra acquired, peptide sequences confidently identified and numbers of proteins identified all illustrate the high reproducibility of our shotgun analysis platform. Third, our analyses only assessed stability in the context of peptide detection by LC-MS/MS. We were unable to detect changes that altered protein folding or denaturation, which could dramatically impact the performance of immunoassays (10, 33–35), which require recognition of epitopes that may depend on protein secondary structures.

Our time course studies for plasma processing indicated a remarkable stability of plasma proteins to extended storage in EDTA tubes. The few changes we observed all involved peptides from highly abundant proteins. This suggests that preprocessing delay affects a small percentage of the copies of any particular protein and that the effect would be expected to be most easily detectable with higher abundance proteins. Most of the observed changes reflected increased detection of certain peptides. This may reflect gradual denaturation of proteins that enabled more complete digestion. Freeze thaw cycles produced little effect on detected peptide and protein inventories. As in the plasma processing time course studies, the few changes observed reflected increased yields of certain peptides, thus suggesting that protein denaturation may have facilitated digestion. Hemolysis affected plasma proteomes primarily through contamination with hemoglobin proteins. Methionine oxidation and semitryptic peptide yields were not significantly affected. We note that enhanced proteolysis of lower abundance proteins would be difficult to detect against the high background of tryptic peptides in our analyses. Nevertheless, we did detect semitryptic peptides from medium- to high-abundance proteins and these were unaffected by preanalytical variables. We did not assess the interaction of hemolysis with either plasma processing time course or freeze-thaw cycles, although it would be reasonable to suggest that this could increase oxidation.

Comparison of plasma and serum proteomes provided an interesting view of the potential impact of collateral damage through activation of proteases. Although it might be expected that activation of proteases of the clotting and complement cascades could affect many other proteins, our data suggest that activated clotting- and complement-related proteases have minimal impact on plasma and serum proteins. This probably reflects the biochemical specificity of these proteases for their physiological substrates, as has been reported in MALDI-based studies of the low molecular weight peptidome (15–17). We did observe greater variability in semitryptic peptides in serum than in plasma, but the difference was not significant. The lack of widespread proteolytic damage is consistent with previous work on MALDI profiling of the low molecular weight serum peptidome, in which the most dynamic changes were limited to physiologic substrates of blood proteases (17). Taken together, our data suggest that individual proteins and peptides in plasma may be protected from damage by both the sheer complexity of the plasma proteome and by a relatively small group of very high abundance plasma proteins. The distribution of damage over such a broad population may reduce the impact on any individual protein.

We do not consider this study a definitive evaluation of the stability of plasma proteins to preanalytical variables. As we noted above, our analyses did not detect a large portion of the plasma proteome and the imprecision of spectral count data could have masked modest changes in certain proteins. Nevertheless, by combining systematic manipulation of preanalytical variables with shotgun and MRM analyses, we were able to examine the stability at the peptide level of a far broader range of plasma proteins than had been previously possible with MALDI/SELDI or two-dimensional gel platforms. Perhaps the ultimate assessment of plasma proteome stability could be done with a large number of MRM assays targeted to hundreds or even thousands of peptides (3), but that would be an enormously expensive and complex undertaking. A more practical follow-up to our study could be done with newly configured MRM assays as they undergo analytical validation (36).

This work does suggest a possible reevaluation of plasma collection protocols in clinical proteomics studies where MRM analyses will be used to analyze biomarkers. Although our data are not sufficient for specific guidance, they do suggest that acceptable timeframes for plasma collection could be expanded from typical standard operating procedures (processing within 1 h of collection) for studies involving MRM analyses. An interesting corollary is the potential utility of discarded blood plasma for MRM-based analyses. At Vanderbilt University Medical Center, DNA is extracted from discarded patient blood specimens taken for clinical tests and then stored together with a de-identified patient medical record (37). This collection (currently over 150,000 samples and records) permits hypothesis-generating studies linking genomic data to clinical phenotypes (38). The stability of plasma proteins under typical clinical laboratory storage conditions (4 °C for up to 48 h prior to sample processing) could make it feasible to assemble a collection of plasma linked to DNA and de-identified medical records for MRM based analyses.

Acknowledgments—We thank Nancy Winters and Misti Martinez for their expert technical assistance.

* This work was supported by the National Cancer Institute Clinical Proteomic Technologies Assessment for Cancer program though National Institutes of Health Grant 1U24CA126479 and by generous gifts from the Jim Ayers Foundation and from the Robert J. Kleberg, Jr. and Helen C. Kleberg Foundation.

[S] This article contains supplemental Figs. S1 to S4 and Tables S1 to S4.

‡‡ To whom correspondence should be addressed: Department of Biochemistry, Vanderbilt University School of Medicine, U1213C Medical Research Building III, 465 21st Avenue South, Nashville, TN 37232-8575. Tel.: 615 322-3063; Fax: 615 343-8372; E-mail: daniel.liebler@vanderbilt.edu.

REFERENCES

1. Anderson, N. L., and Anderson, N. G. (2002) The human plasma proteome: history, character, and diagnostic prospects. *Mol. Cell. Proteomics* **1,** 845–867
2. Rifai, N., Gillette, M. A., and Carr, S. A. (2006) Protein biomarker discovery and validation: the long and uncertain path to clinical utility. *Nat. Biotechnol.* **24,** 971–983
3. Anderson, N. L., Anderson, N. G., Pearson, T. W., Borchers, C. H., Paulovich, A. G., Patterson, S. D., Gillette, M., Aebersold, R., and Carr, S. A. (2009) A human proteome detection and quantitation project. *Mol. Cell. Proteomics* **8,** 883–886
4. Addona, T. A., Abbatiello, S. E., Schilling, B., Skates, S. J., Mani, D. R., Bunk, D. M., Spiegelman, C. H., Zimmerman, L. J., Ham, A. J., Keshishian, H., Hall, S. C., Allen, S., Blackman, R. K., Borchers, C. H., Buck, C., Cardasis, H. L., Cusack, M. P., Dodder, N. G., Gibson, B. W., Held, J. M., Hiltke, T., Jackson, A., Johansen, E. B., Kinsinger, C. R., Li, J., Mesri, M., Neubert, T. A., Niles, R. K., Pulsipher, T. C., Ransohoff, D., Rodriguez, H., Rudnick, P. A., Smith, D., Tabb, D. L., Tegeler, T. J., Variyath, A. M., Vega-Montoto, L. J., Wahlander, A., Waldemarson, S., Wang, M., Whiteaker, J. R., Zhao, L., Anderson, N. L., Fisher, S. J., Liebler, D. C., Paulovich, A. G., Regnier, F. E., Tempst, P., and Carr, S. A. (2009) Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. *Nat. Biotechnol.* **27,** 633–641
5. Whiteaker, J. R., Zhao, L., Abbatiello, S. E., Burgess, M., Kuhn, E., Lin, C., Pope, M. E., Razavi, M., Anderson, N. L., Pearson, T. W., Carr, S. A., and Paulovich, A. G. (2011) Evaluation of large scale quantitative proteomic assay development using peptide affinity-based mass spectrometry. *Mol. Cell. Proteomics* **10,** M110.005645-1-10.
6. Hoofnagle, A. N., Becker, J. O., Wener, M. H., and Heinecke, J. W. (2008) Quantification of thyroglobulin, a low-abundance serum protein, by immunoaffinity peptide enrichment and tandem mass spectrometry. *Clin. Chem.* **54,** 1796–1804
7. Anderson, N. L., Anderson, N. G., Haines, L. R., Hardie, D. B., Olafson, R. W., and Pearson, T. W. (2004) Mass spectrometric quantitation of peptides and proteins using Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA). *J. Proteome Res.* **3,** 235–244
8. Berna, M. J., Zhen, Y., Watson, D. E., Hale, J. E., and Ackermann, B. L. (2007) Strategic use of immunoprecipitation and LC/MS/MS for trace-level protein quantification: myosin light chain 1, a biomarker of cardiac necrosis. *Anal. Chem.* **79,** 4199–4205
9. Christiansen, M., Høgdall, C. K., Andersen, J. R., and Nørgaard-Pedersen, B. (2001) Alpha-fetoprotein in plasma and serum of healthy adults: preanalytical, analytical and biological sources of variation and construction of age-dependent reference intervals. *Scand. J. Clin. Lab. Invest.* **61,** 205–215
10. Hegstad-Davies, R. L. (2006) A review of sample handling considerations for reproductive and thyroid hormone measurement in serum or plasma. *Theriogenology* **66,** 592–598
11. Shih, J., Datwyler, S. A., Hsu, S. C., Matias, M. S., Pacenti, D. P., Lueders, C., Mueller, C., Danne, O., and Möckel, M. (2008) Effect of collection tube type and preanalytical handling on myeloperoxidase concentrations. *Clin. Chem.* **54,** 1076–1079
12. Rai, A. J., Gelfand, C. A., Haywood, B. C., Warunek, D. J., Yi, J., Schuchard, M. D., Mehigh, R. J., Cockrill, S. L., Scott, G. B., Tammen, H., Schulz-Knappe, P., Speicher, D. W., Vitzthum, F., Haab, B. B., Siest, G., and Chan, D. W. (2005) HUPO Plasma Proteome Project specimen collection and handling: towards the standardization of parameters for plasma proteome samples. *Proteomics* **5,** 3262–3277
13. Tuck, M. K., Chan, D. W., Chia, D., Godwin, A. K., Grizzle, W. E., Krueger, K. E., Rom, W., Sanda, M., Sorbara, L., Stass, S., Wang, W., and Brenner, D. E. (2009) Standard operating procedures for serum and plasma collection: early detection research network consensus statement standard operating procedure integration working group. *J. Proteome Res.* **8,** 113–117
14. Gelfand, C. A., and Omenn, G. S. (2011) Preanalytical variables for plasma and serum proteome analyses. In: Ivanov, A. R., and Lazarev, A. V., eds. *Sample Preparation in Biological Mass Spectrometry*, pp. 269–289, Springer, New York
15. Koomen, J. M., Li, D., Xiao, L. C., Liu, T. C., Coombes, K. R., Abbruzzese, J., and Kobayashi, R. (2005) Direct tandem mass spectrometry reveals limitations in protein profiling experiments for plasma biomarker discovery. *J. Proteome Res.* **4,** 972–981
16. Marshall, J., Kupchak, P., Zhu, W., Yantha, J., Vrees, T., Furesz, S., Jacks, K., Smith, C., Kireeva, I., Zhang, R., Takahashi, M., Stanton, E., and Jackowski, G. (2003) Processing of serum proteins underlies the mass spectral fingerprinting of myocardial infarction. *J. Proteome Res.* **2,** 361–372
17. Villanueva, J., Shaffer, D. R., Philip, J., Chaparro, C. A., Erdjument-Bromage, H., Olshen, A. B., Fleisher, M., Lilja, H., Brogi, E., Boyd, J., Sanchez-Carbayo, M., Holland, E. C., Cordon-Cardo, C., Scher, H. I., and Tempst, P. (2006) Differential exoprotease activities confer tumor-specific serum peptidome patterns. *J. Clin. Invest.* **116,** 271–284
18. Paulovich, A. G., Billheimer, D., Ham, A. J., Vega-Montoto, L., Rudnick, P. A., Tabb, D. L., Wang, P., Blackman, R. K., Bunk, D. M., Cardasis, H. L., Clauser, K. R., Kinsinger, C. R., Schilling, B., Tegeler, T. J., Variyath, A. M., Wang, M., Whiteaker, J. R., Zimmerman, L. J., Fenyo, D., Carr, S. A., Fisher, S. J., Gibson, B. W., Mesri, M., Neubert, T. A., Regnier, F. E., Rodriguez, H., Spiegelman, C., Stein, S. E., Tempst, P., and Liebler, D. C. (2010) Interlaboratory study characterizing a yeast performance standard for benchmarking LC-MS platform performance. *Mol. Cell. Proteomics* **9,** 242–254
19. Tabb, D. L., Vega-Montoto, L., Rudnick, P. A., Variyath, A. M., Ham, A. J., Bunk, D. M., Kilpatrick, L. E., Billheimer, D. D., Blackman, R. K., Cardasis, H. L., Carr, S. A., Clauser, K. R., Jaffe, J. D., Kowalski, K. A., Neubert, T. A., Regnier, F. E., Schilling, B., Tegeler, T. J., Wang, M., Wang, P., Whiteaker, J. R., Zimmerman, L. J., Fisher, S. J., Gibson, B. W., Kinsinger, C. R., Mesri, M., Rodriguez, H., Stein, S. E., Tempst, P., Paulovich, A. G., Liebler, D. C., and Spiegelman, C. (2010) Repeatability and reproducibility in proteomic identifications by liquid chromatography-tandem mass spectrometry. *J. Proteome Res.* **9,** 761–776
20. Cortes, H. J., Pfeiffer, C. D., Richter, B. E., and Stevens, T. (1987) Porous ceramic beds supports for fused silica packed capillary columns used in liquid chromatography. *J. High Res. Chromatogr.* **10,** 446–448
21. Licklider, L. J., Thoreen, C. C., Peng, J., and Gygi, S. P. (2002) Automation of nanoscale microcapillary liquid chromatography-tandem mass spectrometry with a vented column. *Anal. Chem.* **74,** 3076–3083
22. Tabb, D. L., Fernando, C. G., and Chambers, M. C. (2007) MyriMatch: highly accurate tandem mass spectral peptide identification by multivariate hypergeometric analysis. *J. Proteome Res.* **6,** 654–661
23. Ma, Z. Q., Dasari, S., Chambers, M. C., Litton, M. D., Sobecki, S. M., Zimmerman, L. J., Halvey, P. J., Schilling, B., Drake, P. M., Gibson, B. W., and Tabb, D. L. (2009) IDPicker 2.0: Improved protein assembly with high discrimination peptide identification filtering. *J. Proteome Res.* **8,** 3872–3881
24. Zhang, B., Chambers, M. C., and Tabb, D. L. (2007) Proteomic parsimony through bipartite graph analysis improves accuracy and transparency. *J. Proteome Res.* **6,** 3549–3557
25. Li, M., Gray, W., Zhang, H., Chung, C. H., Billheimer, D., Yarbrough, W. G., Liebler, D. C., Shyr, Y., and Slebos, R. J. (2010) Comparative shotgun proteomics using spectral count data and quasi-likelihood modeling. *J. Proteome Res.* **8,** 4295–4305
26. Zhang, H., Liu, Q., Zimmerman, L. J., Ham, A. J., Slebos, R. J., Rahman, J., Kikuchi, T., Massion, P. P., Carbone, D. P., Billheimer, D., and Liebler, D. C. (2011) Methods for peptide and protein quantitation by liquid

chromatography-multiple reaction monitoring mass spectrometry. *Mol. Cell. Proteomics* **10**, M110.006593
27. MacLean, B., Tomazela, D. M., Shulman, N., Chambers, M., Finney, G. L., Frewen, B., Kern, R., Tabb, D. L., Liebler, D. C., and MacCoss, M. J. (2010) Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics* **26,** 966–968
28. West-Norager, M., Kelstrup, C. D., Schou, C., Hogdall, E. V., Hogdall, C. K., and Heegaard, N. H. (2007) Unravelling in vitro variables of major importance for the outcome of mass spectrometry-based serum proteomics. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **847,** 30–37
29. Davies, M. J. (2005) The oxidative environment and protein damage. *Biochim. Biophys. Acta* **1703,** 93–109
30. Tu, C., Rudnick, P. A., Martinez, M. Y., Cheek, K. L., Stein, S. E., Slebos, R. J., and Liebler, D. C. (2010) Depletion of abundant plasma proteins and limitations of plasma proteomics. *J. Proteome Res.* **9,** 4982–4991
31. Qian, W. J., Kaleta, D. T., Petritis, B. O., Jiang, H., Liu, T., Zhang, X., Mottaz, H. M., Varnum, S. M., Camp, D. G., 2nd, Huang, L., Fang, X., Zhang, W. W., and Smith, R. D. (2008) Enhanced detection of low abundance human plasma proteins using a tandem IgY12-SuperMix immunoaffinity separation strategy. *Mol. Cell. Proteomics* **7,** 1963–1973
32. Farrah, T., Deutsch, E. W., Omenn, G. S., Campbell, D. S., Sun, Z., Bletz, J. A., Mallick, P., Katz, J. E., Malmström, J., Ossola, R., Watts, J. D., Lin, B., Zhang, H., Moritz, R. L., and Aebersold, R. (2011) A high-confidence human plasma proteome reference set with estimated concentrations in PeptideAtlas. *Mol. Cell. Proteomics* 10, M110 006353
33. Hoofnagle, A. N., and Wener, M. H. (2009) The fundamental flaws of immunoassays and potential solutions using tandem mass spectrometry. *J. Immunol. Methods* **347,** 3–11
34. Ramachandran, R., Patterson, M., Murphy, K. G., Dhillo, W. S., Patel, S., Kazarian, A., Ghatei, M. A., and Bloom, S. R. (2008) Preanalytical factors affecting RIA measurement of plasma kisspeptin. *Clin. Chem.* **54,** 615–617
35. Semjonow, A., Köpke, T., Eltze, E., Pepping-Schefers, B., Burgel, H., and Darte, C. (2010) Pre-analytical in-vitro stability of [-2]proPSA in blood and serum. *Clin. Biochem.* **43,** 926–928
36. Regnier, F. E., Skates, S. J., Mesri, M., Rodriguez, H., Tezak, Z., Kondratovich, M. V., Alterman, M. A., Levin, J. D., Roscoe, D., Reilly, E., Callaghan, J., Kelm, K., Brown, D., Philip, R., Carr, S. A., Liebler, D. C., Fisher, S. J., Tempst, P., Hiltke, T., Kessler, L. G., Kinsinger, C. R., Ransohoff, D. F., Mansfield, E., and Anderson, N. L. (2010) Protein-based multiplex assays: mock presubmissions to the US Food and Drug Administration. *Clin. Chem.* **56,** 165–171
37. Roden, D. M., Pulley, J. M., Basford, M. A., Bernard, G. R., Clayton, E. W., Balser, J. R., and Masys, D. R. (2008) Development of a large-scale de-identified DNA biobank to enable personalized medicine. *Clin. Pharmacol. Ther.* **84,** 362–369
38. Ritchie, M. D., Denny, J. C., Crawford, D. C., Ramirez, A. H., Weiner, J. B., Pulley, J. M., Basford, M. A., Brown-Gentry, K., Balser, J. R., Masys, D. R., Haines, J. L., and Roden, D. M. (2010) Robust replication of genotype-phenotype associations across multiple diseases in an electronic medical record. *Am. J. Hum. Genet.* **86,** 560–572