Published in final edited form as:

Proteomics. 2005 February ; 5(2): 572–584.

Comparative Proteome Analyses of Human Plasma Following *in vivo* Lipopolysaccharide Administration Using Multidimensional Separations Coupled with Tandem Mass Spectrometry

Wei-Jun Qian¹, Jon M. Jacobs¹, David G. Camp II^{1,†}, Matthew E. Monroe¹, Ronald J. Moore¹, Marina A. Gritsenko¹, Steve E. Calvano^{2,†}, Stephen F. Lowry^{2,†}, Wenzhong Xiao^{3,†}, Lyle L. Moldawer^{4,†}, Ronald W. Davis^{3,†}, Ronald G. Tompkins^{5,†}, and Richard D. Smith^{1,†,*}

¹ Biological Sciences Division and Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, Richland WA 99352

² Department of Surgery, UMDNJ-Robert Wood Johnson Medical School, 125 Paterson Street, Suite 7300, New Brunswick, New Jersey 08901

³ Stanford Genome Technology Center, Stanford University School of Medicine, Palo Alto, CA 94304

⁴ Laboratory of Inflammation Biology and Surgical Science, Department of Surgery, University of Florida College of Medicine, 1600 SW Archer Road, Gainesville, FL 32610-0286

⁵ Department of Surgery, Shriners Burn Center and Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114

Abstract

There is significant interest in characterization of the human plasma proteome due to its potential for providing biomarkers applicable to clinical diagnosis and treatment and for gaining a better understanding of human diseases. We describe here a strategy for the comparative proteome analyses of human plasma, which is applicable to biomarker identifications for various disease states. Multidimensional liquid chromatography-mass spectrometry has been applied to make comparative proteome analyses of plasma samples from an individual prior to and 9 h after lipopolysaccharide (LPS) administration. Peptide peak areas and the number of peptide identifications for each protein were used to evaluate the reproducibility of LC-MS/MS and to compare relative changes in protein concentration between the samples following LPS treatment. A total of 804 distinct plasma proteins (not including immunoglobulins) were confidently identified with 32 proteins observed to be significantly increased in concentration following LPS administration, including several known inflammatory response or acute-phase mediators such as C-reactive protein, serum amyloid A and A2, LPS-binding protein, LPS-responsive and beige-like anchor protein, hepatocyte growth factor activator, and von Willebrand factor, and thus constituting potential biomarkers for inflammatory response.

Keywords

human plasma; proteomics; lipopolysaccharide; comparative analysis; LC-MS/MS

[†]Inflammation and the Host Response to Injury Investigators

*Address correspondence to: Dr. Richard D. Smith Environmental Molecular Sciences Laboratory Pacific Northwest National Laboratory P.O. Box 999, MSIN: K8-98, Richland WA, 99352 (rds@pnl.gov)

1 Introduction

human plasma, the soluble portion of blood, may effectively constitute the most complex human sub-proteome, containing not only “classical” plasma proteins, but also “leakage” proteins that potentially originate from virtually all cell types and tissues[1]. Because plasma protein concentrations are generally tightly regulated to maintain their normal physiological functions, changes in concentration provide direct information about disease processes and signaling pathways. There has been significant interest in quantitatively monitoring plasma proteomic changes between different states (i.e., normal vs. diseased states) in order to develop novel biomarkers for clinical diagnosis and treatment. However, the complexity and dynamic range of the plasma proteome has presented enormous challenges for current proteomic technologies, especially for quantitative proteomics.

Traditionally, two-dimensional gel electrophoresis (2-DE) has been the primary technique for separation of complex protein mixtures. Due primarily to the limited dynamic range of this technique, only 289 plasma proteins were reportedly identified prior to 2002[1]. Recent advances in liquid chromatography coupled with mass spectrometry (LC-MS) have greatly improved the dynamic range and sensitivity for analysis of complex protein mixtures[2-6]. Large-scale proteome profiling has been demonstrated for different organisms as well as mammalian tissues and cell lines by using multi-dimensional LC coupled with tandem mass spectrometry (MS/MS) (shotgun sequencing)[3,6-12]. Recently, Adkins *et al.* reported the identification of 490 proteins from an immunoglobulin-depleted human serum sample using a two-dimensional liquid chromatography coupled to tandem mass spectrometry (2D-LC-MS/MS) approach[13].

The use of shotgun sequencing for plasma proteome analysis has also been evaluated with different conventional mass spectrometers[14]. Pieper *et al.* reported the coupling of multidimensional LC, 2-DE, and MS for comprehensive profiling of the human serum proteome and nearly 3700 separated protein spots on gels were observed and 325 proteins were identified by MS analysis of 1800 gel spots[15]. More recently, Shen *et al.* applied a high resolution nano-scale 2D-LC-MS/MS approach for analyzing human plasma, which resulted in the identification of more than 800 plasma proteins[16].

In this work, we describe a strategy using peptide peak areas and the number of peptide identifications from 2D-LC-MS/MS analyses for a semiquantitative comparison of protein concentrations between plasma samples obtained from a human subject prior to (untreated) and 9 h after lipopolysaccharide (LPS) administration (treated). LPS is a purified, bacterial endotoxin released by Gram-negative bacteria and is known to induce a broad range of inflammatory reactions, including cytokine productions, cell migration, and production of acute-phase proteins[17-19]. LPS binds to the cell surface toll-like receptor in conjunction with LPS-binding protein and CD14, activating a cascade of intracellular signaling, and resulting in the activation of the transcription factor NF- κ B and in production and release of cytokines and other inflammatory mediators[18]. One of the aims of this research was to identify acute phase plasma proteomic changes in response to a prototypical inflammatory-challenge, such as LPS administration, with the ultimate goal of achieving a better understanding of the host response to systemic inflammation and sepsis syndrome, currently a substantial health care burden in the US[20].

Although the use of immunoaffinity approaches for removing high abundance proteins from plasma may allow more low abundance proteins to be detected[13,15], the removal of high abundance proteins such as albumin may simultaneously remove specifically or nonspecifically bound low abundance proteins of interest[21]. In this work, plasma samples were analyzed using 2D-LC-MS/MS without major protein depletion. A total of 804 plasma

proteins (not including immunoglobulins) were identified, significantly expanding the number of proteins previously identified in human plasma. Peptide peak areas determined from peptide elution profiles and the number of peptide identifications (peptide hits[22]) for each protein were used to provide two independent semi-quantitative measures of the reproducibility of the LC-MS/MS approach and for the comparison of relative changes in protein concentrations between untreated and treated samples. By using these two approaches, a number of proteins were confidently identified as up-regulated following LPS treatment.

2 Materials and methods

2.1 Human plasma samples

The human plasma samples were supplied by Department of Surgery at the University of Florida College of Medicine (LLM), which serves as the Sample Collection and Coordination Site for a multicentered clinical trial (Inflammation and the Host Response to Injury). The original sample was generated from a volunteer subject at the Department of Surgery at the Robert Wood Johnson Medical School who after signed informed consent, received an intravenous injection of Clinical Center Reference Endotoxin (CCRE, Lot 2) LPS (4 ng/kg body weight administered over five minutes), and arterial or venous blood was collected at various time-points between 0 to 24 h following endotoxin administration. The plasma samples were prepared from whole blood by centrifugation with samples at T= 0 h (untreated, baseline prior to endotoxin administration) and T= 9 h (treated, 9 h following LPS administration) used for this study. Approval for the conduct of this Program was obtained from the Institutional Review Boards of the University of Florida College of Medicine, the Robert Wood Johnson Medical School, and the Pacific Northwest National Laboratory.

2.2 Plasma sample processing and tryptic digestion

200 μ L each of the untreated and treated plasma samples were diluted to 1 mL with 50 mM NH_4HCO_3 , pH 8.2. The plasma proteins were denatured using 8 M urea for 1 h at 37°C and reduced with 10 mM dithiothreitol (DTT) for 30 min at 37°C. Cysteine residues in the protein samples were alkylated with 40 mM iodoacetamide for 90 min at room temperature with shaking in the dark. Following alkylation, the samples were desalted into 50 mM NH_4HCO_3 by gel filtration using a prepacked PD-10 column containing Sephadex G-25 (Amersham Biosciences, Piscataway, NJ). The protein concentrations for the desalted samples were measured using a BCA protein assay (Pierce, Rockford, IL), which gave the total protein amount of 15.0 and 13.9 mg for the untreated and treated plasma samples, respectively. The samples were digested into peptides using sequencing grade trypsin (Promega, Madison, WI) overnight at 37°C with a 1:50 (w:w) trypsin-to-protein ratio. Tryptic activity was quenched by boiling the samples for 5 min and immediately placing the samples on ice.

2.3 Peptide fractionation using strong cation exchange (SCX) chromatography

3.0 mg each of untreated and treated plasma peptides were used for SCX fractionation. Samples were first lyophilized and then resuspended in 1.5 mL of 10 mM ammonium formate, 25% acetonitrile, pH 3.0. Samples were injected onto a Polysulfoethyl A 200 mm $\times$ 4.6 mm (5 μ m, 300 Å) column (Poly LC, Columbia, MD) preceded by a 10 mm $\times$ 4.6 mm guard column with a flow rate of 1 mL/min. The separations were performed with a Shimadzu LC-10A system utilizing a Unicam 4225 (Thermo Electron, Waltham, MA) UV/Vis detector with mobile phases consisting of solvent A: 10 mM ammonium formate, 25% acetonitrile, pH 3.0, and solvent B: 500 mM ammonium formate, 25% acetonitrile, pH 6.8. After sample loading, the run was isocratic for 10 min at 100% solvent A. Peptides were eluted by following linear gradients from 100% solvent A to 50% solvent B over 40 min and from 50% solvent B to 100% solvent B over another 10 min. The mobile phase was held at 100% solvent B for another 15 min. The mobile phase was then switched to 100% nanopure water for 30 min followed by

switching to 100% solvent A to let the column reequilibrate with solvent A for 20 min before the start of another run. One mL fractions (1 min per fraction) were collected using a Shimadzu FRC-10A fraction collector after the start of the gradient. A total of 50 fractions were collected with each fraction lyophilized and used for further reversed-phase LC-MS/MS analysis.

2.4 Reversed-phase capillary LC-MS/MS

LC-MS/MS analyses of peptide samples were performed using a fully automated custom-built capillary LC system (described in detail elsewhere[23]) coupled on-line with an LCQ Deca XP ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA) using an in-house-manufactured ESI interface. The automated LC-MS/MS system allows for the analysis of 6 samples over a 24 h period. The reversed-phase capillary column was made by slurry packing 5 μ m Jupiter C₁₈ bonded particles (Phenomenex, Torrance, CA) into a 65 cm long, 150 μ m i.d. $\times$ 360 μ m o.d. fused-capillary (Polymicro Technologies, Phoenix, AZ) incorporating a 2- μ m retaining stainless steel screen in an HPLC union (Valco Instruments Co., Houston, TX). The mobile phase consisted of 0.2% acetic acid and 0.05% TFA in water (A) and 0.1% TFA in 90% acetonitrile/10% water (B). Mobile phases were degassed on-line using a vacuum degasser (Jones Chromatography Inc., Lakewood, CO).

The SCX fractions were dissolved in 50 μ L 25 mM NH₄HCO₃, pH 8.0. 10 μ L of each peptide sample was injected onto the reversed-phase capillary column for LC-MS/MS analysis. The mobile phase was held at 100% A for 20 min. A non-linear exponential gradient elution was generated by increasing the mobile-phase composition to ~70% B over 150 min using a stainless steel mixing chamber. To identify the eluting peptides, the ion trap mass spectrometer was operated in a data-dependent MS/MS mode (m/z 400-2000), in which a full MS scan was followed by three MS/MS scans using a normalized collision energy setting of 45%. Dynamic exclusion was applied to prevent repeated analysis of the same high abundant ion.

2.5 Data analysis

The SEQUEST algorithm (Thermo Finnigan, San Jose, CA) was used for peptide and protein identification by searching the MS/MS spectra against the NCI-Frederick human protein database[24]. A static mass modification on cysteine residues corresponding to the alkylation with iodoacetamide (57.0215 Da) was applied during the SEQUEST analysis. Stringent criteria were used for the post-SEQUEST analysis for the confident identification of proteins present in the plasma. An initial filter based upon previously published criteria [3,6,7] was performed and only those proteins with ≥ 2 different identifying peptides were retained. Briefly, the initial filter criteria are as follows: SEQUEST correlation score (Xcorr) ≥ 1.8 for charge state 1+ full or partial tryptic peptides, Xcorr ≥ 1.9 for charge state 2+ full tryptic peptides and Xcorr ≥ 2.2 for partial tryptic peptides, and Xcorr ≥ 3.2 for charge state 3+ full tryptic peptides and Xcorr ≥ 3.75 for partial tryptic peptides, all with a DelCN value of ≥ 0.05 . In addition, a normalized elution time (NET) constraint was placed upon peptide identifications based upon the predicted versus observed elution time in the LC separation prior to MS/MS analysis [25]. Such a technique has been previously shown to increase the overall confidence of the dataset[11]. Identified peptides which did not elute with $\pm 10\%$ of their predicted elution time were removed as possible false identifications. For proteins identified by only one identifying peptide, a much more stringent criterion was used, as follows: For charge state 1+, only full tryptic peptides at Xcorr ≥ 2.0 , for charge state 2+ full tryptic peptides at Xcorr ≥ 2.2 and partial tryptic peptides at Xcorr ≥ 3.0 , for charge state 3+ only full tryptic peptides at Xcorr ≥ 3.75 , all with a DelCN value of ≥ 0.10 . These criteria effectively reduced the protein identifications based upon only one peptide by $\sim 90\%$. After filtering, the total number of peptide identifications (peptide hits) from an individual protein was calculated, which includes the repetitive identifications of the same peptide in one or multiple SCX fractions. All

identifications corresponding to the immunoglobulin entries in the database were excluded due to the difficulty in distinguishing between specific antibodies by peptide level identifications.

An in-house developed software tool (MASIC) was used to generate peptide chromatographic elution profiles (selected-ion chromatograms) of the full MS scans and to determine the peak area of each peptide chromatographic peak. If an individual peptide was identified in multiple SCX fractions, the sum of the peak areas from each fraction was assigned as the total peak area for the peptide. When comparing two different samples or analyses, peptide peak area ratios were calculated only for peptides observed in both samples or analyses with the same charge state. Protein abundance ratios based on peak areas were calculated by averaging the peptide peak area ratios for the same protein.

3 Results and discussion

3.1 SCX fractionation and LC-MS/MS analyses

SCX and reversed-phase capillary LC were coupled offline for 2D-separations of plasma peptides to improve the dynamic range of the analysis and increase the number of protein identifications. In an attempt to observe changes in protein concentrations between the two different plasma samples, equal amounts of both samples were processed and analyzed under identical conditions. Following trypsin digestion, peptides were fractionated into 50 fractions using SCX with each fraction analyzed by LC-MS/MS. Fractions rich in peptides (e.g., Fraction 8-31 as shown in Figure 1A and 1B) were analyzed twice by LC-MS/MS. A total of 148 LC-MS/MS analyses were performed for the untreated and treated samples. Figure 1 shows the SCX chromatograms plotted using both UV absorbance and the corresponding numbers of peptides identified in each fraction for the untreated (A) and treated (B) plasma samples. The chromatograms derived from UV absorbance and the number of detected peptides from each fraction correlate well, with the untreated and treated samples showing almost identical chromatographic patterns. This observation suggests good reproducibility of the sample processing and SCX separation. Figure 1C shows the base peak chromatogram of the LC-MS/MS analysis of fraction 11 from the untreated plasma. As shown, a few high abundance species (high intensity peaks) are observed from this fraction, with the majority of other peaks having relatively low intensity. Despite the presence of some very high abundance species, analysis of fraction 11 still resulted in identification of 296 different peptides corresponding to 120 proteins on the basis of database searching. Figure 1D shows two examples of MS/MS spectra originating from species with relatively low intensities (positions 1 and 2 marked with arrows in corresponding Figure 1C). These spectra resulted in confident identification of two peptides, S.CGSAAGGPDLYGTLK.E ($X_{\text{corr}} = 3.81$) and K.GLQYAAQEGLLALQSELLR.I ($X_{\text{corr}} = 4.27$), which correspond to EGF response factor 2 (TISD_HUMAN) and LPS-binding protein (LBP_HUMAN), respectively.

3.2 Plasma Protein identifications

From the 148 LC-MS/MS analyses for the untreated and treated plasma samples, approximately 470,000 MS/MS spectra were acquired, which resulted in a total of 376,664 raw SEQUEST identifications after database searching against the human protein database. After filtering the SEQUEST results with conservative criteria (see Materials and methods), 39,183 SEQUEST identifications were retained, which corresponded to ~10% of the raw identifications. Recently, Shen *et al.* reported various numbers of protein identifications (between 800 and 1682 proteins from plasma) that were obtained using different previously published SEQUEST filtering criteria[16], including several less restrictive criteria. In this work, we have applied more restrictive criteria to reduce the likelihood of false positive protein identifications. These criteria include the use of tryptic peptides only for protein identification, the use of a NET constraint, the requirement of 2 or more different identifying peptides per

protein for most proteins, and the requirement of high SEQUEST scores for proteins identified by only one peptide (see Materials and methods). By combining results for the two plasma samples, a total of 804 non-redundant plasma proteins (excluding immunoglobulins) were identified based upon 5,176 different peptides and following the manual removal of possible redundancy in the identified protein entries. 669 proteins (83%) were identified by at least 2 different tryptic peptides. The applied criteria were also compared to the most conservative filtering criteria reported by Shen *et al.*[16], with 1078 plasma proteins identified using the latter criteria. This result further supports the stringency of the protein identification criteria applied in this work. Table 1 summarizes the total numbers of peptide identifications and protein identifications from the plasma samples obtained from the human subject before and after the LPS administration. The numbers of total peptide hits, different peptides, and proteins identified are comparable between the two plasma samples. Among the 804 plasma proteins, 545 proteins (67%) were common to both samples. All proteins that were only observed in one of the two plasma samples are of low abundances in plasma. The detection of low abundance proteins is based upon fewer peptides and is less reproducible compared to the relatively high abundance proteins largely due to the stochastic element in the process of selecting peaks for MS/MS fragmentation.

The identification of 804 non-immunoglobulin plasma proteins has further expanded the known human plasma proteome. Previously, Adkins *et al.* reported the identification of 490 proteins from an immunoglobulin-depleted serum sample by using a similar 2D-LC-MS/MS approach[13]. Shen *et al.* reported conservative identification of 433 plasma proteins (800 total including immunoglobulins) using a high resolution nanoscale 2D-LC-MS/MS approach[16]. Despite the use of more restrictive criteria in this work, better proteome coverage is observed compared to these similar previous studies. The increased proteome coverage can be attributed to optimization of sample processing, 2D-separations, and MS detection. Approximately 33% of peptides identified are cysteine-containing peptides, compared to only ~6.2% of cysteine-containing peptides reported by Shen *et al.*[16]. The results suggest that the alkylation of plasma proteins provides better coverage of cysteine-containing peptides and more protein identifications. The identified proteins were also compared to previously reported sets of plasma proteins, and 42% of the 433 plasma proteins reported by Shen *et al.* [16], and 67% of 195 proteins observed from two independent sources reported by Anderson *et al.* [26] are also identified in this study. A complete list of the 804 plasma proteins identified is supplied as Supplemental Table 1.

Figure 2 shows the functional categories of the 804 proteins identified. The largest category of these proteins is cellular tissue derived proteins, which account for almost half of the total. Only ~26% of proteins were accepted “classical” plasma proteins or proteins secreted into plasma, which includes circulatory and binding proteins, coagulation and complement factors, proteases and inhibitors, cytokines and related low abundant proteins, and other secreted proteins. A high percentage (23%) of proteins were classified as unknown (either hypothetical, or having no functional annotation).

3.3 Assessing the reproducibility of LC-MS/MS analysis

One of the goals of this study was to compare relative plasma protein concentrations for an individual before and after LPS administration without the use of stable isotope labeling. For this purpose, peptide peak areas and the total number of peptide identifications (peptide hits) [22] for each protein were independently used to compare protein abundances between two different samples. The reproducibility of LC-MS/MS analysis was first evaluated by replicate analyses of the same sample. 46 SCX peptide fractions are analyzed in duplicate, and the collective data obtained from each analysis were treated as if obtained from one combined sample. Peptide peak areas and the number of peptide hits for each protein from the first

“combined” analysis were calculated and compared to the corresponding results from the replicate analysis. Table 2 provides a comparison of results between the two analyses. The results show consistent reproducibility in terms of total peptide hits, the number of different peptides, and the number of different proteins identified between the two analyses. The percentage of proteins that are common to both analyses was 73%, similar to that shown in Table 1, and all non-overlapping proteins had three or fewer peptide hits per protein, indicating the relatively low abundance of these proteins.

The reproducibility of LC-MS/MS was further evaluated by using both peptide peak areas and the number of peptide hits for each protein. Figures 3A and 3B show the comparison of peptide peak areas between the duplicate analyses at the peptide and protein level. In Figure 3A, peak areas of each peptide from the first and the second analysis are plotted on the *x*-axis and *y*-axis, respectively. Peptide peak areas were determined from peptide selected ion chromatograms (see Materials and methods). Only the 2383 detected peptides common to both analyses are plotted for comparison. While some variations in peptide peak areas between the two analyses are observed, there is a general correlation between the two analyses, as indicated by the linear regression fitting. In Figure 3B, the protein abundance ratios (1st analysis/2nd analysis) are plotted against the number of different peptides for each protein. The protein abundance ratios were obtained by averaging peak area ratios for all peptides from each protein (see Materials and methods). Abundance ratios are given for the 374 proteins with peptides in common for both analyses. As shown, most of the relatively high abundance proteins (e.g., with 3 or more different peptides for each protein) have average ratios close to 1. Greater variation in ratios is observed for the proteins detected with only one or two different peptides.

Figures 3C and 3D show the comparison of peptide hits for each protein between the two analyses in terms of number and ratio, respectively. In Figure 3C, peptide hits for each protein from the duplicated analyses are compared by plotting the number of peptide hits for each protein from the first and the second analysis on the *x*-axis and the *y*-axis, respectively. A linear regression was applied to fit the data and a slope of 0.9768 and correlation coefficient (R^2) of 0.9996 were observed, demonstrating the overall reproducibility of the LC-MS/MS approach in terms of peptide hits for each protein. As shown in the zoom-in view in Figure 3C, even for relatively low abundance proteins having fewer peptide hits, the correlation between the two analyses is still relatively good. For those proteins observed in only one analysis (either $x = 0$ or $y = 0$), the number of peptide hits was always 3 or less. In Figure 3D, ratios of peptide hits for each protein from the two analyses are plotted against the average peptide hits for each protein. Ratios are given for those proteins observed in common for both analyses. Compared to the peptide peak area data in Figure 3A and 3B, a better correlation between the duplicate analyses was observed for peptide hits from each protein.

A statistical analysis was applied to the ratio data presented in Figure 3B and 3D, and the averages and standard deviations of the protein abundance ratios and ratios of peptide hits were calculated. The proteins identified from the duplicate analyses were divided into three groups by their relative abundances (Figure 3E), and the averages and standard deviations of the ratio values were generated for each abundance group. These averages and standard deviations from the duplicate analyses provide a basis for comparing changes in protein concentrations between the two different plasma samples.

3.4 Identification of potential inflammatory mediators upon LPS-stimulation

The results from the duplicate LC-MS/MS analyses suggest that peptide peak areas and peptide hits for each protein could be used for semi-quantitative comparison of changes in protein concentration between different plasma samples. In an attempt to determine whether there is a correlation between the number of peptide hits for each protein and the protein concentrations in plasma, a list of 74 proteins along with their concentrations in plasma previously documented

in the literature (see Table 3) was used to evaluate the correlation. Figure 4 shows that there is a general correlation between peptide hits and protein concentration; however, a large variation in peptide hits for any given protein concentration is observed. This variation was expected since the number of peptide hits is dependent on the size and exact sequence of the protein.

Next, we attempted to identify potential changes in plasma protein concentration by comparing the peptide peak areas and the number of peptide hits for each protein between samples obtained from a subject before and after LPS administration. For this comparison, protein abundance ratios based on peak areas and ratios of peptide hits for each protein between the treated and untreated sample were generated similarly to that described for Figure 3B and 3D. The averages and standard deviations of the ratios observed from the duplicate analyses (Figure 3E) were used to determine whether there is a statistically significant change in protein concentration between the two samples. The concentrations for most of the relatively abundant proteins were not statistically different following LPS treatment based on the protein abundance ratio and the ratio of peptide hits for each protein (see Supplemental Table 1). Table 4 shows 32 proteins identified as significantly increased in concentration following LPS treatment based upon either the protein abundance ratios or the ratios of peptide hits. For those proteins only identified in one of the two samples, a protein with a significant change in concentration was identified when the number of peptide hits for the protein was greater than 3 in the other sample. This rule of thumb is based on the observation that the number of peptide hits was always 3 or less if the protein was observed only in one of the duplicated analyses. The two approaches using peptide hits and the peptide peak areas appear to be complementary, since some up-regulated proteins were identified based on only one of the two methods. For 8 out of 9 proteins in Table 4 for which a protein abundance ratio was determined, the increase in concentration following LPS administration was supported by both the protein abundance ratios and the ratios of peptide hits. Several of the proteins on the list (Table 4A) have been previously determined to be inflammatory response markers or mediators whose concentrations are increased during acute phase response. These proteins include LPS-binding protein, C-reactive protein, LPS-responsive and Beige like anchor protein, serum amyloid A, serum amyloid A2, hepatocyte growth factor (HGF) activator, and von Willebrand factor [17,27-29]. This observation demonstrates the efficiency of these approaches for comparing relative changes of protein concentrations between two samples. A set of new proteins (Table 4B) that are potentially involved in the inflammatory response and may serve as novel biomarkers were also identified in this analysis. Several proteins were observed to be significantly decreased in concentrations following LPS treatment, including apolipoprotein M, pleckstrin, megakaryocyte stimulating factor, non-muscle myosin heavy chain. Since it is unclear whether these down-regulated proteins have a role in the inflammatory response, the list of these proteins is provided as Supplemental Table 2.

3.5 Dynamic range challenge for plasma protein identification and comparative analyses

Human plasma harbors thousands of low abundance proteins that make up only ~1% of the total protein in plasma, and originate from all cell types and tissues throughout the body [1]. This complexity and broad dynamic range has significantly limited the ability to identify a large number of low-level plasma proteins. The number of proteins identified in this study still represents only a small fraction of the plasma proteome that might be detectable with greater sensitivity and dynamic range. Recently, Shen *et al.* reported an estimated dynamic range as high as $\sim 10^8$ based on the detection of a low abundance protein from non-depleted plasma [16]. In this work, the dynamic range of detection using the 2D-LC-MS/MS approach was conservatively estimated to be $\sim 10^6$ - 10^7 based on the observation of several known ng/mL level proteins from the non-depleted untreated plasma sample such as HGF activator, coagulation factor VIII, HGF-like protein, and alpha-fetoprotein in the presence of serum albumin (see Table 3 for protein concentrations). However, most of the cytokines known as

key inflammatory mediators such as tumor necrosis factor- α , Interleukin (IL)-1, IL-6, IL-8, IL-12, and interferon- γ are generally present at pg/mL level in plasma[1,17]. Further developments are still needed for study of such low abundance cytokines.

The complexity and broad dynamic range of the plasma proteome has also limited the total number of proteins identified with significant changes in concentration because it is more challenging to determine statistically significant changes for low abundance proteins. By comparing the number of proteins identified between the two samples, nearly 150 low abundance proteins were only identified in the sample obtained following LPS administration, suggesting that many of these proteins may be up-regulated following LPS treatment. Several cytokine and related proteins, previously described as potential inflammatory mediators (such as calgranulin a and calgranulin b[30], adiponectin[31], and intelectin[32]), were observed only in the treated sample or with more peptide hits from the treated than the untreated sample (see Supplemental Table 1). However, due to the challenge in determining changes for low abundance proteins, no statistical differences in peptide hits for many of these proteins have been observed. With improved dynamic range of the proteomic approach, it is expected that many additional low abundance proteins that are up and down regulated will be confidently identified. The coupling of better quantitative techniques such as ^{18}O stable isotope labeling [33] with this approach will also be beneficial for more efficiently detecting abundance changes of low abundance proteins.

The dynamic range and complexity of plasma samples may be reduced by removing high abundance proteins using immunoaffinity approaches[15], or by enriching specific subsets of peptides such as cysteine-containing peptides[34,35] and N-glycosylated peptides[36]. These depletion or enrichment methodologies can enable detection of more low abundance proteins. The dynamic range of a proteomic approach can also be improved by using more sensitive mass spectrometers such as Fourier transform-ion cyclotron resonance (FT-ICR) MS[37] and higher resolution separation techniques such as nano-scale LC[5]. Currently, we are investigating the use of stable isotope ^{18}O labeling[33] coupled with LC-FT-ICR for quantitative plasma proteomics. With the combination of stable isotope labeling and high sensitivity of FT-ICR, we expect to detect and quantify abundance changes of many more low abundance plasma proteins. Several plasma samples from varying time points following LPS administration will be used to study the time-dependent acute phase response to LPS-treatment. Provided that both the dynamic range and the quantitation can be improved, this study will provide a global proteome survey of potential mediators in inflammatory response that may contribute significantly to our understanding of systemic inflammation and sepsis syndrome.

4 Concluding remarks

Global proteomic approaches based upon LC-MS/MS analyses have been applied for the characterization of the plasma proteome. While the quantification of changes in protein concentrations is anticipated to provide a wealth of information related to human diseases, the extreme biological complexity of the plasma proteome presents a challenging analytical task. In this work we demonstrated the use of a 2D-LC-MS/MS approach for identifying plasma proteins and for semi-quantitatively comparing protein concentrations between samples obtained prior to and 9 hrs following LPS administration to a healthy subject. Our study resulted in the confident identification of 804 different plasma proteins, which significantly expanded the known human plasma proteome. The dynamic range of detection using this approach has been estimated to be $\sim 10^6$ - 10^7 .

This work represents the first non-gel based comparative proteomic study of human plasma for two different conditions— before and after administration of LPS to a human subject and represent a step towards achieving quantitative proteomics of human plasma. Using protein

abundance ratios determined from peptide peak areas and the ratio of peptide hits for each protein as a means for semi-quantitative comparison, a list of proteins have been identified as up-regulated following LPS administration, and that may play a role in the regulation of inflammatory response. These proteins observed with up-regulation following LPS administration should be subject to further validation by replicate comparative experiments to test the reproducibility of observed changes as well as other independent approaches such as ELISA. Due to the dynamic range limitation and the semi-qualitative nature of our current approach, information obtained with respect to changes in concentrations represents only preliminary results for the quantitative study of the plasma proteome following LPS treatment. With further improvements, quantitative proteomic approaches will have broad applications in serum or plasma for discovering novel biomarkers for many diseased states (i.e., biomarkers for early detection of cancers).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Portions of this research were supported by the National Institute of General Medical Sciences (NIGMS, Large Scale Collaborative Research Grants U54 GM-62119-02), the NIH National Center for Research Resources (RR18522), and the Environmental Molecular Science Laboratory (a national scientific user facility sponsored by the Department of Energy's Office of Biological and Environmental Research and located at Pacific Northwest National Laboratory). The CCRE was kindly supplied by Anthony Suffredini, M.D., NIH Clinical Center, Bethesda, MD. Pacific Northwest National Laboratory is operated by Battelle Memorial Institute for the U.S. Department of Energy under contract DE-AC06-76RLO-1830.

Abbreviations

LPS, lipopolysaccharide; CCRE, Clinical Center Reference Endotoxin; SCX, strong cation exchange chromatography; NET, normalized elution time; HGF, hepatocyte growth factor; IL, interleukin.

5 References

1. Anderson NL, Anderson NG. Mol. Cell. Proteomics 2002;1:845–867. [PubMed: 12488461]
2. McCormack AL, Schieltz DM, Goode B, Yang S, et al. Anal. Chem 1997;69:767–776. [PubMed: 9043199]
3. Washburn MP, Wolters D, Yates JR. Nat. Biotechnol 2001;19:242–247. [PubMed: 11231557]
4. Han DK, Eng J, Zhou H, Aebersold R. Nat. Biotechnol 2001;19:946–951. [PubMed: 11581660]
5. Shen Y, Zhao R, Berger SJ, Anderson GA, et al. Anal. Chem 2002;74:4235–4249. [PubMed: 1219598]
6. Peng J, Elias JE, Thoreen CC, Licklider LJ, Gygi SP. J. Proteome Res 2003;2:43–50. [PubMed: 12643542]
7. Florens L, Washburn MP, Raine JD, Anthony RM, et al. Nature 2002;419:520–526. [PubMed: 12368866]
8. Koller A, Washburn MP, Lange BM, Andon NL, et al. Proc. Natl. Acad. Sci. USA 2002;99:11969–11974. [PubMed: 12163647]
9. Mawuenyega KG, Kaji H, Yamuchi Y, Shinkawa T, et al. J. Proteome Res 2003;2:23–35. [PubMed: 12643540]
10. Kislinger T, Rahman K, D. R, Cox B, et al. Mol. Cell Proteomics 2003;2:96–106. [PubMed: 12644571]
11. Jacobs JM, Mottaz HM, Yu LR, Anderson DJ, et al. J. Proteome Res 2004;3:68–75. [PubMed: 14998165]
12. Wu CC, MacCoss MJ, Howell KE, Yates JR. Nat. Biotechnol 2003;21:532–538. [PubMed: 12692561]

13. Adkins JN, Varnum SM, Auberry KJ, Moore RJ, et al. *Mol. Cell Proteomics* 2002;1:947–955.
14. Wu S-L, Choudhary G, Ramstrom M, Bergquist J, Hancock WS. *J. Proteome Res* 2003;2:383–393. [PubMed: 12938928]
15. Pieper R, Gatlin CL, Makusky AJ, Russo PS, et al. *Proteomics* 2003;3:1345–1364. [PubMed: 12872236]
16. Shen Y, Jacobs JM, Camp DG, Fang R, et al. *Anal. Chem* 2004;76:1134–1144. [PubMed: 14961748]
17. Van Amersfoort ES, Van Berkel TJ, Kuiper J. *Clin. Microbiol. Rev* 2003;16:379–414. [PubMed: 12857774]
18. Lakhani SA, Bogue CW. *Curr. Opin. Pediatr* 2003;15:278–282. [PubMed: 12806257]
19. Paludan SR. *J. Leukoc. Biol* 2000;67:18–25. [PubMed: 10647993]
20. Martin GS, Mannino DM, Eaton S, Moss M. *N. Engl. J. Med* 2003;348:1546–1554. [PubMed: 12700374]
21. Liotta LA, Ferrari M, Petricoin E. *Nature* 2003;425:905. [PubMed: 14586448]
22. Gao J, Opiteck GJ, Friedrichs MS, Dongre AR, Hefta SA. *J. Proteome Res* 2003;2:643–649. [PubMed: 14692458]
23. Shen Y, Zhao R, Belov ME, Conrads TP, et al. *Anal. Chem* 2001;73:1766–1775. [PubMed: 11338590]
24. Eng JK, McCormack AL, Yates JR. *J. Am. Soc. Mass Spectrom* 1994;5:976–989.
25. Petritis K, Kangas LJ, Ferguson PL, Anderson GA, et al. *Anal. Chem* 2003;75:1039–1048. [PubMed: 12641221]
26. Anderson NL, Polanski M, Pieper R, Gatlin T, et al. *Mol. Cell Proteomics* 2004;3:311–316. [PubMed: 14718574]
27. Ridker PM, Hennekens CH, Buring JE, Rifai N. *N. Engl. J. Med* 2000;342:836–843. [PubMed: 10733371]
28. Jensenius M, Ueland T, Fournier PE, Brosstad F, et al. *J. Infect. Dis* 2003;187:1332–1336. [PubMed: 12696016]
29. Okajima A, Miyazawa K, Naitoh Y, Inoue K, Kitamura N. *Hepatology* 1997;25:97–102. [PubMed: 8985272]
30. Ikemoto M, Tanaka T, Takai Y, Murayama H, et al. *Clin. Chem* 2003;49:594–600. [PubMed: 12651812]
31. Ouchi N, Kihara S, Arita Y, Maeda K, et al. *Circulation* 1999;100:2473–2476. [PubMed: 10604883]
32. Tsuji S, Uehori J, Matsumoto M, Suzuki Y, et al. *J. Biol. Chem* 2001;276:23456–23463. [PubMed: 11313366]
33. Yao X, Afonso C, Fenselau C. *J. Proteome Res* 2003;2:147–152. [PubMed: 12716128]
34. Gygi SP, Rist B, Gerber SA, Turecek F, et al. *Nat. Biotechnol* 1999;17:994–999. [PubMed: 10504701]
35. Liu T, Qian WJ, Strittmatter EF, Camp DG, et al. *Anal. Chem.* 2004In press
36. Zhang H, Li X.-j. Martin DB, Aerbersold R. *Nat. Biotechnol* 2003;21:660–665. [PubMed: 12754519]
37. Pasa-Tolic L, Harkewicz R, Anderson GA, Tolic N, et al. *J. Am. Soc. Mass Spectrom* 2002;13:954–963. [PubMed: 12216736]
38. Craig, WY.; Ledue, TB.; Ritchie, RF. *Plasma Proteins: Clinical Utility and Interpretation*. Foundation of Blood Research (FBR); (www.fbr.org)
39. Abbas, AK.; Lichtman, AH.; Pober, JS. *Cellular and Molecular Immunology*. W.B. Sanders Company; Philadelphia, PA: 1997.
40. Simmons P, Penny R, Goller I. *Med. J. Aust* 1969;2:494–506. [PubMed: 4186516]
41. Lum G, Gambino SR. *Am. J. Clin. Pathol* 1974;61:108–113. [PubMed: 4809144]
42. Putnam, FW., editor. *The Plasma Proteins: Structure, Function, and Genetic Control*. Academic Press; New York, NY: 1975.
43. Gallay P, Barras C, Tobias PS, Calandra T, et al. *J. Infect. Dis* 1994;170:1319–1322. [PubMed: 7963737]
44. Nesheim M, Pittman DD, Giles AR, Fass DN, et al. *J. Biol. Chem* 1991;266:17815–17820. [PubMed: 1917924]
45. Tollefsen DM, Pestka CA. *Blood* 1985;66:769–774. [PubMed: 4041618]

46. Baxter RC. J. Clin. Endocrinol. Metab 1990;70:1347–1353. [PubMed: 1692332]
47. Petersen SV, Valnickova Z, Enghild JJ. Biochem. J 2003;374:199–206. [PubMed: 12737624]
48. Shimomura T, Kondo J, Ochiai M, Naka D, et al. J. Biol. Chem 1993;268:22927–22932. [PubMed: 8226803]

Figure 1.

Strong cation exchange (SCX) chromatograms and LC-MS/MS analysis. SCX chromatograms for untreated plasma (A) and treated plasma (B) are plotted using both UV absorbance and number of peptides identified in each fraction. All peptides included have passed SEQUEST filtering criteria. For those fractions analyzed in duplicate, the number of identified peptides used is the average of the two analyses. C) Base peak chromatogram of LC-MS/MS analysis of fraction 11 from untreated plasma (marked with * in A). D) MS/MS spectra for two low abundance species. Fragment ions that match the predicted ions are labeled as either b- or y-ions. (1) MS/MS spectrum of a parent ion with m/z 770.81 from 28.41 min marked with an arrow on (C). The spectrum is identified as the partial-tryptic peptide S.CGSAAGGPDLYGTLK.E originating from protein TISD_HUMAN. (2) MS/MS spectrum of a parent ion with m/z 1037.20 from 68.78 min marked with an arrow in (C). The spectrum is identified as the fully tryptic peptide K.GLQYAAQEGLLALQSELLR.I, originating from protein LBP_HUMAN.

Figure 2.
Functional categorization of the 804 identified plasma proteins.

(E)

Average $\pm$ standard deviation	High abundance group	Medium abundance group	Low abundance group
Protein abundance ratios from (B)	1.23 ± 0.38 (n = 145)	1.27 ± 0.70 (n = 60)	1.31 ± 1.22 (n = 269)
Ratios of peptide hits from (D)	1.03 ± 0.12 (n = 115)	0.98 ± 0.29 (n = 46)	1.07 ± 0.62 (n = 327)

Figure 3.

Comparison of duplicate LC-MS/MS analyses of a sample that contains 46 SCX fractions. (A) Comparison of peptide peak areas between the two analyses. Each spot represents a peptide with its peak areas from the 1st and 2nd analysis shown on the *x*-axis and the *y*-axis, respectively. (B) Protein abundance ratios (1st analysis/2nd analysis) as a function of the number of distinct peptides from each protein. Each spot represents one protein with the abundance ratio shown on the *y*-axis and the number of different peptides on the *x*-axis. (C) Comparison of peptide hits for each protein between the duplicate analyses. Each spot represents one protein with the number of peptide hits from the 1st and 2nd analysis shown on the *x*-axis and the *y*-axis, respectively. The inset is a zoom-in view of peptide hits in the 0-20 region for both the *x*- and

y-axis. A total of 545 proteins were plotted, with the majority of spots overlapped in the low peptide hits region. (D) Ratio of peptide hits for each protein (1st analysis/2nd analysis) as a function of the average number of peptide hits. Each spot represents one protein, with the average number of peptide hits on the x-axis and the ratio of peptide hits on the y-axis. (E) Results from statistical analysis of the protein abundance ratios from (B) and the ratios of peptide hits from (D). The proteins were divided into three groups: the high abundance group (number of distinct peptides ≥ 4 or number of peptide hits ≥ 10), the medium abundance group (number of distinct peptides = 2-3 or number of peptide hits between 5 and 10), and the low abundance group (number of distinct peptides = 1 or number of peptide hits < 5). The numbers in parentheses indicate the number of proteins in each group.

Figure 4. Correlation between peptide hits for each protein and the plasma protein concentration documented in the literature. The protein concentrations and peptide hits for the selected proteins are listed in Table 3.

Table 1

Numbers of different peptides and proteins identified from plasma samples obtained prior to (untreated) and 9 hrs following LPS administration (treated) to a human subject^a.

	Total peptide identifications	Different peptides identified	Proteins identified
Untreated	19,730	4,000	666
Treated	18,719	4,018	684
Combined number ^b		5,176 (2,783)	804 (546)

^a All included peptides passed the SEQUEST filtering criteria and the normalized elution time (NET) constraint described in the Materials and methods. Numbers in parentheses represent the number of peptides or proteins observed in both samples.

^b Total number of different peptides and proteins combined from the two samples.

Table 2Peptide and protein identifications from duplicate analyses of a sample consisting 46 SCX peptide fractions^a.

	Total peptide identifications	Different peptides identified	Proteins identified
1 st analysis	14,750	2,997	461
2 nd analysis	14,581	3,084	483
Combined number		3,698 (2,383)	545 (399)

^a All included peptides passed the SEQUEST filtering criteria and the NET constraint. Numbers in parentheses represent the number of peptides or proteins observed from both analyses.

Table 3
Selected proteins with known plasma concentrations and corresponding peptide hits observed for each protein^a.

Protein	Concentration (µg/mL)	Peptide Hits	Protein	Concentration (µg/mL)	Peptide hits
albumin	45000	7858	transcortin	70	20
fibrinogen	3000	921	HMW kininogen	70	86
transferrin	2660	979	complement C9	60	35
alpha-2-macroglobulin	2000	1030	complement C7	60	44
complement C3	1500	836	alpha-2-antiplasmin	60	50
apolipoprotein A-I	1375	282	plasma retinol-binding protein	55	28
haptoglobin	1365	337	serum amyloid p-component	55	20
alpha-1-antitrypsin	1235	504	SP-40 (clusterin)	50	82
apolipoprotein B-100	1130	574	prekallikrein	50	17
alpha-1-acid glycoprotein	870	210	angiotensinogen	45	49
alpha-2-hs-glycoprotein	625	155	complement C6	40	21
factor H	500	168	serum carboxypeptidase n	35	20
apolipoprotein A-II	460	184	factor I	35	38
alpha-1-antichymotrypsin	450	94	apolipoprotein E	35	75
vitamin d-binding protein	400	275	apolipoprotein C-II	33	28
ceruloplasmin	400	188	coagulation factor XII	30	19
prealbumin	345	199	coagulation factor CIs	30	42
vitronectin	340	61	properdin	25	4
complement C4	315	532	alpha-1-microglobulin (AMBP)	25	29
fibronectin	300	328	acid-labile subunit (ALS)	24	23
C4BP	250	94	complement C2	20	32
alpha-1B-glycoprotein	225	126	LPS-binding protein	18	7
beta-2-glycoprotein	220	80	beta-2-microglobulin	16	7
factor B	200	127	thyroxine-binding globulin	15	16
C1 inhibitor	200	70	serum amyloid A	10	16
hemoexin	200	169	coagulation factor XIII	10	12
plasminogen	155	64	coagulation factor X	8	5
prothrombin	150	64	von willebrand factor	7.5	3
antithrombin III	145	106	coagulation factor V	7.5	14
apolipoprotein C-III	124	57	apolipoprotein C-I	7	37
histidine-rich glycoprotein	100	82	PEDF	5	35
complement C1r	100	40	coagulation factor IX	5	6
complement C5	95	134	factor D	2	10
kininogen	90	99	HGF activator	0.4	2
zinc-alpha-2-glycoprotein	80	39	coagulation factor VIII	0.2	6
heparin cofactor II	80	56	HGFL	0.02	2
complement C1q	75	37	alpha-fetoprotein	0.005	7

^aThe plasma protein concentrations are approximate average values based on previous literature [14,38-48]. The number of peptide hits for each protein was from the plasma sample prior to LPS administration, since most of the literature values were based on the un-stimulated plasma samples.

Table 4

Potentially up-regulated plasma proteins following LPS administration^a.

(A) Proteins previously known to be up-regulated.		Protein name	Peptide hits (untreated)	Peptide hits (treated)	Ratio of peptide hits (treated/untreated)	Abundance ratio (treated/untreated)
Reference ID						
SW:SAA_HUMAN		serum amyloid A protein	16	36	2.3	5.9
PIR1:YLHUA		serum amyloid A2 protein	2	8	4	4.3
SW:HGFA_HUMAN		hepatocyte growth factor activator (hgf activator)	2	8	4	3.8
SW:LBP_HUMAN		lipopolysaccharide-binding protein	7	27	3.9	2.6
SW:VWF_HUMAN		von Willebrand factor	3	13	4.3	1.1
PIR2:I39456		serum amyloid A2-beta	0	8		
SW:CRP_HUMAN		C-reactive protein	0	4		
GP:AF216648_1		LPS responsive and Beige-like anchor protein	0	5		
(B) Potential novel up-regulated proteins.						
GP:AB023226_1		KIAA1009 protein	1	7	7	2.9
SW:A2GL_HUMAN		leucine-rich alpha-2-glycoprotein (lrg)	50	75	1.5	2.87
GP:AB037722_1		KIAA1301 protein	2	8	4	2.67
GP:AF207881_1		tumor-associated hydroquinone (NADH) oxidase tNOX mRNA	8	21	2.6	1.95
SW:LMA4_HUMAN		laminin alpha-4 chain	0	4		
SW:LRP2_HUMAN		low-density lipoprotein receptor-related protein 2	0	4		
SW:GCST_HUMAN		aminomethyltransferase, mitochondrial	0	4		
GP:AY057448_1		MARK4 serine/threonine protein kinase	0	6		
SW:MM20_HUMAN		matrix metalloproteinase-20 (mmp-20)	0	5		
SW:BMR2_HUMAN		bone morphogenetic protein receptor type II	0	4		
SW:ANR3_HUMAN		serine/threonine-protein kinase ankrd3	0	4		
SW:MACF_HUMAN		actin cross-linking family protein 7 (macrophilin)	0	7		
SW:KF5C_HUMAN		kinesin heavy chain isoform 5c	0	4		
SW:CPT7_HUMAN		cytochrome p450 17	0	4		
SW:TF1B_HUMAN		transcription intermediary factor 1-beta	0	4		
SW:CHLE_HUMAN		cholinesterase	0	10		
GP:AB058686_1		KIAA1783 protein	0	4		
PIR2:S25409		transcription factor znf6	0	5		
SW:ELFL_HUMAN		ets-related transcription factor elf	0	4		
SW:MLH3_HUMAN		dna mismatch repair protein mlh3	0	5		
SW:NOP5_HUMAN		nucleolar protein nop5	0	4		
SW:RPB1_HUMAN		dna-directed rna polymerase ii largest subunit, mitochondrial	0	5		
SW:SIP1_HUMAN		zinc finger homeobox protein 1b	0	4		
SW:VDR_HUMAN		vitamin d3 receptor (vdr) (1,25-dihydroxyvitamin d3 receptor)	0	4		

^a Abundance ratio was calculated based on peptide peak areas. A protein is considered as up-regulated only if the protein abundance ratio or the ratio of peptide hits is 4 standard deviations greater than the average ratio. The averages and standard deviations used for this analysis were listed in the table of Figure 3E. If a protein is only observed in the treated sample and the number of peptide hits is >3, the protein is also considered as up-regulated.