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Research Article

Relative Quantification of Several Plasma Proteins during Liver Transplantation Surgery

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Plasma proteome is widely used in studying changes occurring in human body during disease or other disturbances. Immunological methods are commonly used in such studies. In recent years, mass spectrometry has gained popularity in high-throughput analysis of plasma proteins. In this study, we tested whether mass spectrometry and iTRAQ-based protein quantification might be used in proteomic analysis of human plasma during liver transplantation surgery to characterize changes in protein abundances occurring during early graft reperfusion. We sampled blood from systemic circulation as well as blood entering and exiting the liver. After immunodepletion of six high-abundant plasma proteins, trypsin digestion, iTRAQ labeling, and cation-exchange fractionation, the peptides were analyzed by reverse phase nano-LC-MS/MS. In total, 72 proteins were identified of which 31 could be quantified in all patient specimens collected. Of these 31 proteins, ten, mostly medium-to-high abundance plasma proteins with a concentration range of 50–2000 mg/L, displayed relative abundance change of more than 10%. The changes in protein abundance observed in this study allow further research on the role of several proteins in ischemia-reperfusion injury during liver transplantation and possibly in other surgery.

1. Introduction

Blood plasma is one of the most complex biological fluids in humans. It contains hundreds of physiological circulatory proteins and hundreds to thousands of condition-specific proteins that change in concentration in response to pathophysiological changes in the body [1]. This richness of proteins and its accessibility makes plasma one of the most commonly used sources for medical diagnostics [2–4]. Currently used routine immunodiagnostic tests are able to identify and quantify numerous plasma biomarkers with great precision and sensitivity. The problem with modern immunodiagnostic methods is that they are based on antibody interactions with the proteins being investigated, requiring a separate test for each biomarker, thus increasing the workload and costs in medical healthcare. To overcome the limitations of single ELISA assay, several chip-based antibody microarray methods have been developed [5–7]. In antibody microarrays the antibodies are attached to the surface of the assay chip and the sample is flushed over the chip allowing the proteins to bind to the antibodies on the chip. After washes the bound proteins can be detected with either direct labeling [8] or indirect sandwich type immunoassays [9]. The main benefit of antibody microarrays is the sensitivity and precision of traditional antibody assays combined with the speed and efficiency of analyzing numerous proteins simultaneously. The disadvantage is the requirement for specific antibodies targeted to the desired proteins or against each posttranslationally modified version of the protein. In addition, the dynamic range of antibody microarrays is generally similar to normal ELISA-type assays, ranging three to four orders of magnitude [6, 10, 11] complicating the

analysis of complex, high dynamic range samples such as plasma. Consequently, the current antibody arrays are most suitable for targeted analysis of disease or condition-specific biomarkers [7, 12–14] rather than examining changes in the whole proteome or discovering novel biomarkers.

Advances in the field of mass spectrometry (MS) have enabled an alternative way of investigating cellular and plasma protein biomarkers [15–17]. Current MS machinery and bioinformatics software are rapidly approaching the sensitivity and dynamic range limits of immunodiagnostic tests [18, 19]. The main benefit of the MS-based assay is the ability to characterize tens to hundreds of proteins simultaneously [20] without the need to target any specific posttranslational modification, subproteome, or disease. Further, parallel analysis of several proteins in a single MS run greatly reduces the time and costs of diagnostic analyses. The drawback on using plasma in MS-based assays is the high dynamic range of protein abundances [3]. Albumin, the most abundant protein in plasma, constitutes ca. 60% of the entire amount of proteins in plasma at approximate concentration of 50 mg/mL. On the other hand, cytokines are found in plasma at the level of picograms per milliliter or lower. Such variability in protein abundances is a problem for modern mass spectrometers with a dynamic range of 10^3–10^4 [21]. In this study we examined the applicability of iTRAQ peptide labeling technology [22] to characterize relative changes in the levels of several plasma proteins during liver transplantation (Figure 1).

The success of liver transplantation is significantly influenced by both preservation and reperfusion injury. During procurement, liver is disconnected from circulation, resulting in interrupted oxygen supply to cells. Ischemia induces several physiological effects such as activation of apoptosis and necrosis pathways [23]. Upon graft implantation, the cells suffer from reperfusion injury. Reactive oxygen species are generated after the sudden increase in cellular oxygen levels leading to DNA, plasma membrane, and protein damage [24]. Further, inflammation and coagulation cascades become activated damaging both the graft and other end-organs. Characterization of protein abundance changes during liver transplantation could be extremely helpful in patient prognosis and care. In this pilot study we characterized several plasma proteins that change in abundance during graft reperfusion in liver transplantation using iTRAQ peptide labeling technology.

Blood samples were drawn simultaneously from portal and hepatic veins and from radial artery. Following immunodepletion of the six high-abundant human plasma proteins, the plasma proteome was digested to peptides with trypsin. The digests were then labeled with iTRAQ 8plex mass tags. The iTRAQ technology is based on small amine-reactive isobaric mass tags that can be attached to the N-terminus and lysine residues of digested peptides. The tag consists of a reporter ion used for quantification and a balancer group balancing the reporter masses of each iTRAQ tag to equal mass. During collision-induced fragmentation within the mass spectrometer the peptide bound mass tag is fragmented, along with the peptide backbone, to produce a balancer group and a reporter ion of known mass (113, 114, 115, 116, 117, 118, 119, and 121 daltons in iTRAQ 8plex system). Each reporter ion produces a distinct MS fragment where the intensity of the reporter ion fragment corresponds to the amount of the peptide. The relative abundances of the peptides, and ultimately proteins, which are derived from different iTRAQ labeling pools (i.e., different samples), can be deduced from these spectra.

Samples were fractionated by means of strong cation-exchange and reverse phase chromatography and finally analyzed by quadrupole time-of-flight mass spectrometry. Protein identification and quantification was performed using the Mascot search engine. We were able to identify 72 different proteins and to quantify 31 of those proteins in all patient specimens.

2. Materials and Methods

2.1. Patients and Sampling. Three male patients undergoing liver transplantation surgery for primary sclerosing cholangitis (PSC) in the transplantation and liver surgery clinic of the surgical hospital participated in this study (Table 1). Patients with PSC were recruited to ensure similar surgical technique and comparable cold ischemia and anhepatic times and perioperative bleeding. The patients were transplanted within a time period of two months.

The study was approved by the Ethics Committee of the Department of Surgery, Hospital District of Helsinki and Uusimaa (DNr. 33/13/03/02/08). All grafts were obtained from brain-dead donors and were preserved with University of Wisconsin solution. The classic surgical technique of transplantation was used without bypass or piggyback. In brief, the patient's native liver is removed after clamping of supra- and infrahepatic inferior caval vein. Then the portal vein and hepatic artery are clamped. The suprahepatic caval anastomosis is completed first. Just before completion of portal anastomosis, the liver is flushed via the portal vein with 1000 mL of Ringer's solution with the suprahepatic caval vein clamped, followed by rinse with 400 mL of portal venous blood. After graft rinsing, the suprahepatic caval and portal veins are declamped, followed by completion of the infrahepatic caval anastomosis and declamping. Finally, the hepatic arterial anastomosis is completed.

Three separate 10 mL blood specimens were taken from each patient during early graft reperfusion. A sample representing blood flow from the liver was drawn from hepatic vein during graft flushing with portal blood before permanent connection to circulation. Simultaneously, a sample representing blood entering the liver was drawn from portal vein. To monitor changes in systemic circulation, an additional sample was drawn from radial artery. Samples from hepatic and portal veins were drawn by puncture directly from the vessel whereas the sample from radial artery was drawn from an indwelling arterial cannula. All samples were drawn into sterile syringes and transferred immediately to sodium citrate anticoagulated tubes (Vacutainer Systems Europe, Becton Dickinson, Plymouth, UK) and placed on ice until further processing. Within 30 minutes, plasma was separated by centrifugation at 2000 g for 10 minutes at +4°C and aliquotted to 150 μL fractions. The aliquots were then

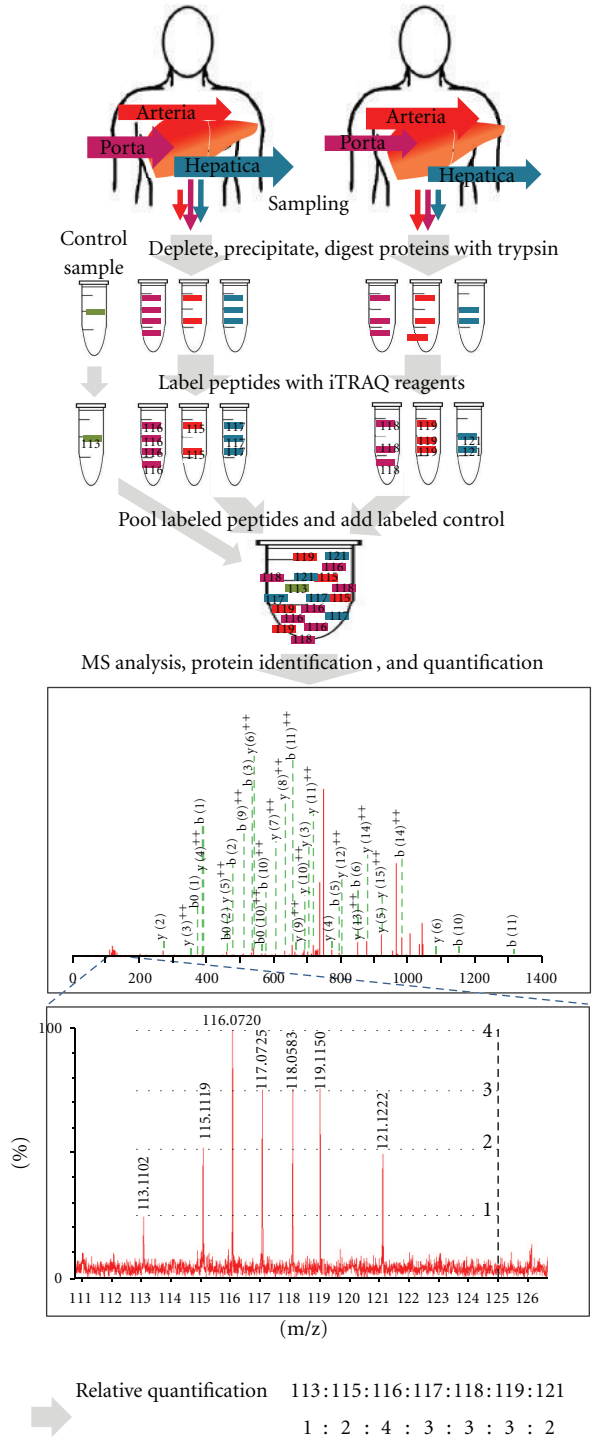

Figure 1: General workflow for patient sampling, iTRAQ labeling, and protein quantification. All patient samples are depleted and trypsin digested separately. Each sample is then labeled with a different iTRAQ mass tag whereafter the samples are pooled. The pooled samples are fractionated with strong cation exchange and reverse-phase chromatography and analyzed by quadrupole time-of-flight mass spectrometry. The resulting Mascot peptide fragmentation spectra (upper spectra image) provide the peptide sequence and the low mass end (highlighted in lower spectra) shows the iTRAQ reporter ion region used for quantification.

Table 1: Patient characteristics.

	Patient 1	Patient 2	Patient 3
Patient weight (kg)	68	74	62
Patient age (yrs)	35	39	47
Liver cold ischemia time (min)	273	295	279
Anhepatic time (min)	51	52	57
Weight of the transplanted liver (grams)	1400	750	1100
Total bleeding in the operation (liters)	1.8	2.3	3.6

stored at −80°C until analysis. Samples from each patient were analyzed at the same time, approximately 6 months after sampling. The entire protocol for each patient was performed twice from depletion to MS analysis.

2.2. Plasma Depletion. To pellet the precipitated material, 450 μL of plasma were thawed on ice and centrifuged at 16 100 g at +4°C for 5 minutes. The supernatant was diluted 1 : 5 with Agilent Multiple Affinity Removal Buffer A and proteinase inhibitor was added to the samples (complete EDTA free mini, Roche Diagnostics GmbH, Mannheim, Germany). The samples were filtered with 0.22 μm syringe filters (low protein binding Durapore, Millipore Corporation, Billerica, MA). Protein concentrations measurements were done from all samples using BCA protein concentration measurement kit (Pierce, Rockford, IL). In order to maintain the original concentration ratios throughout the processing of the samples, the volumes were kept equal in all samples, 455 μL of diluted plasma from each sample were diluted to 2 mL with Agilent Buffer A. Six high-abundant plasma proteins, including albumin, IgG, IgA, transferrin, haptoglobin, and antitrypsin, were depleted using Agilent Multiple Affinity Removal LC Column-Human 6 (Agilent Technologies, Palo Alto, CA) using Waters 666 pump and 600S controller HPLC system coupled to Waters 2487 Dual wavelength absorbance detector (Waters Corporation, Milford, MA). The injection volume was 200 μL, 1.75 mL of flow thru fraction containing low abundance proteins was collected. Injection and collection was performed three times to monitor the reproducibility of the depletion (representative chromatograms of repeatability and sample-to-sample comparison are shown in Supplemental File 4 in supplementary Material available online at doi: 10.1155/2011/248613). A control sample was prepared by pooling and then aliquotting the arterial, hepatic, and portal vein samples from one patient. The control sample was used in iTRAQ quantification as a benchmark to which all three patient samples were compared in order to acquire the quantification values. All samples were then stored at −80°C.

2.3. Protein Precipitation, Digestion, and iTRAQ Labeling. Portal, hepatic, and arterial blood samples from two patients were analyzed simultaneously with the control sample. 150 μL of the low abundance protein fraction, containing an average protein mass of 51.75 μg in arterial sample, 51.05 μg in portal sample, and 40.4 μg in hepatic sample, was precipitated by adding 100% trichloroacetic acid to a final concentration of 25% (v/v) and incubated on ice for 30 minutes with periodic vortexing. Precipitated proteins were pelleted by centrifugation (16 100 g, +4°C, 5 minutes) using a table top centrifuge. The protein pellet was washed once with 500 μL of −20°C acetone with 0.1 M hydrochloric acid and centrifugated (16 100 g, +4°C, 5 minutes), and the wash was repeated using −20°C acetone.

Digestion was performed by the iTRAQ peptide labeling protocol by AB Sciex (Concord, Canada). All used buffers were from iTRAQ Reagent 8plex Buffer Kit from AB Sciex. First, the protein samples were dissolved into 50 μL of iTRAQ dissolution buffer. Two microliters of iTRAQ denaturant were added to the samples and mixed vigorously to solubilize the protein pellet. Then 2 μL of reducing reagent were added and samples were incubated at +60°C for 1 hour. After reduction, 1 μL of cysteine blocking reagent was added followed by incubation at room temperature for 10 minutes. Two micrograms of trypsin (Promega sequencing grade modified trypsin, Promega, Madison, WI) were added to the samples, which were then incubated at +37°C for approximately 16 hours.

iTRAQ reagents were dissolved in 50 μL of isopropanol and transferred to the digested peptide samples. The labeling reaction was performed at room temperature for 2 hours before quenching by 10 μL of 1 M ammonium bicarbonate. Quenching was allowed to progress at room temperature for 30 minutes. After labeling, the samples were pooled into a new sample tube (the full iTRAQ labeling scheme is given in Supplemental File 4).

2.4. Peptide Fractionation and Mass Spectrometry. To reduce the complexity of peptide samples for MS, a fractionation step was performed with a cation-exchange cartridge system (AB Sciex). The cartridge was washed and conditioned according to the Manufacturer's instructions. The pooled sample was evaporated to below 30 μL, diluted to 1 mL of cation-exchange load buffer and pH was adjusted to 2.5–3.5 with HCl. The sample was slowly injected into the cartridge and washed with 1 mL of cation-exchange load buffer. Elution of peptides was performed by increasing the concentration of KCl. Eleven 500 μL elution fractions were collected (5 mM, 10 mM, 15 mM, 25 mM, 35 mM, 50 mM, 75 mM, 100 mM, 150 mM, 200 mM, and 350 mM KCl in 10 mM KH_2HPO_4, pH = 3, with 25% acetonitrile). All fractions were dried in a SpeedVac vacuum concentrator (Thermo Savant, Holbrook, NY) and solubilized to 100 μL of MS phase A buffer (5% acetonitrile with 0.1% formic acid). Fractions were stored at −20°C until mass spectrometric analysis.

On-line reverse phase separation was performed using Waters Symmetry300, C18, 5 μm NanoEase Trap Column coupled to an LC Packings-Dionex Acclaim PepMap C18 column, 75 μm × 250 mm (LC Packings/Dionex, Amsterdam, The Netherlands) on a Waters-Micromass nanoLC CapLC (passive stream-split) coupled to a Q-TOF Ultima Global mass spectrometer. Eluent A contained 0.1% formic

acid in 5% acetonitrile, eluent B 0.1% formic acid in 95% acetonitrile. The flow rate during the LC-run was set to 300–400 nL/minute depending on the amount of organic solvent, with a gradient ranging from 95% to 5% of eluent A. MS data was acquired for 90 minutes in positive ion mode by data-dependent acquisition collecting the precursor MS and six MSMS spectra during one duty cycle. The threshold for switching to MSMS was set to ten counts per second for each individual ion and 5 counts per second for switching back to MS scan. The scan time was 2.4 seconds with a 0.1 second interscan delay. The scan range for MS scan was set to 300–2000 m/z and 50–2500 for MSMS. No lock-mass correction was used (Exact Waters-Micromass nanoLC CapLC and Q-TOF Ultima Global mass spectrometer settings and an example chromatogram of MS run are shown in Supplemental File 4).

2.5. Protein Identification and iTRAQ Quantification. Each MS run was performed twice to gather more data and to compensate for the stochastic nature of the data-dependent acquisition. The peak lists were calculated using Mascot Distiller Software (version 2.3.1.0, Matrix Science, London, UK) and the data from each KCl elution fraction was merged. Peptides were identified with Mascot search engine (version 2.2.04, Matrix Science) against SwissProt database (version 57.7) with *Homo sapiens* as the specified organism. The parameters for search were trypsin digested peptides with maximum of two missed cleavages, iTRAQ-8plex on lysine and N-terminus as well as methylthio-cysteine as fixed modifications, and oxidation of methionine as variable modification. Default Mascot iTRAQ correction factors were used to compensate for the iTRAQ reagent isotope impurities. Peptide tolerances for the search were ±0.2 Da for parent and ±0.1 Da for fragment ions resulting in false discovery rate of less than 1.4% when searched against false decoy database. The significance threshold for peptides was set to "Identity" and probability value of 0.05. For peptide identification, a Mascot peptide score of more than 20 was required and "Require bold red" designation was used for protein identification.

After peptide identification, an exclusion list was constructed from the identified peptides. This exclusion list was used in subsequent identical MS run to search for unidentified peptides. The resulting peak lists were again merged and searched. A second exclusion list was created from these results and combined with the first list. The fractions were then similarly analyzed with MS for the third time. The benefit of using exclusion lists is clearly illustrated in Figure 2 representing the total distinct peptides that were identified in first iTRAQ experiment. The first exclusion MS run identified 97 and the second 38 additional novel peptides. On the average, the first exclusion run increased the distinct peptide count by 28% and the second run by 10%. All peak lists, from the no-exclusion to two exclusion runs were finally combined and searched with the same search parameters.

Protein identification and quantification was performed with Mascot search engine using MudPIT scoring for protein identification. Normalization was done using summed peptide intensities and automatic outlier removal. This corrects for the possible dilution effect of rinsing the graft with Ringer solution before flushing with portal blood. This normalization step is based on the assumption that the overall plasma protein concentration does not change in the liver. Protein ratios were calculated as median ratio of each patient sample to control sample with at least three peptides required for quantification, (full combined Mascot results are given in Supplemental Files 1–3).

The quantification results were copied to Microsoft Office Excel (Microsoft Corporation, Redmond, WA) and differences between samples were calculated as percent changes. Change across the hepatic circulation (portal to hepatic venous change) was calculated as hepatic vein quantification value subtracted by the portal vein value and divided by the original hepatic vein value. Similarly, change from systemic circulation to portal venous blood was calculated as portal vein quantification values subtracted from arterial value and divided by the original portal value. To calculate the change between arterial and hepatic venous samples, the hepatic vein values were subtracted from arterial value and then divided by hepatic vein value. Finally the resulting values were multiplied by 100 to gain percentage changes.

All quantification values of patient samples and repeat runs were then averaged. Due to the small number of patients and quality of the data, a significance threshold of 10% was used to filter the results.

3. Results

The blood samples were obtained from patients undergoing liver transplantation surgery during the early phase of reperfusion. Samples were drawn from the portal vein (blood entering the liver), hepatic vein (blood exiting the liver), and the radial artery (systemic circulation).

After sampling blood was centrifuged to yield plasma. The protein concentrations were measured before high abundance plasma protein depletion. Generally the portal venous and systemic arterial samples had equal concentrations, 76.4 mg/mL in portal venous and 74.5 mg/mL in arterial blood, while the hepatic venous samples had a markedly lower concentration, an average of 53.6 mg/mL. The dilution is probably due to surgical procedures as the liver is first rinsed with Ringer solution before flushing with portal blood and subsequent sampling of this effluent blood from hepatic vein. To reduce the complexity of the samples by removing the highly abundant albumin and five other high-abundant proteins (IgG, IgA, transferrin, haptoglobin, and antitrypsin), a small aliquot of each sample was subjected to Agilent Multiaffinity Removal column. This depletion step reduced the protein content of the samples by 65 to 75%.

After depletion, trypsin digestion, iTRAQ labeling, extensive peptide fractionation, and mass spectrometer runs, the data files of each patient and repeat runs were combined. This overall list was then searched against Swiss-Prot human database and 72 proteins were identified with good

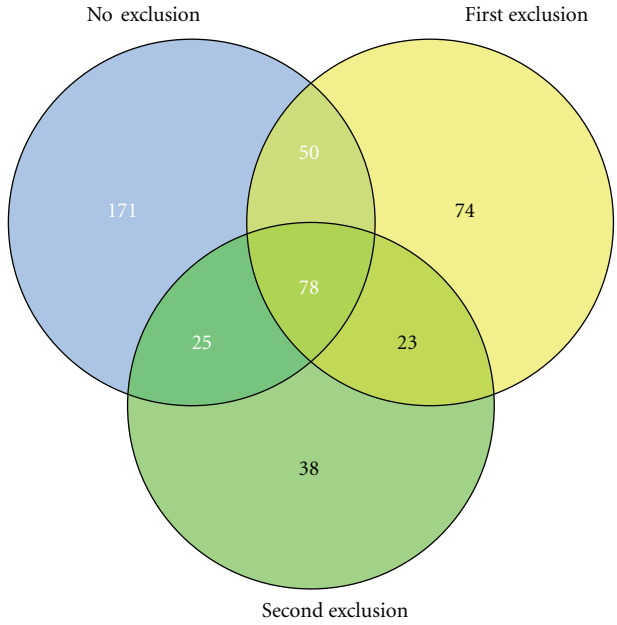

Figure 2: The benefit of using multiple exclusion MS runs. Venn diagram of the peptides identified in the first iTRAQ experiment. The no-exclusion run identified 324 distinct peptides. Of these 128 were identified in the first exclusion run together with 74 novel peptides. The second exclusion run produced 38 additional novel peptides that had not been identified in the no-exclusion or in the first exclusion MS runs.

confidence (Table 2. A complete list of all identifications and quantification is found in Supplemental Files 1–3).

Most of the identified proteins (53 of 72) are found on top of the 150 most abundant proteins in plasma (HUPO Plasma Proteome Project 3020 confirmed identifications [25]). This demonstrates the limitations in sensitivity and dynamic range of the mass spectrometer used in this study. Of the 72 identified proteins, 31 could be quantified in all patient specimens and repeat runs (Figure 3).

As the identified proteins belong to the medium-to-high abundance plasma protein category and major changes in protein abundance are improbable, we decided to consider significant only those changes where the average change of all MS runs was more than 10%.

For changes within the liver from portal to hepatic venous blood, eight proteins changed more than 10%. Five of these were reduced in concentration: histidine-rich glycoprotein (HRG, Swiss-Prot:P04196), antithrombin-III (SERPINC, Swiss-Prot:P01008), fibrinogen alpha chain (FGA, Swiss-Prot:P02671), apolipoprotein E (APOE Swiss-Prot: P02649), and beta-2-glycoprotein 1 (APOH Swiss-Prot: P02749)) and three had increased across the liver: alpha-1-acid glycoprotein 1 (ORM1 Swiss-Prot: P02763), inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4 Swiss-Prot: Q14624), and alpha-2-macroglobulin (A2M Swiss-Prot: P01023). The differences in plasma proteome in systemic arterial to hepatic venous sample were similar to the portal to hepatic venous change: HRG, SERPINC, and FGA had lower and ORM1, ITIH4, and A2M higher concentrations within the liver. Additionally two other proteins, serum paraoxonase/arylesterase 1 (PON1 Swiss-Prot: P27169) and angiotensinogen (AGT Swiss-Prot: P01019) increased in concentration. The changes observed in the systemic arterial to portal venous blood were not as extensive as portal to hepatic vein changes. Only two proteins, PON1 and AGT, increased more than 10%.

4. Discussion

Using plasma depletion, mass spectrometry based protein identification and iTRAQ labeling of peptides, we were able to identify 72 different, mostly medium to high abundance plasma proteins in blood samples taken from patients undergoing liver transplantation. This bias of medium-to-high proteins is not unexpected as the depletion column removes only six of the most abundant proteins. Additionally the sensitivity of the used mass spectrometer limits the dynamic detection limit to the high concentration plasma proteins. For this reason major changes in the detected proteins are unlikely. Of the 72 identified proteins 31 were quantified in all patients. Of these 31 proteins, ten proteins changed more than the 10% confidence limit. We then compared the changes in protein abundances from portal venous to hepatic venous blood, systemic arterial to hepatic venous blood, and systemic arterial to portal venous blood. The changes in portal to hepatic venous blood and systemic arterial to hepatic venous blood were quite similar. The observed changes in arterial to portal blood are quite small as only two proteins were shown to differ.

4.1. Sample Characteristics. We used several methods to reduce the inherent complexity of the plasma sample. Six high-abundant proteins were removed from plasma using the Agilent Multiple Affinity Removal system reducing

Table 2: All identified proteins.

Protein hit no.	Swiss-Prot accession number	Protein description	Protein score	Protein mass (Da)	Protein sequence coverage	Number of unique peptides
1	P01023	Alpha-2-macroglobulin A2M	23708	190553	35.8	46
2	P01024	Complement C3 C3	21127	221709	49.9	68
3	P02671	Fibrinogen alpha chain F	9678	108299	29.6	26
4	P04114	Apolipoprotein B-100 AP	8935	624146	14.9	55
5	P02647	Apolipoprotein A-I AP	8930	37756	65.9	24
6	P02652	Apolipoprotein A-II AP	6461	14514	44	6
7	P01011	Alpha-1-antichymotrypsin SERPINA3	5929	55834	45.4	16
8	P02675	Fibrinogen beta chain F	2192	67148	36.9	17
9	P02774	Vitamin D-binding protein	2072	66618	16.7	9
10	P05155	Plasma protease C1 inhibitor SERPIN	1533	64246	22.6	10
11	P02679	Fibrinogen gamma chain F	1472	62126	25.6	10
12	P02766	Transthyretin TTR	1306	18615	38.8	6
13	P00450	Ceruloplasmin CP	1254	142509	16.7	12
14	P0C0L4	Complement C4-A C4A	1224	216378	10	12
15	P06727	Apolipoprotein A-IV AP	1041	54193	37.4	13
16	P01008	Antithrombin-III SERPINC1	989	64129	30.6	10
17	P04217	Alpha-1B-glycoprotein A1B	971	57889	28.1	12
18	Q14624	Inter-alpha-trypsin inhibitor heavy chain H4 ITIH4	920	116070	18	11
19	P04196	Histidine-rich glycoprotein HR	874	66538	8	3
20	P08697	Alpha-2-antiplasmin SERPINF2	872	60615	8.1	2
21	P01871	Ig mu chain C region I	837	56272	21.2	8
22	P01834	Ig kappa chain C region I	739	14340	17	1
23	P00751	Complement factor B CFB	650	101906	12.8	12
24	P10909	Clusterin CLU	650	61587	8.9	4
25	P02649	Apolipoprotein E AP	543	40391	40.4	10
26	P01019	Angiotensinogen A	537	59813	15.1	5
27	P19823	Inter-alpha-trypsin inhibitor heavy chain H2 ITIH2	407	126474	7.6	5
28	P01042	Kininogen-1 KN	375	87731	11.2	8
29	P27169	Serum paraoxonase/arylesterase 1 P	332	46113	16.1	4
30	P04003	C4b-binding protein alpha chain C4BPA	299	77637	2	1
31	P25311	Zinc-alpha-2-glycoprotein AZ	297	40239	12.4	2
32	P02656	Apolipoprotein C-III AP	295	12975	16.2	1
33	P68871	Hemoglobin subunit beta HBB	241	19639	36.7	4
34	P04264	Keratin, type II cytoskeletal 1 KRT1	239	74821	1.9	1
35	P51884	Lumican LUM	237	46314	11.8	2
36	P02760	Protein AMBP AMBP	219	44754	3.4	1
37	P02790	Hemopexin HPX	206	58336	10.2	6
38	P19827	Inter-alpha-trypsin inhibitor heavy chain H1 ITIH1	198	115927	5.8	4
39	P04004	Vitronectin VTN	194	60659	5.6	2
40	P02763	Alpha-1-acid glycoprotein 1	167	28060	19.4	5
41	P00734	Prothrombin F2	149	79118	6.9	3
42	P08603	Complement factor H CFH	123	163645	2.5	3
43	P02765	Alpha-2-HS-glycoprotein AHS	121	44471	6.5	3
44	P02655	Apolipoprotein C-II AP	113	13406	32.7	3
45	P01842	Ig lambda chain C regions I	110	13967	26.8	2

Table 2: Continued.

Protein hit no.	Swiss-Prot accession number	Protein description	Protein score	Protein mass (Da)	Protein sequence coverage	Number of unique peptides
46	P02751	Fibronectin FN1	103	286474	2	4
47	P01031	Complement C5 C5	88	224691	1.6	2
48	P02735	Serum amyloid A protein SAA1	84	15349	11.5	1
49	P10643	Complement component C7 C7	73	108363	1.1	1
50	P36955	Pigment epithelium-derived factor SERPINF1	65	54831	7.4	2
51	P43652	Afamin AFM	63	84843	7.2	3
52	Q9NP78	ATP-binding cassette subfamily B member 9 ABCB9	56	92635	0.9	1
53	P06681	Complement C2 C2	54	94470	2.4	2
54	P01876	Ig alpha-1 chain C region I	49	41890	8.2	3
55	P00747	Plasminogen PL	49	105720	5.4	5
56	P02747	Complement C1q subcomponent subunit C C1QC	46	29712	3.7	1
57	P02743	Serum amyloid P-component APCS	46	29326	2.7	1
58	P13645	Keratin, type I cytoskeletal 10 KRT10	45	66119	3.9	2
59	P02753	Retinol-binding protein 4 RBP4	43	26646	5	1
60	P00915	Carbonic anhydrase 1 CA1	42	34632	4.6	1
61	P02749	Beta-2-glycoprotein 1 AP	39	47703	2.6	1
62	P05452	Tetranectin CLEC3B	38	27724	5.9	1
63	P15169	Carboxypeptidase N catalytic chain CPN1	35	57729	4.1	2
64	P02452	Collagen alpha-1(I) chain C	34	156470	1	2
66	P02746	Complement C1q subcomponent subunit B C1QB	32	31310	15	2
67	P07357	Complement component C8 alpha chain C8A	32	75160	3.1	1
65	Q5XKE5	Keratin, type II cytoskeletal 79 KRT79	32	66900	2.2	1
69	P04278	Sex hormone-binding globulin SHB	31	47706	6	1
68	P05546	Heparin cofactor 2 SERPIND1	31	67377	1.4	1
71	P35858	Insulin-like growth factor-binding protein complex acid labile chain I	30	69340	2.6	1
70	P06396	Gelsolin	30	99638	2.2	1
73	Q5T8A7	Protein KIAA0649 KIAA0649	29	150089	1	1
72	Q2WGJ9	Fer-1-like protein 6 FER1L6	29	259655	0.3	1

the protein content of the samples by more than 65%. The depletion allows the identification of more proteins in the sample [26] by reducing the dynamic range and by increasing the amount of medium-to-low abundance proteins that can be labeled and injected to MS. Even though depletion reduces the complexity and dynamic range of the analysis, it also removes those plasma proteins that bind to albumin, immunoglobulins, or other high abundance proteins [27]. This carrier effect may cause some differences in quantification results especially with low abundance proteins. This issue was not considered significant in this study as the quantified proteins belong to the medium-to-high abundance plasma protein category.

After trypsin digestion and iTRAQ labeling the peptides were purified and fractionated offline using strong cation exchange cartridge followed by online separation with reverse phase chromatography coupled to MS. Such two-dimensional fractionation setup is extremely important in iTRAQ-based peptide quantification [28]. First, the complexity is reduced allowing a more detailed MS analysis as the mass spectrometer can only analyze a certain amount of ions during one duty cycle. Second, the prefractionation decreases the chances of false peptide quantification due to simultaneous fragmentation of coeluting peptides with equal mass that originate from different proteins. Third, two fractionation steps allow for a greater amount of sample to be loaded onto the column, thus increasing the chance of detecting lower abundance proteins. The drawback of several fractionation steps is the cost in time and possible sample loss during fractionation.

Despite its proven reliability, iTRAQ-based protein quantification has some inherent problems that may affect the results. Different protein isoforms, various glycosylation, and other posttranslational modifications may cause discrepancy in peptide-based quantification. These issues are fundamental in plasma proteomics, but they are not discussed in here. It should be noted that the physiological status of the recipient and donor as well as medication and care before and during surgery may also affect general liver functions.

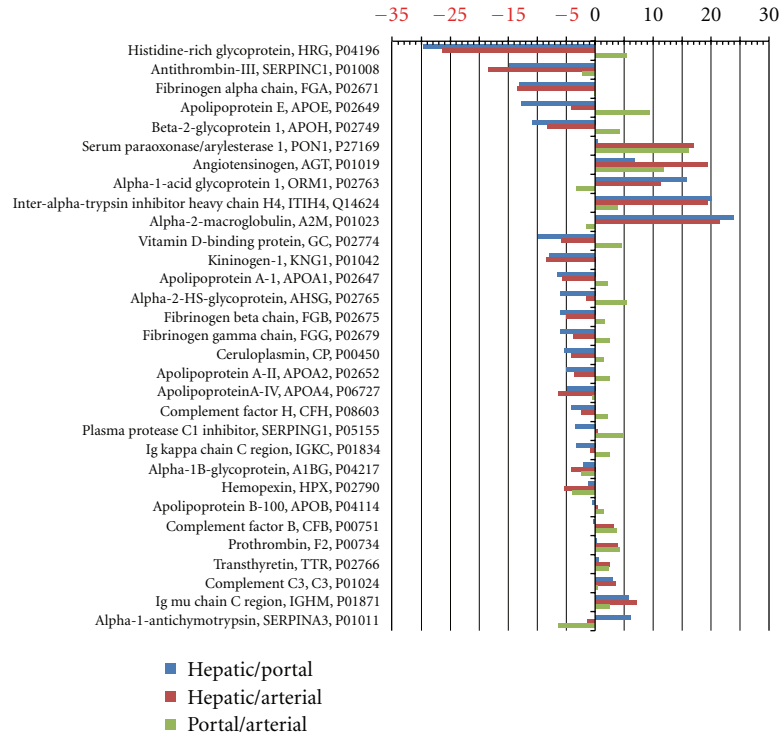

Figure 3: All quantified proteins and percent changes. The Mascot quantification results from all patients and MS repeat runs were averaged and the percent change values were calculated. All changes above 10% were considered significant. The proteins are represented as UniProtKB protein and gene names and accessions.

Protein degradation during sample collection, handling, and storage is of major concern when quantifying plasma proteins [29–31]. Protein half-lives in normal circulation are controlled by production by liver or other tissues, clearance through various organs, or degradation by circulatory proteases [32]. After sampling, the degradation is continued by plasma proteases as well as unspecific proteolysis of labile proteins through physicochemical fragmentation. Therefore, the speed of sample handling, length of storage and temperature, and as simultaneous sample analysis as possible, are crucial factors in quantifying plasma proteome samples [29]. Although blood samples were immediately kept at +4°C after sampling, long-term stored at −80°C [29, 33], and kept on ice with proteinase inhibitors added prior to depletion, we cannot rule out the effect of nonspecific protein degradation during sample handling or storage on the final results. However, in our opinion the effect of such degradation is moderate as our experiment identified mainly medium-to-high abundance proteins. It has been shown that sample storage temperature and time do not have a profound effect on the low-molecular-weight proteome [33] or the high-abundant proteins [29].

Further, we assume that while each protein species has a unique half-life or degradation time, it is constant between samples (e.g., albumin in arterial sample degrades at the same rate as in portal or hepatic venous samples). Even though the proteins are constantly degraded, affecting the absolute abundances of proteins in samples, the relative abundances of each protein species between the samples remains more or less the same. As all three samples from one patient were analyzed always at the same time using the same protocol, the effect of protein degradation on relative iTRAQ quantification is likely minor.

We observed a major reduction in overall protein concentrations in hepatic venous samples as compared to those taken from radial artery and portal vein. This significant reduction of almost 30% was considered to be caused by rinsing the graft with Ringer solution before rinsing with portal blood and subsequent sampling. This dilution effect is compensated by the normalization step in Mascot iTRAQ quantification method.

4.2. Changes in the Hepatic Circulation. As the hepatic venous samples contain the first blood rinsed through the transplanted liver, the changes in protein abundances between portal and hepatic venous blood reflect the severity of reperfusion injury. At this point, hepatic arterial blood flow is not yet reconstructed. Therefore the hepatic venous protein content is not influenced by the systemic arterial proteome. In this study five proteins were found to decrease across the hepatic circulation. These were histidine-rich glycoprotein (HRG), antithrombin-III (SERPINC1), fibrinogen alpha chain (FGA), apolipoprotein E (APOE), and beta-2-glycoprotein 1 (APOH).

The most notable reduction of nearly 30% in the concentration was observed with HRG. HRG is a glycosylated plasma protein with histidine and proline rich regions [34]. Produced by the liver, HRG is found in plasma at a relatively

high concentration of 100 mg/L and at a much lower concentration in platelets [35]. Although the main function of HRG is not entirely clear, it is suggested to participate in immunoregulation, metal homeostasis, and blood coagulation [36]. HRG interacts with plasminogen, heparin, and thrombospondin [37–39]. Its role in coagulation may explain the reduction in HRG levels in liver transplantation as the coagulation system is activated in the ischemia-reperfusion injury. Alternatively, HRG may be retained in the liver in immunoregulative functions as the graft is recognized as foreign material by the innate immune system.

Additionally, the levels of three other proteins related to coagulation decreased across the hepatic circulation, implying protein consumption within the graft. Antithrombin-III [40] is an important regulator of coagulation [41]. It inhibits thrombin and several other serine proteases of the clotting cascade [42]. Fibrin, on the other hand, is the main component of fibrin clots. It is formed by cleavage of fibrinopeptides from fibrinogen alpha and beta chains [43]. Another potential coagulation inhibitory protein is beta-2-glycoprotein-1 or apolipoprotein H (APOH). APOH binding to the exposed phospholipids of the damaged tissues may inhibit activation of the coagulation cascade [44].

Hepatic consumption of these four proteins indicates that blood coagulation is activated immediately during graft reperfusion. Innate immune system recognizes the postischemic liver tissue and beta-2-glycoprotein attaches to the surface of the broken cells. Reperfusion rapidly initiates blood coagulation, leading to fibrinogen conversion to fibrin and fibrin clots, whereas SERPINC1 is consumed in the liver to inhibit excessive thrombin formation. Further, HRG is retained in the liver to promote coagulation or to exert immunoregulative functions. This proposed cascade leads to the observed uptake of these proteins into the liver.

The levels of Apolipoprotein E (APOE), a major component of chylomicrons and other low-density lipoprotein particles, were lower in the hepatic venous samples when compared to those from the portal vein. APOE is produced in a variety of tissues, including the liver. APOE mediates functions of the chylomicron internationalization process, in which the lipids and cholesterol of chylomicrons are transported from intestines to tissues. APOE also mediates the removal of chylomicron remnants in hepatocytes [45]. This detected APOE uptake reflects the chylomicron and APOE clearance from the blood into the liver.

Three proteins were found to increase in concentration across the hepatic circulation. These include alpha-1-acid glycoprotein (ORM1), inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4), and alpha-2-macroglobulin (A2M). All three are high-abundant plasma proteins produced by the liver, ITHI4, and ORM1 almost exclusively. A2M is a protease inhibitor that inhibits a wide range of proteases including thrombin, plasmin, and kallikrein [46]. ITIH4 and ORM1 both function in acute-phase reactions [47, 48].

The profile of changes in HRG, SERPINC1, FGA, ORM1, ITIH4, and A2M levels was similar when systemic arterial samples were compared to either hepatic or portal venous samples. This suggests that the major changes in these proteins indeed take place in the liver and not in other parts of the body.

5. Conclusions

Using protein depletion and LC-MS/MS we have analyzed the very early ischemia-reperfusion changes in plasma proteome during liver transplantation surgery. Despite the sensitivity limit imposed by the used mass spectrometer and analysis methods we were able to characterize differences in several plasma proteins. Major changes were observed in proteins involved in coagulation, especially in histidine-rich glycoprotein. Further studies are needed to validate these results. Nevertheless, our data demonstrates that MS-based protein identification and quantification is a feasible option when examining the effects of major surgery or other organ-related shock to plasma proteome.

Conflict of Interests

The authors declare that they have no competing interests.

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