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Exploratory study of proteins in urine of patients with histoplasma antigenuria☆

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ABSTRACT

Disseminated histoplasmosis is an invasive fungal infection that can be fatal in patients with weak immune system. The goal of our exploratory study was to evaluate differences in urinary protein profiles among samples of healthy individuals, patients with proteinuria (PRU), and histoplasma antigenuria (HIS), and to identify physiological pathways associated with the excreted proteins. Urine samples were depleted of abundant proteins, deglycosylated, digested with trypsin, fractionated and analyzed by nano-LC-QTOF. The total number of human proteins identified in the samples was 117, of which 20 and 23 were unique to the samples from patients with PRU and HIS, respectively. Pathway analysis of proteins identified in samples of PRU and HIS patients suggested increased levels of proteins associated with acute response signaling, coagulation system, prothrombin activation, glucocorticoid regulation and the lipid antigen presentation signaling pathway networks. The obtained data provide information on protein expression associated with HIS, and suggest that further more rigorous studies aimed at the identification of proteins associated with proteinuria of different causes are feasible.

1. Introduction

Kidneys remove waste products and toxins from the blood while preventing loss of proteins in the urine [1]. In healthy individuals, higher molecular weight proteins do not cross the glomeruli and lower molecular weight proteins are filtered but then reabsorbed in the tubules; when glomeruli are damaged, proteins appear in urine. Excretion of proteins in urine in amounts greater than 150 mg per day is called proteinuria. Proteinuria can be associated with kidney damage, nephropathies, genetic disorders, malignancies and infectious diseases [1,2].

One of the infectious diseases that cause proteinuria is an invasive mycosis caused by the dimorphic fungus *Histoplasma capsulatum*. After spores of *H. capsulatum* are inhaled, the organism converts to a yeast phase, capable of disseminating through the bloodstream to various parts of the body [3,4]. Disseminated histoplasmosis can become severe and potentially fatal in patients with weakened immune systems (e.g., infants, elderly, severely ill, organ transplant recipients, and HIV-infected individuals) [3–6]. Proper treatment of proteinuria requires determining the cause of the condition in the patient, and its relationship with other possible diseases.

Identification of urine biomarkers associated with proteinuria of different causes may assist with differential diagnosis, elucidation of the pathogenesis of diseases, and potentially assist with discovery of novel diagnostic markers and treatments. Many studies of the human urinary proteome were aimed at the investigation of kidney-associated diseases [7–11]; however, urinary proteins originate not only from glomerular filtration, but also from tubular secretion, shedding of epithelial cells, and invading pathogens [12]. Thus, potentially, urine may contain biomarkers for a wide range of diseases causing proteinuria.

The goals of this exploratory study were to evaluate differences among excretory proteins in urine from patients with histoplasma antigenuria (HIS), healthy individuals, and patients with proteinuria of other causes (PRU). A further goal was to access the effect of sample fractionation on the enhancement of the number of peptides that can be identified in urine samples. We sought to identify common human proteins among the conditions, proteins that are unique to HIS, and to determine the physiological pathways, associated with the identified proteins.

2. Materials and methods

2.1. Patient samples

Urine samples used in the study were from 20 to 50 years old men and were mid-stream voids collected in sterile urine

Abbreviations: HIS, histoplasma antigenuria; PRU, proteinuria; H, histoplasma; IPA, ingenuity pathway analysis; GO, gene ontology.

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collection cups. Samples for the control group were from self-reported healthy individuals and were collected between 8 am and 10 am. Proteinuria samples were from an in-patient hospital population. Samples from patients that tested positive for histoplasma antigen and samples of patients with PRU were remaining aliquots of samples sent to ARUP laboratories (Salt Lake City, UT) for diagnostic testing. Three pools of samples were prepared: (i) pool of urine from healthy individuals, (ii) pool of urine samples from patients with proteinuria and negative for histoplasma antigen, and (iii) pool of urine samples from patients positive for histoplasma antigen (HIS-1) as determined using a sandwich type immunoassay [13] that employs a polyclonal antibody (ImmunoMycologics, Inc., Norman, OK). Each sample pool contained equal aliquots of urine from four individuals. The samples were tested for total urinary protein and creatinine using the Roche Modular Analytics (Roche, Basel, Switzerland). Accuracy of the measurements of total protein, creatinine, and histoplasma antigen was within ±10% and imprecision was less than 10%. To account for the difference in the content of proteins in the samples from the control and the disease groups, sample volumes were adjusted to contain a total amount of protein per pool of 0.85 mg. A pool of urine samples from patients with a positive test for histoplasma antigen (HIS-2) was used to determine if unique proteins identified in pool HIS-1 would be present in an independent set of samples, and to verify potential association of these proteins with HIS. Urine pool HIS-2 contained 0.5 mL aliquots of seven urine samples of patients tested positive for histoplasma antigen using immunoassay [13]. All samples were stored frozen at −70 °C until use, and thawed immediately before experiments. The study was approved by the Institutional Review Board of the University of Utah (IRB #7275 and #20023). All personal identifiers were removed from the patient samples; written informed consent was obtained from healthy individuals participated in the study.

2.2. Reagents and supplies

Trypsin was purchased from Princeton Separations (Adelphia, NJ). Dithiothreitol, 2,2,2-trifluoroethanol, formic acid, and the deglycosylation kit EDEGLY were obtained from Sigma–Aldrich (St. Louis, MO). Zorbax SB-C18 Chip (40 nL trap, 75 μm × 150 mm, 5 μm particles) and the Multiple Affinity Removal System (MARS) for the depletion of six abundant proteins (albumin, transferrin, haptoglobin, IgG, IgA, and alpha-1 antitrypsin) were purchased from Agilent Technologies (Santa Clara, CA). Ultrafilters Amicon Ultra-4 with molecular weight cutoff of 5, 30, 50 and 100 kDa were purchased from Millipore (Billerica, MA). Solid phase extraction (SPE) columns Strata X were purchased from Phenomenex (Torrance, CA). All other reagents used were of highest purity commercially available. Solvents were of HPLC grade, purchased from JT Baker (Phillipsburg, NJ).

2.3. Sample preparation

Urine pools were concentrated and desalted using ultrafilters with a 5 kDa cutoff to the volume of approximately 100 μL. To the retained fraction, 3.5 mL of molecular grade purity water was added and the solution was filtered a second time to the final volume of 100 μL. The desalted samples were depleted of six abundant proteins using the MARS depletion kit, and the depleted fraction was collected. The depletion protocol for urine samples was previously optimized and published [14]. The depleted samples were ultrafiltered using a 5 kDa cutoff membrane, then proteins were denatured, reduced and deglycosylated using the EDEGLY kit. The denaturing, reduction and deglycosylation was performed according to the manufacturer instructions using five enzymes, each with specificity to a different type of glycosylation. The resulting samples were filtered consecutively using ultrafilters with cutoff of 100, 50, 30 and 5 kDa; the ultrafiltration was performed by centrifugation at 3,500 RCF (20 °C) for 15 min. These four fractions were further processed by denaturing the proteins with 2,2,2-trifluoroethanol (25 μL), reducing with DTT alkylating cysteine residues with iodoacetamide, followed by in-solution digestion with trypsin at 37 °C for 18 h. Peptides from the digests were then extracted using SPE Strata X according to the manufacturer instructions, and five fractions were eluted from the SPE columns using solvents of different strength (10%, 20%, 30%, 40% and 70% of acetonitrile in water; last solvent contained 1% formic acid). The samples were dried and reconstituted in 20 μL of solvent.

2.4. Instrumental analysis

Sample fractions were analyzed on the Agilent 6510 QTOF equipped with a ChipCube and series 1200 nano-HPLC system (Agilent Technologies, Santa Clara, CA). Peptide separation was performed on a Zorbax SB-C18 HPLC Chip. Mobile phase consisted of 5% acetonitrile in water containing 0.1% formic acid, and 5% water in acetonitrile containing 0.1% formic acid. An aliquot of each sample (0.5 μL) was injected onto loading trap of the chip at flow rate of 4 μL/min with the effluent directed to waste. After 1 min, the flow was diverted to the analytical column and acquisition was started. Mobile phase for the analytical separation was delivered at a flow rate of 0.4 μL/min, with a gradient of 5% to 60% acetonitrile in 15 min, followed by conditioning and re-equilibration to initial conditions. Data acquisition (2 GHz extended dynamic range) was performed with the MassHunter® software rev.B.02 (Agilent Technologies, Santa Clara, CA) with an acquisition rate of 3 scans/s followed by MS/MS scans of the five most intense ions with the exclusion set for 60 s after five consecutive MS/MS scans. Each sample was analyzed three times using different mass ranges for the precursor ion selection (m/z 400–620 Da, 620–800 Da and 800–1600 Da). The QTOF analyzer was tuned to a resolution of 12,000 and calibrated before each experiment for a mass accuracy of <2 ppm. The capillary voltage was 1800 V, fragmenter 175 V, skimmer 65 V, and a nebulizer gas flow was 6 L/min. The mass-dependent collision energy was used for the product ion scans. Two reference masses were used for the real time mass correction. The samples were analyzed over the course of six days. Two quality control samples were analyzed at the beginning and end of each set of samples.

2.5. Data analysis

Acquired data were searched against the NCBI human database using Spectrum Mill® rev.A.03.03 (Agilent, Santa Clara, CA). Search settings included: enzyme trypsin, precursor mass tolerance 20 ppm, product ion tolerance 50 ppm, variable modifications: oxidized methionine (15.9949 Da), carbamidomethylation (57.0340 Da), ubiquitination GG (114.0429 Da) [15,16]. Similar spectra that were acquired within 30 s were merged and one missed cleavage was allowed. Spectrum Mill search results were summarized with acceptable score for peptides (>8) and proteins (>11). The error rate (as approximated using a scrambled database) for the identification of peptides with score above 10 was <1%; for peptides with score above 7, the error was <5%. For proteins identified with single peptide, chromatograms and mass spectra were manually evaluated for the presence and the intensity of peptide peaks, presence of the product ions, and the mass accuracy of the parent and the product ions.

The composite results from individual pools were then compared using the Spectrum Mill software, and lists of proteins and peptides, as well as quantitative information were exported for further evaluation. Proteins were quantified using spectral counting, where the relative abundance of a protein was assumed

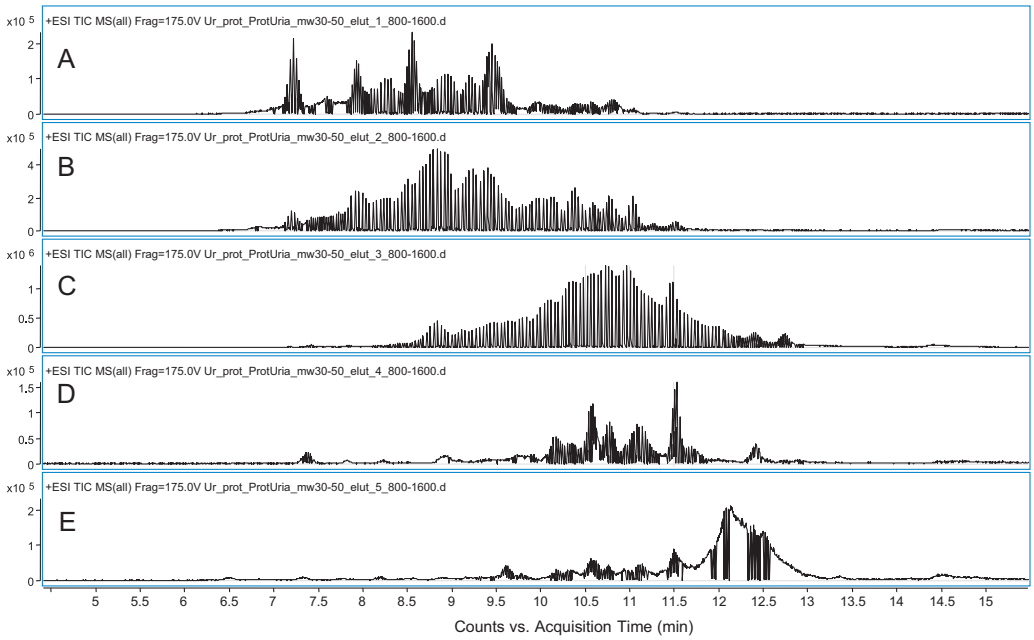

Fig. 1. Total ion chromatograms (overlays of MS and MS/MS scans) of five SPE elutions of the tryptic digest of the PRU sample fraction (molecular weight 30–50 kDa). Peptides were eluted from SPE column using elution solvents containing water with 10% (A), 20% (B), 30% (C), 40% (D), and 70% (E) of acetonitrile (elution solvent E also contained 1% formic acid).

proportional to the number of individual spectra acquired from a protein [17]. Pathway analysis was performed using Ingenuity Pathway Analysis (IPA) v8.7 (Ingenuity, Redwood City, CA).

Individual samples used in the PRU pool were tested for beta-2-microglobulin using an electrochemiluminescent immunoassay performed on the IMMULITE® 2000 analyzer (Siemens Diagnostics, Deerfield, IL). Chromogranin A was tested by an enzyme-linked immunosorbent assay (ALPCO Diagnostics, Salem, NH).

3. Results

3.1. Effect of ultrafiltratioon, fractional elution of peptides, and precursor ion selection

Sample preparation used in this study involved extensive fractionation that lead to an increase in the number of peptides that were identified in the samples (total number of identified peptides was 1796). After depletion of six abundant proteins and deglycosylation, samples were separated in four fractions by ultrafilteration using filters with molecular weight cutoff of 5, 30, 50 and 100 kDa. The percent of peptides identified in the individual fractions (5–30, 30–50, 50–100 and above 100 kDa), was 21%, 13%, 25%, and 41%, respectively. After digestion, peptides were purified using SPE, and five fractions were eluted using elution solvents with progressively increasing strength (10%, 20%, 30%, 40% and 70% of acetonitrile in water). Fig. 1 shows total ion chromatograms of five SPE elutions (PRU sample, molecular weight fraction of 30–50 kDa), and demonstrates the reduction in the data complexity through the use of fractional elution. The percent of peptides identified in the elution fractions was 16%, 24%, 24%, 14%, and 22%. The percent of peptides identified in the injections that used different mass ranges for the precursor ion selection (m/z 400–620 Da, 620–800 Da and 800–1600 Da), was 34, 32, and 33%, respectively.

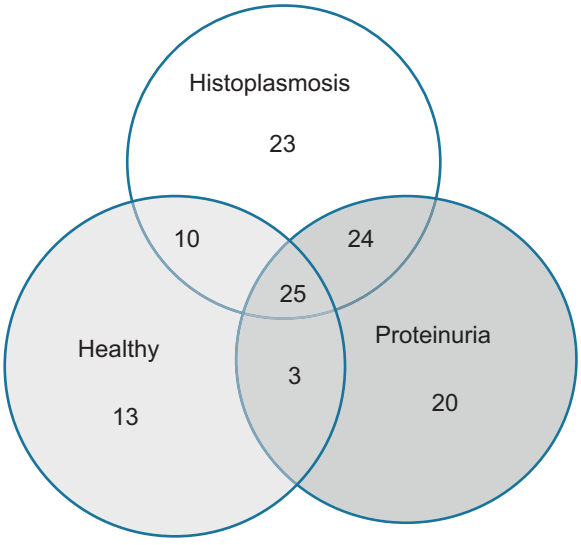

Fig. 2. Venn diagram: proteins unique for each group and proteins in common among the groups.

3.2. Total urinary protein, creatinine, and histoplasma antigen tests

Median (range) of the total protein concentration in the samples from healthy men, men with PRU and men with HIS, were 90 μg/mL (10–260), 1210 μg/mL (1010–1320) and 440 μg/mL (350–1410), respectively. The pH of the samples ranged between 5.5 and 7.5. The median (range) for the creatinine concentration in the corresponding groups were 1320 μg/mL (980–2630), 970 μg/mL (910–1020) and 650 μg/mL (480–1190), respectively. The distributions of the total protein concentration in individual samples were not statistically different between the healthy and HIS patients, and both were statistically different from the PRU patients (evaluated with the Tukey-Kramer HSD test). Median concentrations of creatinine were not statistically different among the groups. Concentrations of the histoplasma antigen in the samples of the HIS patients ranged between 17 and 48 (HIS-1); and 11 and 30 (HIS-2) enzyme immunoassay units.

Table 1
List of proteins in common among the urine samples of healthy individuals, proteinuria and histoplasmosis patients, and relative abundance of the proteins.

Protein name	Accession number	Da	Scan count				Fold change		
			Total	HIS-1	PRU	Healthy	HIS-1 vs. healthy	PRU vs. healthy	HIS-1 vs. PRU
Alpha-1-microglobulin/bikunin precursor	4502067	39,000	244	85	90	69	1.23	1.30	0.94
Prostaglandin H2 D-isomerase	32171249	21,029	67	25	22	20	1.25	1.10	1.14
Coagulation factor II precursor	4503635	70,037	59	33	18	8	4.13	2.25	1.83
Prosaposin	11386147	58,113	52	42	1	9	4.67	0.11	42
Uromodulin	56550049	69,761	49	1	1	47	0.02	0.02	1.00
Alpha-2-HS-glycoprotein	4502005	39,325	49	21	22	6	3.50	3.67	0.95
Secreted phosphoprotein 1 isoform a	91206462	35,423	38	1	12	25	0.04	0.48	0.08
Insulin-like growth factor 2	108796063	20,141	28	6	4	18	0.33	0.22	1.50
Inter-alpha (globulin) inhibitor H4 (plasma Kallikrein-sensitive glycoprotein)	31542984	103,358	25	14	2	9	1.56	0.22	7.00
WAP four-disulfide core domain 2 isoform 1 precursor	56699495	12,993	22	11	3	8	1.38	0.38	3.67
CD14 antigen precursor	4557417	40,076	22	20	1	1	20	1.00	20
Ribonuclease, RNase A family, 2 (liver, eosinophil-derived neurotoxin)	4506549	18,354	18	6	1	11	0.55	0.09	6.00
Natural killer cell-specific antigen KLIP1	49472828	38,248	15	3	4	8	0.38	0.50	0.75
HGFL protein	51317358	28,248	14	4	4	6	0.67	0.67	1.00
Heparan sulfate proteoglycan 2	62859979	468,828	14	3	7	4	0.75	1.75	0.43
Gelsolin isoform a precursor	4504165	85,698	12	7	4	1	7.00	4.00	1.75
amylase, alpha 2A pancreatic precursor	4502085	57,707	11	2	1	8	0.25	0.13	2.00
kininogen 1	4504893	47,884	11	2	1	8	0.25	0.13	2.00
CD74 antigen isoform b	10835071	26,399	9	6	2	1	6.00	2.00	3.00
Vitronectin precursor	88853069	54,306	7	2	2	3	0.67	0.67	1.00
Regenerating islet-derived 1 alpha precursor	29725633	18,731	5	1	2	2	0.50	1.00	0.50
Apobec-1 complementation factor isoform 2	20357575	65,203	5	1	3	1	1.00	3.00	0.33
Cadherin 13 preproprotein	4502719	78,287	4	1	1	2	0.50	0.50	1.00
PREDICTED: hypothetical protein LOC23052 isoform 1	51470760	55,017	4	2	1	1	2.00	1.00	2.00
Vitamin D-binding protein precursor	32483410	52,918	3	1	1	1	1.00	1.00	1.00

3.3. Proteins identified in urine samples

A typical extracted ion chromatogram and product ion spectrum of a peptide are shown in Supplemental Data Fig. 1. The total number of human proteins identified with high confidence in the samples was 117. Supplemental Data Table 1 summarizes the identified proteins and a Venn diagram (Fig. 2) shows distribution of proteins among the groups. To better understand the changes in the global patterns of protein abundances among the urine pools of healthy individuals and patients diagnosed with PRU and HIS, proteins were parsed into groups: (i) proteins in common for all groups (Table 1), (ii) proteins unique to the pathologic samples (Table 2), (iii) proteins in the HIS-1 pool that were increased or decreased by over three-fold.

The total number of proteins in common among the three groups was 25. Proteins identified in all three groups, along with scan count, and the ratio of the number of scans assigned to each of the proteins are listed in Table 1. The number of unique proteins identified in the samples from healthy individuals, patients with PRU, and HIS, were 13, 20 and 23, respectively (Fig. 2). Among the proteins that were common for all groups (Table 1), 14 proteins in PRU pool and 15 proteins in HIS-1 pool showed over two-fold change in the abundance compared to the control group. Compared to urine of healthy individuals, urine of the HIS patients showed greater than a three-fold increase for prosaposin, coagulation factor II, alpha-2-HS-glycoprotein, CD14 antigen and gelsolin; and greater than a three-fold decrease in the abundance of uromodulin, secreted phosphoprotein 1, insulin-like growth factor 2, natural killer cell-specific antigen KLIP1, amylase, and alpha 2A pancreatic precursor.

Out of the proteins identified in sample HIS-1, 76% (63/83) were also present in a urine pool HIS-2 (Supplemental Data Table 1); in addition 80% of proteins (67/83) that were identified in HIS samples in this study were identified in our earlier study [14]. In addition, presence of two proteins was confirmed using commercial immunoassays. Individual samples used in the PRU pool were analyzed, using immunoassays for beta-2-microglobulin precursor and chromogranin A. The median concentrations of the above proteins in the samples were 5.287 ng/mL; and 1.426 ng/mL, respectively.

Table 2
Proteins identified in the pathologic samples, which had over 3-fold difference in abundance between the HIS and PRU samples.

Protein name	Accession number	MW (Da)	Scan count		Fold change: HIS-1 vs. PRU
			HIS-1	PRU	
Hypothetical protein LOC651928	91206444	26,246	35	8	4.4
Anti-RhD monoclonal T125 gamma1 heavy chain	91206434	52,287	20	1	20
SH3 domain binding glutamic acid-rich protein like 3	13775198	10,438	17	2	8.5
Complement component 3 precursor	4557385	187,165	14	3	4.7
Glutaredoxin (thioltransferase)	4504025	11,776	14	0	Absent in PRU
PREDICTED: similar to Ig kappa chain V–I region HK102 precursor	89077015	13,146	13	0	Absent in PRU
Ubiquitin C	67191208	77,029	12	2	6
Retinol-binding protein 4, plasma precursor	55743122	23,010	11	32	0.34
GM2 ganglioside activator precursor	39995109	20,839	7	1	7
Secretoglobin, family 1A, member 1 (uteroglobin)	4507809	9994	6	1	6
Fatty acid binding protein 1, liver	4557577	14,209	6	1	6
Profilin 1	4826898	15,054	6	0	Absent in PRU
PREDICTED: similar to Ig heavy chain V region 102 precursor	89037914	13,908	6	0	Absent in PRU
Cathepsin D preproprotein	4503143	44,553	5	1	5
PREDICTED: similar to Ig kappa chain V–I region HK102 precursor	89066083	13,546	5	0	Absent in PRU
Transthyretin	4507725	15,887	1	5	0.2
Alpha 1B-glycoprotein	21071030	54,254	1	4	0.25
Albumin precursor	4502027	69,367	0	152	Absent in HIS
Serum amyloid A2	13540475	13,508	0	23	Absent in HIS
Chromogranin A precursor	4502805	50,689	0	17	Absent in HIS
Serine (or cysteine) proteinase inhibitor, clade A	50363217	46,737	0	12	Absent in HIS
Fibrinogen, alpha polypeptide isoform alpha-E preproprotein	4503689	94,974	0	5	Absent in HIS

3.4. Protein oxidation and ubiquitination

Two of the variable modifications used during the database searches were oxidized methionine and ubiquitination [15,16]. The number of spectra of the peptides containing oxidized methionine and the ubiquitin-specific tag was compared among the groups. The number of peptides with oxidized methionine in samples corresponding to the urine pools of healthy individuals, PRU, and HIS patients were 27, 49, and 48, respectively. The number of spectra of the peptides containing a ubiquitin-specific tag [15] was 38, 24, and 62, respectively.

3.5. Gene ontology and pathway analysis

Proteins identified in the samples were mapped according to their cellular localization using Gene Ontology (GO) (Fig. 3). Metabolic and cell signaling pathways, biological processes, and networks of the protein-protein interaction were interpreted using IPA [18,19]. Results of the GO and IPA analysis are summarized in Figs. 3 and 4 and Supplemental Data Table 2 and Figs. 2–4.

Based on the results of the GO analysis, no difference was observed among the groups in the percentage of the membrane and cytoplasm proteins, while a greater percentage of the extracellular and lower percentage of the nucleus proteins were observed in the HIS patients. A substantial difference among the groups was observed in the percentage of proteins corresponding to different cellular functions. In the samples of the HIS patients, the percent of the transport proteins was twice as high compared to the control group, and percentage of signaling and binding proteins was 12% lower.

The Fisher's test was used by the IPA to identify statistically significant pathways and networks ($p < 0.05$). The IPA analysis of proteins identified in the study revealed 6, 7 and 5 statistically significant pathway networks in urine samples of PRU and HIS patients and controls, respectively. Supplemental Data Table 2 summarizes the networks, genes and proteins that correspond to the nodes in Supplemental Data Figs. 2–4. For six of the biological pathways identified in the data analysis with IPA (Fig. 4), the associated p-values were lower, and number of the associated proteins were higher in the HIS samples compared to the healthy controls, namely: acute phase response signaling pathway, coagulation system pathway, intrinsic prothrombin activation pathway, extrinsic prothrombin activation pathway, lipid antigen presentation pathway, and macrophage migration inhibitory factor (MIF) mediated glucocorticoid regulation pathway. Proteins corresponding to the antigen presentation pathway and neuroprotective role of thimet oligopeptidase pathway were absent in the control group, while were present in the HIS-1 sample.

4. Discussion

Earlier studies demonstrated an increase in the number of identified proteins in urine through the use of antibody-based depletion of abundant proteins [14] and deglycosylation of urinary proteins [20]. In this study, in addition to the depletion and the deglycosylation, we utilized ultrafiltration of the depleted urine samples, and fractional elution of the peptides from SPE adsorbent (Fig. 1). Additionally, an increase in the number of the identified peptides was gained through the use of the narrow mass ranges for the selection of the precursor ions during the data-dependent acquisition. The number of identified peptides was approximately equally distributed among the precursor ions selected from the mass ranges (m/z 400–620 Da, 620–800 Da and 800–1600 Da). Incorporation of the above-discussed techniques in the sample preparation, along with the use of narrow mass ranges for the precursor ion selection during the QTOF analysis, allowed reducing complexity of the samples and key to identification of overall larger number of peptides and proteins.

In this study we have used differential proteomics techniques to identify and semi-quantify proteins present in significantly different abundance in urine excreted by HIS patients, when compared to urine samples of PRU patients and healthy individuals. Among the most abundant proteins that were found only in the HIS samples (Supplemental Data Table 1) there were six enzymes (glutaredoxin, carbonic anhydrase, kallikrein, acid alpha-glucosidase, ribonuclease T2 precursor, quiescin Q6), four plasma membrane proteins (peptidoglycan recognition protein, brain abundant signal protein, carcinoembryonic antigen-related cell adhesion molecule, vascular cell adhesion molecule), and five immunoglobulins (Ig kappa chain). Among the proteins in common of the studied groups, 15 proteins had over two-fold difference in abundance in the HIS group, compared to the other two groups. Prominent among these

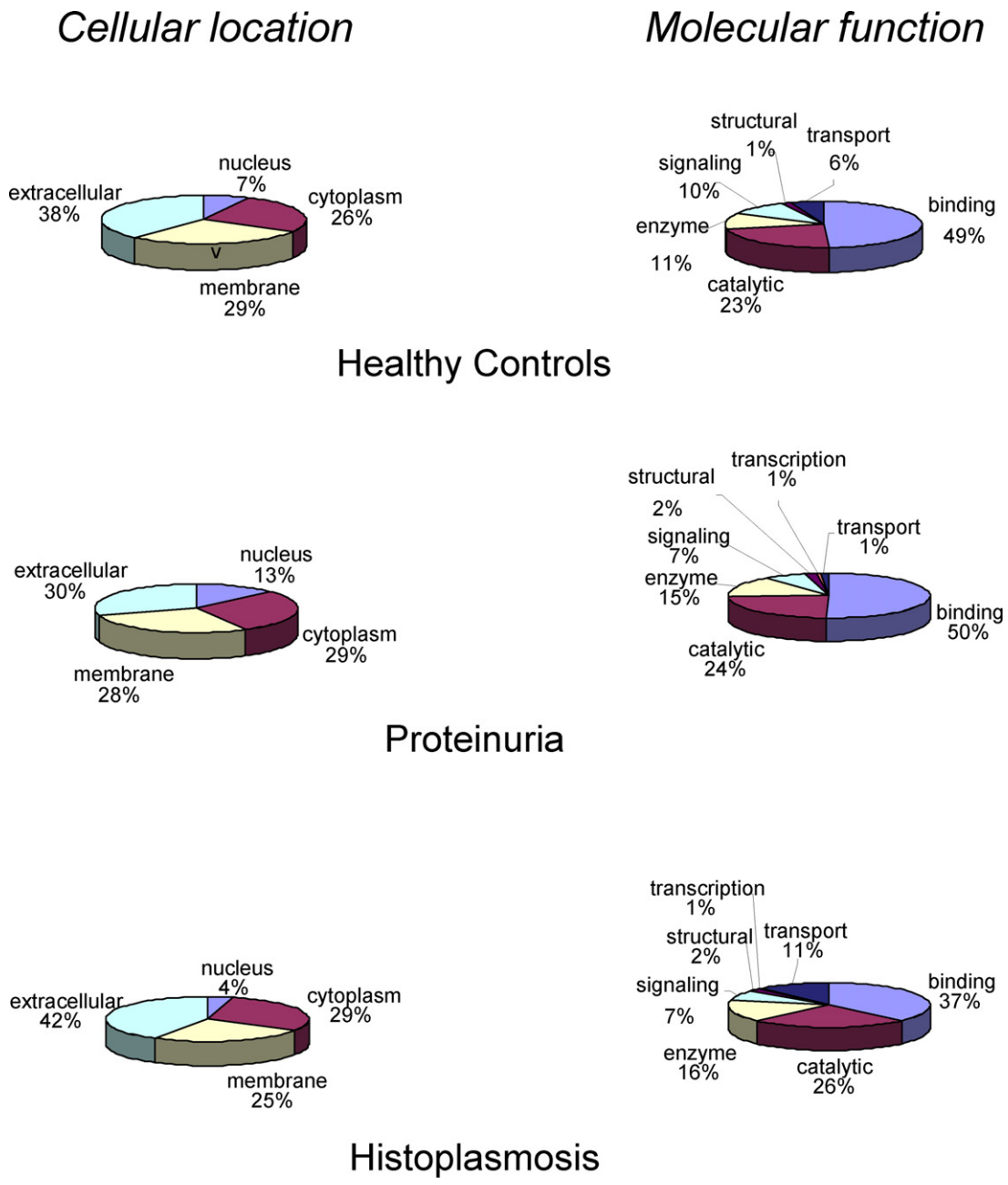

Fig. 3. Distribution of proteins by their cellular location and molecular function in urine of healthy individuals, proteinuria and histoplasmosis patients.

were changes in seven proteins known to be localized in extracellular space (transthyretin, secretoglobin, retinol binding protein, alpha-1-B glycoprotein, gelsolin, prosaposin, and coagulation factor II); six proteins localized in cytoplasm (ubiquitin, cathepsin, fatty acid binding protein, glutaredoxin, GM2 ganglioside activator, profilin 1); and two proteins characteristic of plasma membrane (CD14 and CD74).

Uromodulin is one of the major proteins in urine of healthy individuals [10,11]; in our study, peptides of uromodulin corresponded to 14% (45/322) of the total number of identified peptides in the control group, while in samples of HIS and PRU patients, they corresponded to less than 0.2% (1/800 in HIS and 1/664 in PRU) of the number of identified peptides.

The effect of disease was also observed in the distribution of protein modifications identified in the samples. Compared to the control group, twice as many peptides containing oxidized methionine were detected in the PRU and HIS pools, suggesting increased oxidative stress [21]; and three times as many ubiquitinated peptides were detected in the HIS pool, suggesting increased rate of cell death and protein degradation. The ubiquitin system is a well characterized pathway involved in regulation of the majority of cellular processes in eukaryotes [22,23]. It was shown that many diseases, including infectious diseases, are associated with abnormalities in the ubiquitination process [22,23].

In addition to the differences in the identified specific proteins, a difference among the groups was also observed in the percentage of transport, signaling, binding proteins, and enzymes that were excreted in urine (Fig. 3).

In this study we used a bioinformatics pathway analysis to identify the significant signaling pathways and networks associated with HIS. IPA inferred that a large number of proteins in urine of HIS patients were associated with acute-phase response, coagulation

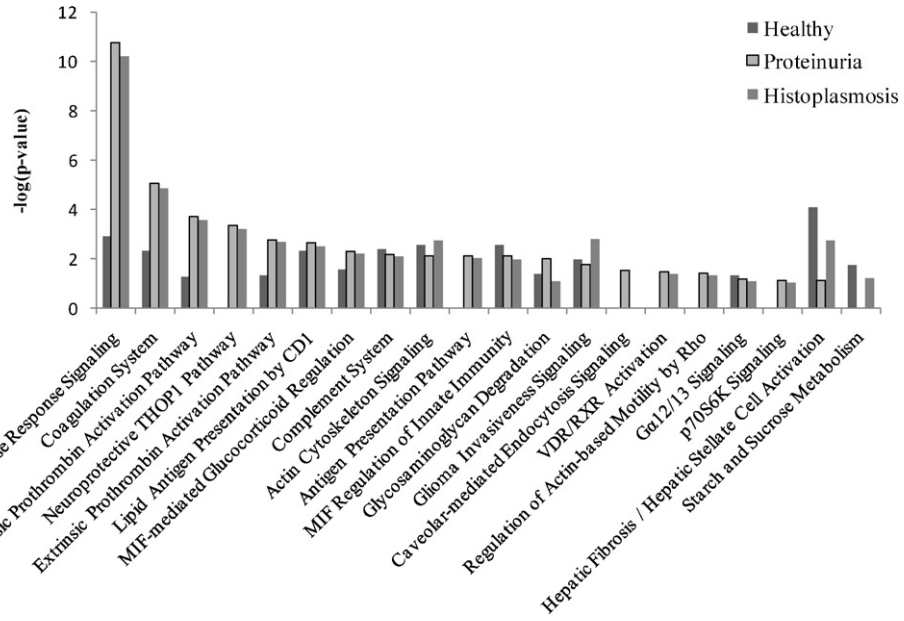

Fig. 4. Major metabolic and cell signaling pathways associated with proteins identified in urine samples of healthy individuals, proteinuria and histoplasmosis patients.

system, inflammation, and immune response pathways. While it is impossible to interpret from these data whether identified proteins were derived from distinct cellular processes or are a component of the urinary milieu, these data suggest the importance of cell death and turnover in response to HIS.

We realize potential limitations of this study; it was based on a limited number of samples, with each of the samples prepared as a pool of urine samples from four individuals. Furthermore, the samples from HIS group may have undergone different specimen storage and handling prior to being received for testing (compared to the other two groups). Sample pooling helped to minimize effects of storage/handling variation, as suggested in other studies [24,25]. One of the steps during the sample preparation was immunodepletion of abundant proteins; because of nonspecific binding, some of the proteins that were not targeted for the depletion could be removed from the samples through co-depletion with the abundant proteins. Because of the limited number of samples used in the study and normalization of the sample volume based on the total protein content in urine samples, no quantitative conclusions could be made, related to the expected concentration of identified proteins in individual urine samples. Samples utilized in the study were from 20 to 50 years old men; some of the proteins in urine samples from women could be different from the proteins observed in samples from men.

In summary, we report a large number of differentially expressed human proteins in the urine of HIS patients. The identified differentially expressed proteins are associated with the acute response signaling pathway, coagulation system pathway, intrinsic and extrinsic prothrombin activation pathways, MIF-mediated glucocorticoid regulation and the lipid antigen presentation pathway. Knowledge of the signaling pathway networks provides important information for the protein expression changes associated with the HIS and potentially could assist with better understanding of the pathophysiology of histoplasmosis. Our data suggest that further, more rigorous studies, aimed on identification of proteins in urine associated with proteinuria of different causes, and studies of cellular processes, responsible for excretion of these proteins, are warranted. The MS-based approach for identification of the ubiquitination sites [16] through detection of the k-GG tagged lysine residues proved to be useful for identification of ubiquitinated proteins excreted in urine. Our methodology has allowed us to detect potentially unique proteins excreted in urine of patients suffering from histoplasmosis. The combination of techniques that we used in this study is a promising approach for future studies aimed toward identification of biomarkers in urine.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.09.006.

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