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Label-free mass spectrometric profiling of urinary proteins and metabolites from paediatric idiopathic nephrotic syndrome

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ABSTRACT

Idiopathic nephrotic syndrome (INS) is caused by renal diseases that increase the permeability of the glomerular filtration barrier without evidence of a specific systemic cause. The aim of the present work was to reveal inherent molecular features of INS in children using combined urinary proteomics and metabolomics profiling. In this study, label-free mass spectrometric analysis of urinary proteins and small molecule metabolites was carried out in 12 patients with INS *versus* 12 sex- and age-matched control subjects with normal renal function. Integration and biological interpretation of obtained results were carried out by Ingenuity IPA software. Validation of obtained proteomics data was carried out by Western blot method. Proteomics data have been deposited to the ProteomeXchange Consortium with the data set identifier PXD000765. This study indicates for the first time that paediatric INS is associated with up-regulation of afamin, hydroxyphenylacetate and uridine, and concomitant down-regulation in glutamine and phenylalanine levels, and many of these molecular species were previously shown to be involved in oxidative stress. Further studies in larger patient population are underway to investigate the role of oxidative stress in renal injury in paediatric INS.

1. Introduction

Nephrotic syndrome (NS) can be generally divided into primary (idiopathic nephrotic syndrome, INS) reflecting glomerular diseases intrinsic to the kidney and unrelated to any systemic cause, and secondary NS that can be induced by several different pathological conditions (e.g. autoimmune and vasculitic diseases, infectious diseases, etc.). INS is considered the most common form of NS in children, wherein more than 90% of cases are between 1 and 10 years of age, and around 50% after 10 years of age [1]. Major clinical features that characterise INS develop as a consequence of compromised integrity of the glomerular filtration barrier, in particular altered cell morphology and motility of podocytes, terminally differentiated cells that line the outer portion of the glomerular basement membrane. Although the molecular trigger for the onset of INS still remains largely unknown, several lines of evidence demonstrate that altered T-lymphocyte responses could play a central role in the INS pathogenesis. It has been postulated that T cells produce a circulating permeability factor that interferes with the expression, function or both of the key podocyte proteins to induce proteinuria [2]. However, the nature of this circulating factor is not known, and many cytokines and inflammatory molecules have been implicated including interleukins, Interferon-γ, TGF-β, vascular permeability factor, NF-κB and TNF-α.

Urinary proteomics has garnered much attention lately as a novel tool for biomarker discovery in kidney diseases, because urine can be collected easily, in large amounts and non-invasive manner. Additionally, urinary proteome closely mirrors pathological changes associated with the function of the kidney and the urogenital tract, and is generally more stable than the blood proteome [3]. Similarly, metabolomics has emerged as a valuable analytical platform for identification of urinary and serum biomarkers for renal diseases. The comprehensive investigation of the metabolome has thus proved as an important tool to study phenotype and changes in phenotype triggered by environmental factors, pathological condition, or alterations in genotype [4].

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A limited number of previous proteomics studies in paediatric idiopathic nephrotic syndrome were directed towards the identification of either differentially expressed proteins in urine and plasma during NS compared with remission, or of predictive biomarkers for steroid therapy response in the treatment of idiopathic nephrotic syndrome. Currently, there is neither a report on the urinary proteomics nor on the metabolomics profiling to discriminate between paediatric idiopathic nephrotic syndrome patients and healthy subjects with functional kidneys to identify inherent molecular features of INS. In the present study, we applied for the first time a combined -omics approach using label-free mass spectrometry-based profiling of urinary proteins and metabolites to reveal molecular signatures underlying INS and identify putative pathogenic factors of this disease.

2. Material and methods

2.1. Patients

Twelve subjects with INS and twelve sex- and age-matched patients with normal renal function (control subjects) were recruited at Division of Nephrology, Department of Paediatrics, Zagreb University Hospital Centre, Croatia (Supplementary Table 1). Diagnosis of INS was established by standard laboratory tests and histological examinations of renal biopsy specimens. Both, INS and control patients did not receive any therapy prior to the commencement of this study. The University Hospital Centre Zagreb Ethical Committee approval was received for the study, and the informed consent of children's parents was signed. First morning urine samples were collected from all subjects using sterile urine containers, portioned into 1 ml aliquots to avoid freeze/thaw cycles in repeated experiments of the same sample, and stored at −20 °C for further use.

2.2. Urinary metabolomics analysis

For metabolomics analysis, urine samples were purified using Oasis HLB solid phase extraction (SPE) cartridges (Waters Corporation). SPE was performed according to manufacturer's instructions with slight alterations using 1 ml of paediatric urine as a starting amount. Water/methanol (90/10) washes were performed followed by neat methanol analyte elution. The resultant eluent solutions were evaporated to dryness using a vacuum centrifuge concentrator, reconstituted in 200 μL aqueous 0.1% formic acid solution and vortexed prior to LC–MS analysis. The chromatographic separation of the metabolite samples was conducted with an ACQUITY system equipped with a 1.7-μm bridged ethylene hybrid (BEH), 10 cm × 2.1 mm C18 column (Waters Corporation) and the column was maintained at 45 °C. The metabolites were resolved with a 10 min gradient from 10% to 50% acetonitrile (0.1% formic acid) at 500 μL/min. 5 μL of each sample was injected in triplicate on column and analysed in a random order. A quality control (QC) sample was created from a pool of all samples and injected every tenth injection. MS data were acquired in positive ion mode using a hybrid ion mobility-oaToF Synapt G2 mass spectrometer (Waters Corporation) operated in v-mode of analysis with a resolving power of 20,000 FWHM. Data were real-time lock mass corrected using the singly charged precursor ion of Leu-Enkephalin, which was acquired with a sampling frequency of 30 s. The capillary and cone voltages were 4 kV and 40 V, respectively. Accurate mass data were collected in a data-independent acquisition (DIA) mode [5,6] by alternating the energy applied to the collision cell between a low and elevated state. In low energy MS mode, data were collected at constant collision energy of 4 eV (per unit charge). In the elevated energy mode, the collision energy was ramped from 15 eV to 40 eV (per unit charge) during each integration. The spectral acquisition time in each mode was 0.18 s with a 0.02 s interscan delay. One cycle of low and elevated energy data was acquired every 0.4 s. The quadrupole mass analyser was operated in non-resolving mode and the LC-DIA-MS acquisition range from 20 to 1000 *m/z*. The metabolomics LC–MS data were aligned and normalized using CoMet (Nonlinear Dynamics, Newcastle upon Tyne, United Kingdom). Total ion current normalization was conducted using a two group experimental design with each group the data from thirty-six (patient)/thirty-three (control) (three technical replicates per twelve (patient)/eleven (control) subject samples) LC-DIA-MS runs. The complete data set was imported into EZinfo (Umetrics, Umeå, Sweden) for multivariate statistics, using orthogonal partial least squares-discrimination analysis (OPLS-DA) to examine the multidimensional data and identify group differences. Pareto scaling was used in which each variable was centered and multiplied by $1/\sqrt{S_k}$, where S_k is the standard deviation of the variable. Identification of major metabolic perturbations within the pattern recognition models was achieved by analysis of the corresponding contrasting loadings OPLS-DA results plots. Identification was based on accurate mass and fragmentation spectra using a combination of compound databases (Human Metabolite Database version 3.0 and ChEBI 2012).

2.3. Urinary proteomics analysis

For proteomics analysis, urine samples for protein digest analysis were prepared as previously described with minor modifications using 1 ml of sample as a starting amount [7]. The samples were treated with 1% RapiGest at 80 °C for 45 min prior to reduction and alkylation. The proteins were reduced in the presence of 5 mM dithiothreitol at 60 °C for 30 min and alkylated in the dark with 10 mM iodoacetamide at room temperature for 30 min. The aliquots were incubated with anti-HSA resin and centrifuged using Vivaspin 5,000 MWCO filters (Millipore, Billerica, MA). A series of washes using water were implemented to ensure adequate recovery followed by a final wash with 0.1% RapiGest in 50 mM ammonium bicarbonate (pH 8.5). The supernatants were collected and proteolytic digestion initiated by adding modified trypsin at a ratio of 1:10 (w:w) followed by incubation overnight at 37 °C. The RapiGest surfactant was hydrolyzed by the addition of 2 μl of trifluoroacetic acid to the sample. The samples were incubated at 37 °C for 30 min, centrifuged for 30 min at 13,000 rpm and the supernatants collected. Label-free LC–MS was used for qualitative and quantitative protein digest analyses. The experiments were conducted using a 90 min gradient from 5% to 40% acetonitrile (0.1% formic acid) at 300 nL/min using a nanoACQUITY system and a BEH 1.7 μm C18 reversed phase 75 μm × 20 cm nanoscale LC column (Waters Corporation). Prior to separation, peptides were focused and desalted on a 5 μm Symmetry C18, 180 μm × 2 cm trapping cartridge (Waters Corporation). 1 μL of each sample was injected in triplicate on column and analysed in random order. The column was interfaced to the source of the mass spectrometer, which was maintained at 70 °C, using an electrospray ionization emitter tip (New Objective, Woburn, MA). MS data were acquired in positive ion mode using a hybrid ion mobility-oaToF Synapt G2 mass spectrometer, operated in v-mode of analysis with a resolving power of 20,000 FWHM. Data were post-acquisition lock mass corrected using the doubly charged precursor ion of [Glu^1]-Fibrinopeptide B, which was acquired with a sampling frequency of 60 s. The capillary and cone voltages were 3.5 kV and 30 V, respectively. Peptide data were acquired in ion mobility assisted data independent analysis (IM DIA) mode [8]. In low energy MS mode, data were collected at constant collision energy of 4 eV (per unit charge). In the elevated energy mode, the collision energy was ramped from 15 eV to 40 eV (per unit charge) during each integration. The spec-

tral acquisition time in each mode was 0.48 s with a 0.02 s interscan delay. One cycle of low and elevated energy data was acquired every 1 s. The quadrupole mass analyser was operated in non-resolving mode such that ions from *m/z* 300 to 2000 were efficiently transmitted, ensuring that any ions observed in the LC–MS data less than *m/z* 300 were known to arise from dissociations in the collision cell. The LC-IM-DIA-MS acquisition range was from 50 to 2000 *m/z*. ProteinLynx GlobalSERVER version 2.5.2 (Waters Corporation) was used to process the proteomics LC-IM-DIA-MS data. The data were lock mass corrected, centroided, deisotoped, and charge-state-reduced to generate accurate monoisotopic masses for every detected precursor and product ion. Initial correlation of the precursor and product ions is achieved by means of retention and drift time alignment [8]. Further correlation is obtained during the database search based on the physicochemical characteristics of peptides when they undergo collision induced fragmentation [9].

Protein identifications were obtained by searching the reviewed entries of a *Homo sapiens* UniProt database (2011_09, 20,239 entries), which was *in silico* N-terminally processed using Database on Demand [10]. Peptide and fragment ion tolerances were determined automatically, one missed cleavage site allowed, and fixed carbamidomethylation modification of Cys and variable oxidation of Met and N-linked glycosylation of Asn-X-Ser/Thr modification considered. The proteomics LC–MS data were aligned normalized and quantified using Progenesis LC–MS (Nonlinear Dynamics) for label-free quantification. Median abundance normalization was conducted using a two group experimental design with each group the data from thirty-six (patient)/thirty-three (control) (three technical replicates per twelve (patient)/eleven (control) subject samples) LC-IM-DIA-MS runs. The ProteinLynx Global SERVER search results were appended to the quantitative data, excluding human serum albumin annotated features for data normalization. Proteomic perturbations, i.e. relative protein abundance levels and associated reliability of the measured differences, were considered significant with a minimum fold change of two between both biological groups and an ANOVA *p*-value $\leqslant 0.05$. The results have also been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [7] with the dataset identifier PXD000765.

2.4. Western blot analysis

For Western blot validation of afamin, total urine proteins (50 μg) were resolved on 12% SDS–PAGE gels and transferred to PVDF membranes. After blocking in 4% non-fat milk in TBST for 1 h at room temperature, membranes were incubated overnight with primary antibody raised against afamin (diluted 1.5 μg/ml; Abcam, UK) at 4 °C. After washing with TBST three times, the membranes were further incubated with horseradish peroxidase-conjugated secondary antibody (polyclonal goat anti-rabbit IgG; DAKO, USA) diluted 1:2000 in 1% BSA in TBST for 1 h at room temperature. Subsequently, membranes were washed again three times with TBST. Chemiluminescent detection was performed using the Western Lightning Plus ECL kit (PerkinElmer, USA) and ImageQuant LAS 500 imaging system (GE Healthcare, USA). Experiments were repeated twice.

2.5. Pathway analysis

Ingenuity Pathway Analysis program (IPA) (Ingenuity Systems, Redwood City, CA) was used to find the pathways associated with the proteins and metabolites identified in the mass spectrometry analysis. The program uses computational algorithms to identify local networks that are particularly enriched in the data sets. Such local networks contain the most highly connected focus analytes that specifically interact with other components in the network. The filters and general settings for the core analysis were set to consider all the molecules as well as direct and indirect relationships. Networks of focus genes were algorithmically generated on the basis of their connectivity and ordered by score. This score reflects the relevance of the network based on a p-value calcula-

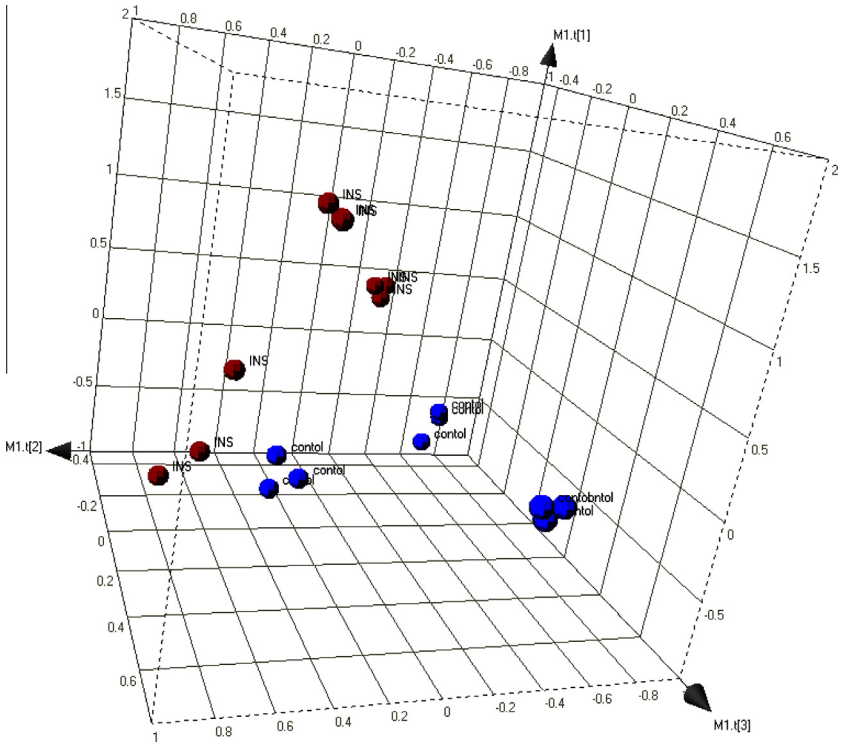

Fig. 1. Three dimensional protein PCA scores plot from OPLS-DA analysis of paediatric INS pre-treated (red) *vs.* control (blue).

tion, i.e. the probability that the network-eligible molecules have been found in a network by chance alone. Networks were also associated with biological functions (and/or diseases) that were most significant for the molecules in the network. Fisheŕs exact test was used to calculate a *p*-value determining the probability that the assignment of each biological function and/or disease to that network was due to chance alone.

3. Results and discussion

Comparative proteomics analysis of urine from INS *versus* control subjects led to the identification of 77 proteins that showed different abundance levels with a fold change of approximately 2 or higher (Supplementary Table 2). Among these, 74 and 3 proteins were over-expressed in INS and control subjects, respectively. Principal component analysis showed significant changes between control and diseased urine samples (Fig. 1). Similar clustering patterns were observed for protein and metabolite datasets, which clearly demonstrates the potential of such integrated approach in the development of non-invasive diagnostics for INS and/or in routine clinical screening procedures for renal diseases. It is important to note that the major bottleneck in the translational efforts of biomarker screening procedures relying on -omics approaches are data analysis and interpretation. The approach presented here for data acquisition in combination with data analysis proved highly efficient, since obtained urinary protein profiles of INS patients correlated well with observed clinical features and encompassed a large number of proteins that had been previously described in the INS pathology including inflammatory mediators (e.g. interleu-

Table 1
Potential protein biomarkers identified only in urine from children with INS.

Accession No.	Protein name	Protein score	Protein average mass (kDa)	Number of matched peptides
P00441	Superoxide dismutase [Cu–Zn]	990	16.0	5
P01019	Angiotensinogen	671	50.0	7
P08185	Corticosteroid-binding globulin	2014	42.7	9
P43652	Afamin	4015	68.5	17
P53004	Biliverdin reductase A	341	33.5	3
Q13522	Protein phosphatase 1 regulatory subunit 1A	1538	19.1	4
Q6P587	Acylpyruvase FAHD1, mitochondrial	1298	25.1	1
Q9NQW7	Xaa-Pro aminopeptidase 1	909	70.5	6
Q9P1Z3	Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 3	143	86.5	2

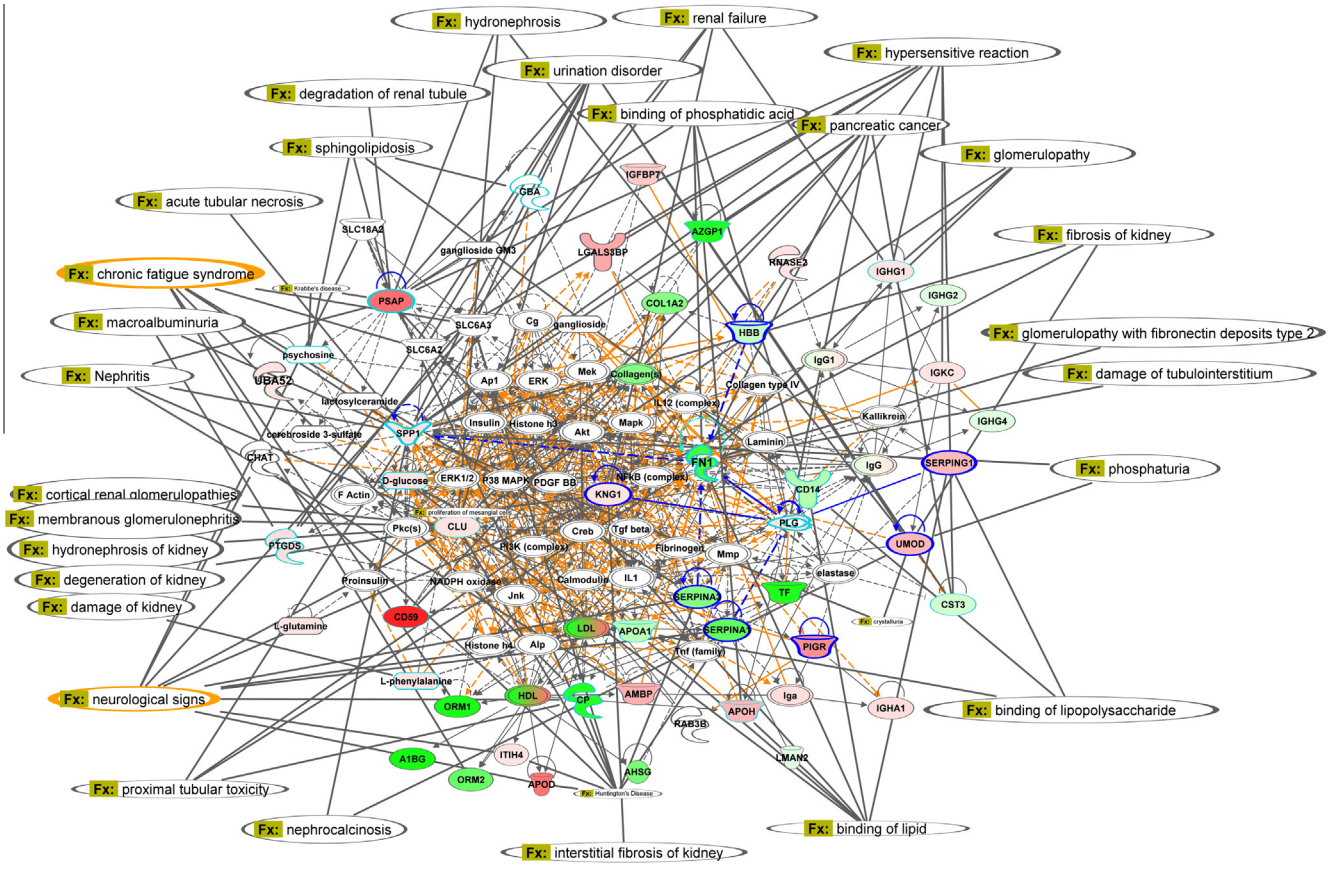

Fig. 2. Combined proteomics and metabolomics data within the biological pathways by the use of pathway analysis software IPA. Colour intensity correlates with a differential expression level (brighter–stronger differential expression). Dashed lines denote regulatory interactions. Solid lines denote protein interactions. Border colours highlight selected groups explained for each network.

kins, and immunoglobulins) [11], glycoproteins (e.g. fetuin, vitronectin, cell surface glycoproteins and α 1-B glycoprotein) [12], and metabolic molecules (e.g. vitamin D metabolites, apolipoprotein A, homocysteine and glutathione metabolism mediators) [13].

INS pathogenesis is strongly related to abnormalities of the glomerular barrier and increased permeability to albumin, and a number of renal membrane proteins and receptors are regulated by glycosylation. Similarly, we detected for the first time the expression of glycoprotein afamin (AFM) in urines from INS patients (Table 1), which was additionally confirmed by Western blot analysis (Supplementary Fig. 1). It was also evident that functional kidneys normally do not excrete afamin in urine. Its detection in INS was rather intriguing due to its previously demonstrated role in neuronal function. For example, Heiser et al. [14] showed that the addition of afamin to cortical chicken neurons exposed to oxidative stress induced by H_2O_2 or beta-amyloid peptide protects cortical neurons from cell death. The findings from the above mentioned study indicate that AFM might be an important component of cellular anti-oxidative defence. Since oxidative stress is known to regulate the magnitude of renal sympathetic nerve activity [15], afamin expression might be associated with oxidative damage-related dysregulation of neuronal pathways that control the major structural and functional components of the kidney leading to changes in the regulation of renal blood flow, glomerular filtration rate, renal tubular epithelial cell solute and water transport. Indeed, integrated network analysis of obtained proteomics and metabolomics data also confirmed the involvement of neural component in the INS pathogenesis (Fig. 2).

Additional evidence for protein and metabolite biomarkers of oxidative stress in INS urine raises the prospect of impaired function of cells and their membranes as a consequence of oxidative damage. For example, we detected biliverdin reductase A in urines from INS patients, an important cytoprotectant against oxidative stress and hypoxia whose expression *in vivo* and *in vitro* is induced by reactive oxygen species, especially hydroxyl radicals [16]. The BVR activity is also induced by TNF-α, the pro-inflammatory cytokine whose levels were found to be significantly elevated in the plasma and urine from patients with primary nephrotic syndrome in comparison with healthy control subjects [17]. Overexpression of BVR enhances activation of the NF-κB transcription factor [18], a master regulator of immune and inflammatory processes in response to injury. *In vivo* study in mice showed that inhibition of the NF-κB signalling in podocytes resulted in the absence of proteinuria and lack of overt changes in kidney morphology [19]. After induction of glomerulonephritis, mice with abrogated podocyte-specific NF-κB signaling (NEMO(pko)) and control mice both developed marked proteinuria, but NEMO(pko) mice recovered much faster, which was manifested by rapid remission of proteinuria and restoration of podocyte morphology. This study clearly shows that pro-inflammatory activity of NF-κB in podocytes increases proteinuria in experimental glomerulonephritis in mice. Similarly, up-regulation of NF-κB in the podocytes of transgenic mice resulted in glomerulosclerosis and proteinuria, providing thus additional evidence for the NF-κB activation as a causative factor for podocyte injury [19]. With this in mind, it seems tempting to believe that increased expression of BVR in INS could be a consequence of oxidative stress and/or immunological dysfunction that precipitates podocyte damage and affects the glomerular permeability.

Importantly, metabolomics data (Table 2) revealed for the first time that hydroxyphenylacetate and uridine were up-regulated, whereas glutamine and phenylalanine were down-regulated in INS patients. Hydroxyphenylacetate is a natural substrate for OAT1, a transmembrane protein mainly expressed in barrier epithelia including renal tubules. Interestingly, hydroxyphenylacetate was detected in the colon cancer rat model, where its urinary levels

Table 2
List of significantly altered urinary metabolites in pediatric INS.

ID	Retention time (min)	*m/z*	Neutral mass	Formula	Co-variance	MVA correlation	ANOVA (*p*)	Factor of change	Regulation in DP group	Average (DP)	Average (C)	Std. dev (DP)	Std. dev (C)
Creatinine	0.58	114.0691	113.0589		0.10	0.17	1.22E−01	1.1	–	607.5	532.4	237.7	217.3
Indoline	2.83	120.0838	119.0735	C_8H_9N	−0.13	−0.52	2.59E−07	1.6	Down	70.0	110.0	25.2	40.9
Creatine	0.62	132.0803	131.0695		0.12	0.22	3.06E−02	1.4	Up	267.4	187.7	193.0	148.3
L-Glutamine	2.45	147.0514	146.0691		−0.11	−0.61	2.43E−10	3.4	Down	10.6	36.2	6.1	23.2
Hydroxyphenyl acetate	2.59	153.0696	152.0473	$C_8H_8O_3$	0.21	0.64	7.62E−12	2.3	Up	164.7	72.2	73.3	28.5
Phenylalanine	2.82	166.0902	165.0790	$C_9H_{11}NO_2$	−0.12	−0.51	4.59E−07	1.6	Down	70.5	109.5	26.1	40.3
3 or 7 methylxanthine*	2.71	167.0601	166.0491		−0.11	−0.27	1.20E−02	1.5	Down	133.1	196.9	120.9	114.9
Glucose (or other sugar)	3.13	181.0760		$C_6H_{12}O_6$	−0.14	−0.41	1.13E−04	2.4	Down	46.7	110.3	51.7	92.0
Uridine	2.55	245.0770	244.0695	$C_9H_{12}N_2O_6$	0.12	0.51	4.56E−07	Infinity	Up	36.0	0	44.3	0
2,6 Dimethyl-heptanoyl carnitine	5.57	302.2399	301.2253		0.11	0.34	6.46E−04	2	Up	92.7	46.0	76.0	45.3

* 7-Methylxanthine is a methyl derivative of xanthine, found occasionally in human urine. 7-Methylxanthine is one of the purine components in urinary calculi.

decreased when rats were fed a diet supplemented with strong antioxidant resveratrol, indicating the correlation between levels of hydroxyphenylacetate and oxidative stress [20]. Similarly, uridine had been identified as a urinary oxidative stress biomarker of mail fertility [21].

The kidney plays an important role in glutamate metabolism, and glutamate is present in high concentrations in renal tubular cells [23]. Glutamate receptors are found in the juxtaglomerular apparatus and proximal tubules in kidney, and are possibly involved in regulating the entry of glutamate to regulate cell volume in response to osmotic stress [24]. Studies in mice showed that lack of metabotropic glutamate receptor 1 function can be associated with albuminuria, podocyte foot process effacement, and reduced levels of nephrin and other proteins known to contribute to the maintenance of podocyte cell structure [25]. Glutamate plays a central role in the synthesis of alanine and glutamine, two most important nitrogen carriers that are released from the muscle. Significant reduction in the level of urinary glutamine in INS patients could result from impairment of glutamate metabolism leading to decreased net synthesis of glutamine. Similarly, lower glutamate levels were previously observed in serum of Immunoglobulin A nephropathy patients [22].

Alteration in urine phenylalanine levels in INS was partially expected, as previous studies showed that the urinary excretion of phenylalanine is reduced or even absent in end stage renal failure [26]. More specifically, in chronic kidney failure patients, there is impairment in the conversion of phenylalanine to tyrosine resulting in accumulation in plasma of metabolites of phenylalanine and tyrosine as a consequence of their impaired urinary excretion and presumably due to their accelerated metabolism *via* other biochemical pathways [27].

In conclusion, combined proteomics and metabolomics approach based on label-free mass spectrometry applied in the present study revealed for the first time the involvement of the glycoprotein afamin and several specific metabolites, namely hydroxyphenylacetate, uridine, glutamate and phenylalanine in pathogenesis of paediatric idiopathic nephrotic syndrome. Oxidative stress may be one of the pathogenic factors associated with dysregulation of neural control of renal function and tissue damage characterised by abnormalities in the podocytes and their structural changes or loss in the renal glomerulus.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.016.

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