

# Diagnostic Urinary Proteome Profile for Immunoglobulin A Nephropathy

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**Keywords.** IgA glomerulonephritis, urine, proteomics, mass spectrometry

**Introduction.** Immunoglobulin A (IgA) nephropathy, the most common type of glomerulonephritis, is only diagnosed by invasive kidney biopsy. Urine proteome panel might help in noninvasive diagnosis and also better understanding of pathogenesis of IgA nephropathy.

**Materials and Methods.** Second mid-stream urine samples of 13 patients with biopsy-proven IgA nephropathy and 8 healthy controls were investigated by means of nanoscale liquid chromatography tandem mass spectrometry. Multivariate analysis of quantified label-free proteins was performed by the principal component analysis and partial least squares models.

**Results.** A total number of 493 unique proteins were quantified by nanoscale liquid chromatography tandem mass spectrometry, of which 46 proteins were considered as putative biomarkers of IgA nephropathy, after multivariate analysis and additional filter criterion and comparing the patients and the controls. Some of the significant differentially expressed proteins were CD44, glycoprotein 2, vasorin, epidermal growth factor, CLM9, protocadherin, utreoglobin, dipeptidyl peptidase IV, NHL repeat-containing protein 3, and SLAM family member 5. These proteins were related to various involved pathogenic pathways of inflammatory response and complement system.

**Conclusions.** This proteome profile could be utilized in the diagnosis of IgA nephropathy. In addition, providing a noninvasive diagnostic tool, it may shed light on the pathogenesis of IgA nephropathy.

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## INTRODUCTION

As the most common primary glomerulonephritis worldwide, immunoglobulin A nephropathy (IgAN) is not as benign as it was thought.<sup>1</sup> Nearly, 20% of patients with IgAN would reach end-stage renal disease over 20 years of follow-up.<sup>2</sup> Renal biopsy is required for definite diagnosis of IgAN. Diagnosis of IgAN is based on histopathologic features, especially

mesangial deposition of IgA immune complexes with a spectrum of morphologic characteristics: from mesangial expansion and proliferation to crescent formation and sclerosis and fibrosis.<sup>3</sup> Several factors affect pathophysiology of IgAN such as complement factor C3, galactosyltransferase, and serum uric acid, the latter of which has been well reviewed by Nasri and colleagues.<sup>4</sup>

Four decades after the first description of IgAN by Berger,<sup>5</sup> there is ambiguity in the pathogenesis of the disease. Apart from the genetic predisposition, a wide range of immunologic processes (both innate and adaptive), cytokines, and growth factors are involved in pathogenesis of IgAN.<sup>6-8</sup> Complement systems,<sup>9</sup> Toll-like receptors,<sup>10,11</sup> and monocytes are the main players of innate immunity, while B cells, T cells (regulated by vitamin D3),<sup>12</sup> and immune complex formation are components of adaptive immune system known to be involved.<sup>13</sup>

Urine proteomic analysis suggests a noninvasive test to evaluate changes in multiple proteins expression simultaneously, which could replace renal biopsy in the diagnosis of IgAN. In addition to its use in diagnosis, the urine proteome panel might offer means to assess pathways involved in the pathogenesis of IgAN.<sup>14</sup> In this study, we applied proteomic technique, nanoscale liquid chromatography tandem mass spectrometry, on urine samples of patients with IgAN to identify novel biomarkers for diagnosis and shed light on the pathogenesis pathways.

## MATERIALS AND METHODS

### Patients and Samples

At Shahid Labbafinejad Medical Center, 13 patients (11 men and 2 women) aged between 18 and 52 years old (mean age, 33 years) with biopsy-proven IgAN and 8 healthy volunteers (6 men and 2 women) between 28 and 45 years old (mean age, 34.5 years) were consecutively enrolled in this study. Patients with signs of systemic involvement suggesting Schonlein-Henoch purpura, systemic lupus erythematosus, or chronic liver disease were excluded. Age, sex, and demographic data of the patients were noted and patients with concurrent diseases such as diabetes mellitus were excluded. None of the patients had gross hematuria at the time of sampling. At presentation, each patient was evaluated for serum creatinine level, estimated glomerular filtration rate (based on the CKD-EPI equation), presence of hypertension, and amount of proteinuria. All of the patients were from the same ethnicity.

### Sample Preparation and Liquid Chromatography Tandem Mass Spectrometry

Approximately 20 mL to 40 mL of a second morning midstream urine from patients was

collected. Urine proteome was extracted, digested, and prepared for liquid chromatography coupled with mass spectrometry according to Kalantari and colleagues' protocol.<sup>15</sup> Protein concentrations of samples were determined using the bicinchoninic acid protein assay (Pierce, Thermo Scientific, USA).<sup>16</sup>

Nanoscale liquid chromatography tandem mass spectrometry analyses were performed on an Easy-nLC system coupled online to a Q Exactive mass spectrometer (Thermo Scientific, Bremen, Germany). A 10-cm fused silica tip column (SilicaTip, New Objective Inc, Woburn MA, USA) in-house packed with Reprosil-Pur C18-AQ 3- $\mu$ m resin (Dr Maisch GmbH, Ammerbuch-Entringen, Germany) used for peptide separation. Mobile phases consisted of 0.1% formic acid in water v/v (buffer A) and 0.1% formic acid in acetonitrile v/v (buffer B). The liquid chromatography gradient was set up as follows: 5% to 35% buffer B in 89 minutes, 48% to 80% buffer B in 5 minutes, and 80% buffer B for 8 minutes, all at a flow rate of 300 nL/min. Approximately 2.3  $\mu$ g of total protein were injected via a temperature-controlled autosampler.

### Mass Spectrometry Data Analysis

The mass spectrometry acquisition method was comprised of 1 survey full scan ranging from m/z 300 to m/z 1650 acquired with a resolution of R = 70 000 at m/z 400, followed by data-dependent high-energy collision dissociation tandem mass spectrometry of maximum 10 most abundant precursor ions with a charge state  $\geq 2$ . The tandem mass spectrometry spectra were acquired with a resolution of R = 17 500.

Tandem mass spectra were extracted using Raw2MGF (in-house-written program) and searched against a concatenated SwissProt protein database (Human taxonomy) using Mascot 2.3.0 search engine (Matrix Science Ltd, London, UK). Carbamidomethylations of cysteins was set as a fixed modification, and deamidation of asparagine and glutamine as well as oxidation of methionine were set as variable modifications. Up to 2 missed tryptic cleavages were allowed and the mass tolerance was set to 10 ppm and to 0.05 Da for the precursor and fragment ions, respectively.

Relative abundance of proteins identified with 2 or more unique peptides and a significance threshold of E less than 0.05 was determined

using Quanti (an in-house developed software package)<sup>17</sup> which performs accurate label-free peptide and protein quantification with correction for instrumental response fluctuations. The list of quantified proteins was further filtered to 1% false discovery rate, which corresponded to the protein Mascot score of 20.49.

### Statistical Analysis

The quantitative proteome data was subjected to multivariate statistical analysis using SIMCA (SIMCA-p 13.0, Umetrics, Umeå, Sweden). Unsupervised principal component analysis (PCA)<sup>18,19</sup> was performed without consideration of group information for observing the overview of the data structure, detecting the clusters of the data and recognizing the outliers if any. Partial Least Squares (PLS) analysis<sup>20</sup> was applied using SIMCA in order to introduce a predictive model for diagnosis of patients from normal cases based on obtained differential biomarkers by PCA.

### Protein Gene ontology Term Enrichment and Pathways Analysis

Gene ontology enrichment analysis as well as pathways analysis was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) open-source software tool,<sup>21</sup> for general characterization of properties of the proteins in the data set. The proteins were also matched against the database of well-known metabolic pathways (Kyoto Encyclopedia of Genes and Genomes; KEGG). The cutoff for the enrichment score was set to 1.3 and the redundant hits were excluded.

## RESULTS

### Patients

A cohort of 13 patients with biopsy-proven IgAN and 8 healthy controls were enrolled in the study. The mean estimated glomerular filtration rate level was 67.7 mL/min/1.73 m<sup>2</sup> and the mean 24-hour protein excretion was 3.01 g/d among the patients, with 10 having a urine protein excretion rate higher than 1 g/d. Clinical and laboratory data on patients are provided in Table 1.

### Principal Component Analysis

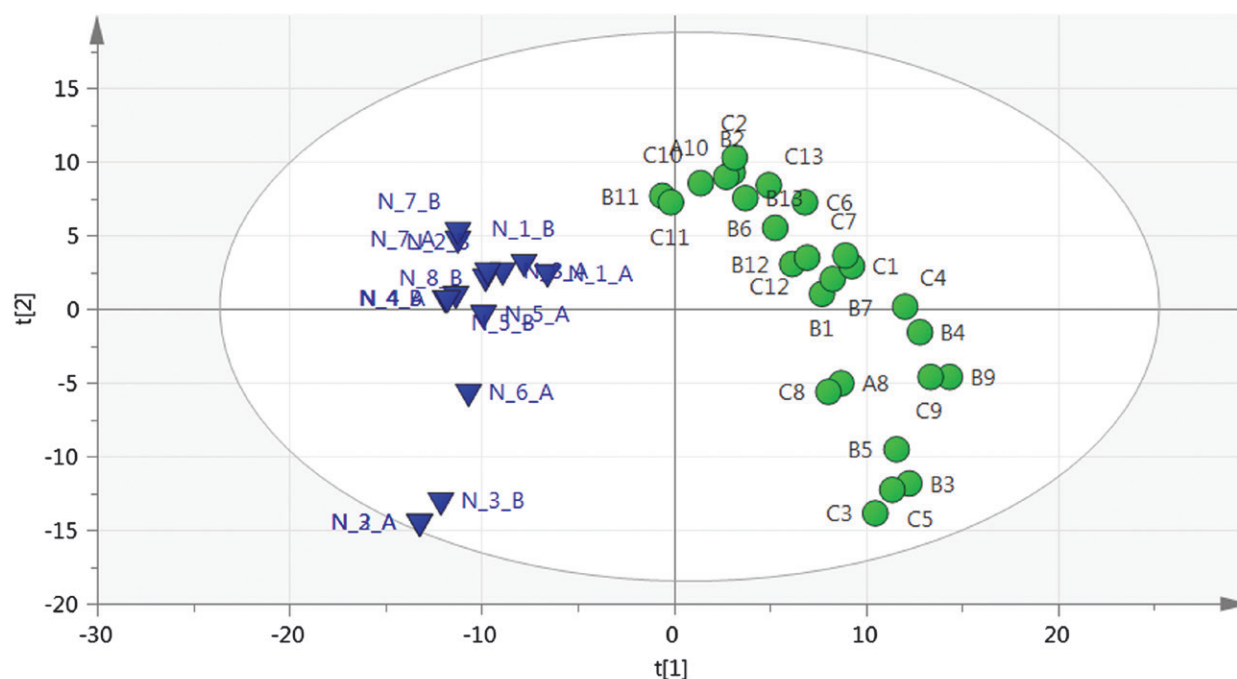
A total number of 493 unique proteins were identified and quantified by the nanoscale liquid chromatography tandem mass spectrometry. The proteins were considered as primary variables, while patients and controls (with 2 replicates for each one) were set as observations. Score plot of PCA indicated 2 clusters, which corresponded correctly with our clinical groups separated along the main principal coordinate, PC1 (Figure 1). All the replicates were close to each other and there were no statistical outliers. The confidence level of the model and significant level of Hotelling T<sup>2</sup> were considered 99% and 0.05, respectively. Of the 493 proteins used for model construction, 147 proteins had the most significant changes, and thus were recognized to be the most important markers responsible for the observed clustering (Supplementary Table 1).

### Predictive Model

Based on the significant proteins obtained from PCA analysis (147 proteins), a predictive model could be designed to distinguish patients and

**Table 1.** Demographic and Laboratory Characteristics of Patients With IgA Nephropathy

Patient	Age, y	Sex	Serum Creatinine, $\mu\text{mol/L}$	Proteinuria, g/d	Glomerular Filtration Rate, mL/min/1.73 m <sup>2</sup>
1	52	Male	154.7	4.6	44.10
2	18	Male	82.2	1.0	119.42
3	29	Male	680.6	6.0	8.58
4	42	Male	97.2	6.4	79.52
5	29	Male	442.0	7.0	46.60
6	28	Female	61.8	1.7	117.91
7	32	Male	158.2	4.1	49.04
8	28	Male	406.6	2.3	16.11
9	23	Female	106.0	0.8	63.65
10	34	Male	88.4	1.3	97.76
11	45	Male	119.3	0.7	68.01
12	34	Male	203.3	2.6	35.71
13	42	Male	45.0	0.5	133.51



**Figure 1.** Two-dimensional score plot of principal component analysis. Unsupervised analysis could cluster our clinical groups correctly. Black and open circles represent patients and normal samples, respectively.

healthy controls (Figure 2). This model was built using the PLS method. The plot of X and Y loading weights ( $w^*$  and  $c$ ) of PLS component 1 against component 2 demonstrated how the X-variables correlated with Y-variables, where X-variables were quantified proteins and Y-variables were differential proteins in the PCA models (Supplementary Table 1). Confidence level on parameters and significant level for Hotelling T2 were set as 99% and 0.05, respectively. Only proteins within this range considered as significant proteins in the model. To avoid overestimation, 7-fold cross-validated scores were calculated for PLS model and applied for calculating the accuracy of the model using receiver operating characteristic curve.<sup>22</sup> The accuracy of predictive model was obtained 97%.

By the PLS predictive model, only 147 proteins remained significant, of which some were different from the set of proteins resulted from PCA (Supplementary Table 2).

#### Differential Urinary Proteins in IgA Nephropathy

The 147 significant proteins resulted from the PLS model were subjected to further filtration based on fold change ( $\geq 1.5$  fold) in order to reach more reliable biomarker candidates. After applying the additional filter criterion, 46 proteins

remained (Figure 2), of which 13 proteins were upregulated (overrepresented; Supplementary Table 3) and 33 proteins were downregulated in IgAN (underrepresented; Supplementary Table 4).

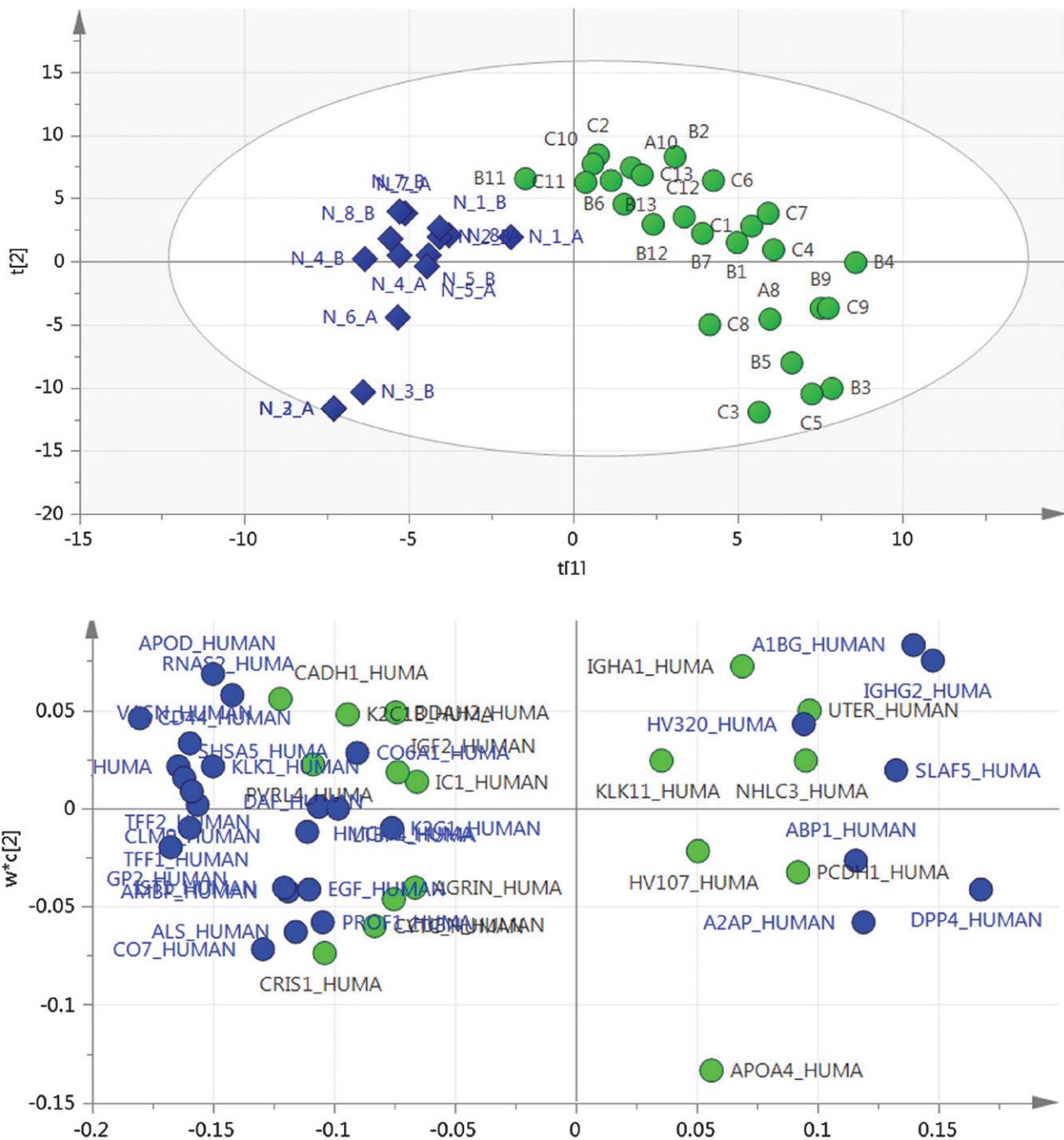
The 10 most significant differentially expressed proteins (upregulated or downregulated) are demonstrated in Table 2 as the valuable diagnostic biomarkers involved in pathogenesis of IgAN.

#### Gene Ontology Enrichment and Pathway Analysis

The DAVID gene ontology analysis yielded “acute inflammatory response” as the most significant biological process ( $P < .001$ ; Figure 3; see details in Supplementary Table 5). The only significant cellular compartment was “extra cellular space” ( $P < .001$ ), and no molecular function enriched in our dataset. Pathway analysis using the DAVID with the KEGG database showed a major pathway “complement and coagulation cascades” ( $P < .001$ ) encompassing decay accelerating factor, complement component C7,  $\alpha$ -2-antiplasmin, IC1.

#### DISCUSSION

Urine proteome might act as a fingerprint of kidney disease and could be used to diagnose the cause of renal injury. Apart from being a



**Figure 2.** Top, Two-dimensional score plot of predictive partial least squares model. The clusters of both patients and healthy controls are similar to score plot of principal component analysis. Black and open circles represent patients and normal samples respectively. Bottom, Loading plot of predictive partial least squares model corresponds to score plot. The significant differential proteins after filtration have been shown. The proteins cluster in right is important in patients and proteins cluster in left is important in controls.

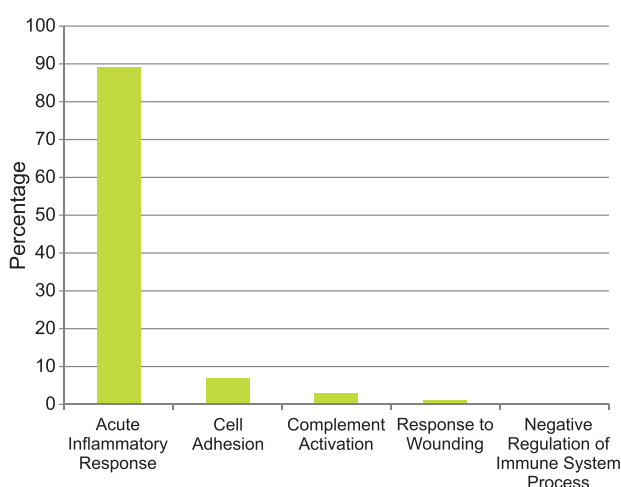
diagnostic tool, it might reveal the mechanism of kidney disease. We demonstrated a set of 46 proteins that could differentiate patients with IgAN from healthy individuals. These proteins participate in various biologic processes such as acute inflammatory response and complement and

coagulation cascades.

When compared with healthy individuals, CD44 had a 10.6-fold lower abundance in individuals with IgAN. CD44 is a transmembrane glycoprotein expressed on leukocytes, epithelial and endothelial cells and is involved in cell-cell and cell-matrix

**Table 2.** Differentially Expressed Urine Proteins in IgA Nephropathy and Healthy Individuals

Protein ID	Protein Name	Biological Process	Change	Change Magnitude
CD44	CD44 antigen	Positive regulation of peptidyl-serine, tyrosin phosphorylation, negative regulation of apoptotic process	Downregulation	10.6
APOD	Apolipoprotein D	Lipid metabolic process, response to reactive oxygen species	Downregulation	6.8
P3IP1	Phosphoinositide-3-kinase-interacting protein 1	Negative regulation of phosphatidylinositol 3-kinase cascade	Downregulation	3.9
GP2	Pancreatic secretory granule membrane major glycoprotein 2	Antigen transcytosis by M cells in mucosal-associated lymphoid tissue	Downregulation	3.7
VASN	Vasorin	Inhibitor of transforming growth factor-β signaling	Downregulation	3.7
KLK1	Kallikrein-1	Proteolysis	Downregulation	3.6
EGF	Epidermal growth factor	Innate immune response, positive regulation of cell proliferation	Downregulation	3.1
PVR	Poliovirus receptor	Cell-cell adhesion, susceptibility to T-cell mediated cytotoxicity, susceptibility to natural killer cell-mediated cytotoxicity	Downregulation	3.1
SHSA5	Protein shisa-5	Induction of apoptosis, positive regulation of I-kappaB kinase, NF-kappaB cascade	Downregulation	3.1
CLM9	CMRF35-like molecule 9	Immunity	Downregulation	2.9
PCDH1	Protocadherin-1	Cell-cell signaling	Upregulation	8.9
A1BG	Alpha-1B-glycoprotein	Immune system	Upregulation	5.6
UTER	Uteroglobin	Regulation of inflammatory response	Upregulation	3.5
DPP4	Dipeptidyl peptidase 4	Positive regulation of cell proliferation, T cell activation	Upregulation	3.5
IGHG2	Ig gamma-2 chain C region	Complement activation, classical pathway	Upregulation	2.9
SLAF5	SLAM family member 5 (CD84)	Blood coagulation, leukocyte migration	Upregulation	2.7
A2AP	Alpha-2-antiplasmin	Positive regulation of cell differentiation, positive regulation of ERK1 and ERK2 cascade	Upregulation	2.0
ABP1	Amiloride-sensitive amine oxidase	Amine metabolic process, response to drug	Upregulation	2.0
6PGL	6-Phosphogluconolactonase	Pentose-phosphate shunt, oxidative branch	Upregulation	1.9
NHLC3	NHL repeat-containing protein 3	Possibility of involvement in a variety of enzymatic processes, including protein modification through ubiquitination	Upregulation	1.9



**Figure 3.** Gene set enrichment analysis of biological Process by the Database for Annotation, Visualization, and Integrated Discovery (DAVID) using significant proteins obtained from the predictive partial least squares model.

interaction, and cascades of inflammation, fibrosis, and tissue repair.<sup>23,24</sup> Under normal condition, CD44 is not detected in renal tissue, but cytokine release following injury results in CD44 upregulation on tubular epithelial cells. The interaction of hyaluronic acid with CD44 makes tubular epithelial cells to secrete proinflammatory cytokines and leads to inflammation.<sup>24</sup> In addition, matrix metalloproteinase-9 binds to CD44 and cleaves and activates pro-transforming growth factor-β1, which results in tissue fibrosis. On the other hand, hepatocyte growth factor (a renoprotective growth factor) is presented to its receptor by CD44, thus CD44 also has prosurvival activity.<sup>25</sup> It seems that CD44 plays a pivotal role in the balance between transforming growth factor-β and hepatocyte growth factor signaling pathways. Increased expression of CD44 in basolateral surface of tubular epithelial cells was demonstrated by Florquin and

colleagues.<sup>24</sup> The decreased urinary expression of CD44 among IgAN patients in our dataset could be due to different specimens used for analysis (kidney tissue versus urine) or as Qiaoling and colleagues showed might be due to significantly lower CD44 expression in the more advanced pathologic stages.<sup>26</sup> In our previous study, there was a decreased expression of CD44 in those with more advanced IgAN.<sup>15</sup>

ApoD is a lipocalin with a role in lipid transport. Its exact function is yet to be defined. A wide range of ligands binds to ApoD such as arachidonic acid, progesterone, retinol, and cholesterol. Increased level of ApoD has also been reported in response to oxidative stress.<sup>27</sup> ApoD is expressed in kidney tissue and has been identified in tubular proteinuria.<sup>28</sup> In a study by Aregger and coworkers, ApoD was identified as a prognostic marker of recovery after acute kidney injury (upregulated in those with late or never recovered kidney function).<sup>29</sup> Vivekanandan-Giri and colleagues reported upregulated urinary levels of ApoD in patients with chronic kidney disease compared with healthy individuals.<sup>30</sup> However, there was a 6.8-fold decreased in urinary expression of ApoD among our patients.

One of the pathogenic hypotheses in development of IgAN is reduced transcytosis of mucosal polymeric IgA and increased serum level of polymeric IgA-antigen complexes. These complexes are then filtered and deposit in renal mesangium.<sup>31</sup> Abnormalities of mucosal immunity have been reported to play a role in pathogenesis of IgAN.<sup>32</sup> Pancreatic secretory granule membrane major glycoprotein is a molecule with a role in antigen transcytosis in mucosal associated lymphoid.<sup>33</sup> Its downregulation (3.7 fold) in urine sample of patients with IgAN in our dataset might be applicable as a diagnostic and probably pathogenic marker.

Vasorin is a member of Slit family of proteins. Its extracellular domain inhibits transforming growth factor- $\beta$  signaling and protects against apoptosis and fibrosis.<sup>34</sup> Moon and associates showed decreased level of urinary vasorin precursor as one of the possible biomarkers of IgAN when compared with healthy volunteers.<sup>35</sup> Our data also demonstrated the diminished urinary expression of vasorin (3.7-fold changes) among IgAN patients, which could serve as a biomarker to distinguish IgAN from healthy individuals.

Epidermal growth factor has been downregulated in urine sample of patients with IgAN with about 3-fold change. Epidermal growth factor is a trophic factor, produced by ascending part of Henle loop, necessary for growth modulation of tubular epithelial cells, and reduced urinary expression of epidermal growth factor has been shown to be a prognostic factor in kidney diseases with tubulointerstitial injuries.<sup>36</sup> Our data were in line with Ranieri and colleagues' work that showed not only diminished urinary epidermal growth factor excretion in IgAN patients compared with normal, but also the prognostic value of the decreased level of epidermal growth factor and higher urinary ratio of interleukin-6 to epidermal growth factor in more advanced disease.<sup>36</sup> In addition, Torres and colleagues found a low ratio of epidermal growth factor to monocyte chemoattractant protein-1 as an independent risk factor for adverse outcome in IgAN patients.<sup>37</sup>

Downregulation of poliovirus receptor (CD155) was observed in patients with IgAN (3.1 fold). CD155 is a transmembrane glycoprotein that is involved in cell-cell and cell-matrix adhesion. It also increases cell proliferation via platelet-derived growth factor and fibroblast growth factor.<sup>38</sup> Decreased expression of poliovirus receptor was demonstrated in patients with autosomal dominant polycystic kidney disease compared with healthy controls.<sup>39</sup> Thus, this decreased expression might be due to kidney damage and not an indicator of specific disorder.

Protocadherins are a family of nonclassical cadherins with different motifs and cytoplasmic domain. Protocadherin 1 has adhesive properties and plays a role in cell-cell signaling and slit junction. Protocadherin 1 has been shown to be expressed in glomerular endothelium and proximal tubule epithelium.<sup>40,41</sup> Studies showed that lack of protocadherins would lead to abnormalities in podocyte morphology and clinical presentations like lack of nephrin.<sup>41</sup> In our study, there was an 8.9-fold increase in urinary protocadherin 1, which has been reported for the first time in IgAN, to the best of our knowledge, and might be due to shedding of tight junction proteins during kidney injury.

Uteroglobin is a steroid-inducible cytokine-like protein with anti-inflammatory and immunomodulatory properties possibly via

inhibiting phospholipase A2 and inhibiting neutrophil chemotaxis.<sup>42,43</sup> Uteroglobulin has a high affinity to fibronectin and interferes with IgA-Fibronectin interaction. Studies have demonstrated evidence of IgAN in mice lacking uteroglobulin.<sup>42,44</sup> However, human studies on uteroglobulin gene polymorphisms in patients with IgAN and urine or blood concentrations of it showed no decrease in uteroglobulin level and even an increase in uteroglobulin expression.<sup>43,45,46</sup> As it is the case in our study (3.5-fold increase in urinary uteroglobulin), a meta-analysis by Yong and colleagues<sup>46</sup> showed no significant association between uteroglobulin polymorphism and development of IgAN, and its expression was an upregulated. Due to its anti-inflammatory properties, this increased expression might be a defense mechanism to decrease formation of IgA-fibronectin aggregates.

Dipeptidyl peptidase IV is a glycoprotein that is expressed in glomerular epithelial and endothelial cells and the brush border of proximal tubules, and on the T-cells. Apart from its role in cleavage of incretin hormones, dipeptidyl peptidase IV has a positive effect on T-cell activation and proliferation. The expression of dipeptidyl peptidase IV is increased in response to inflammation and also in diabetic nephropathy.<sup>46-48</sup> Mitic and associates demonstrated increased urine and serum dipeptidyl peptidase IV in patients with IgAN and suggested these factors as a diagnostic and maybe prognostic factors.<sup>49</sup> As it was the case in our study, a 3.5-fold upregulation in urinary expression of dipeptidyl peptidase IV might play an important role in pathogenesis of IgAN.

In addition to above discussed proteins, the expression of some of the remained proteins is upregulated due to kidney damage or shedding and is not disease-specific such as immunoglobulin  $\gamma$ -2 chain C region,  $\alpha$ -2-antiplasmin, and amiloride-sensitive amine oxidase.<sup>30,50</sup> Increased urinary expression of  $\alpha$ -2-antiplasmin has been shown in diabetic patients with macroalbuminuria and also in patients with acute poststreptococcal glomerulonephritis. Thus, the upregulation may mostly be the consequence of kidney injury than the cause.<sup>51,52</sup>

The most significant biological process obtained by gene ontology term analysis, which is implicated in pathogenesis of IgAN was "acute inflammatory response." Implication of this process in IgAN is

well established,<sup>53,54,55</sup> and our data confirmed this finding also on urine proteome level. It is suggested that activation of inflammatory response could be due to IgA-contained immune complexes (as a hallmark of IgAN) and it has a close relationship with complement system,<sup>56</sup> which also appeared in our pathway analysis.

According to our data, by using disease-related differentially expressed proteins, a diagnostic panel of urine proteome could be suggested; it seems that decreased urinary expression of glycoprotein 2, vasorin, epidermal growth factor, and CLM9, and increased urinary expression of protocadherin, uteroglobulin, dipeptidyl peptidase IV, NHL repeat-containing protein 3, and SLAM family member 5 could be used as urinary proteome profile of IgAN; however, further validation of these candidates and applying other confirmatory techniques would be necessary in larger cohort.

## CONCLUSIONS

A panel of urine proteomes rather than a specific biomarker could be utilized in diagnosis of IgAN (Table 2). If confirmed by larger studies, this panel may provide a diagnostic tool, which is much less invasive than kidney biopsy, and may shed light on the pathogenesis of IgAN.

## CONFLICT OF INTEREST

None declared.

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