Complement components, proteolysis-related, and cell communication-related proteins detected in urine proteomics are associated with IgA nephropathy

Krzysztof Mucha¹, Magdalena Bakun², Radosław Jaźwiec², Michał Dadlez², Michał Florczak¹, Małgorzata Bajor², Kamila Gala¹, Leszek Pączek¹

¹ Department of Immunology, Transplantology and Internal Medicine, Transplantation Institute, Medical University of Warsaw, Warsaw, Poland
² Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland
³ Department of Immunology, Centre of Biostructure Research, Medical University of Warsaw, Warsaw, Poland

INTRODUCTION IgA nephropathy (IgAN) is the most common primary glomerulonephritis. The first symptoms of IgAN are erythrocyturia or hematuria, proteinuria, and decline in renal function, or any combination of the above. One of the promising diagnostic methods is urine proteomics.

OBJECTIVES We studied urine proteomics in patients with IgAN and age- and sex-matched healthy controls. To minimize the risk of protein degradation, we proposed a new protocol for urine collection and preparation.

PATIENTS AND METHODS A total of 30 patients with IgAN and 30 controls were enrolled into the study. Thirty urine samples of the IgAN group were divided into 3 disease pooled samples (DPS I, II, and III) and 30 urine samples of the control group were divided into 3 control pooled samples (CPS I, II, and III). We used isoelectric focusing/liquid chromatography–mass spectrometry/mass spectrometry (IEF/LC-MS/MS) to detect all proteins larger than 10 kDa.

RESULTS Using qualitative analysis, we identified 761, 951, and 956 proteins in each of the 3 IEF/LC-MS/MS experiments. The results were combined, yielding a dataset with 1238 proteins identified by at least 2 peptides. The statistical analysis of the quantitative results revealed 18 proteins that were differently populated in the urine of IgAN patients compared with healthy controls. We found increased urinary concentrations of complement components, coagulation factors, extracellular matrix, intracellular, transmembrane, and other proteins in patients with IgAN. Some of them have never been linked to IgAN before.

CONCLUSIONS We demonstrated that urine proteomics is a promising tool for diagnosing and monitoring patients with IgAN.
could be a good indicator of treatment response are still being sought.

It was previously reported that the presence of urinary proteins is indicative of glomerular damage and interstitial fibrosis. Therefore, one of the most promising diagnostic tools is urine proteomics, particularly because the biological material can be obtained easily and comes directly from the diseased organ, the kidney. During the last decade, several valuable studies have linked proteomics to IgAN, and a number of urine proteins considered IgAN-specific have been reported. However, the quality of the final results was limited by differences in the methodology of urine collection, the number of patients and control subjects studied, and their proteinuria and GFR values. Those factors could considerably confound data interpretation in the previous studies.

The aim of our research was to study urine proteomics in patients with IgAN in comparison with age- and sex-matched healthy controls. Considering the challenges in methodology used in other studies, we proposed a new protocol for urine collection and preparation. The key aim was to limit the risk of protein degradation. First, all samples were collected from the second- or third-morning midstream urine, 1 to 3 h after previous urination. Second, as the presence of low-molecular-weight (LMW) proteinuria in IgAN indicated a less favorable outcome with higher incidence of chronic renal failure (47%) compared with those patients who did not have LMW proteinuria (10%); therefore, we also searched short peptides (≥2). To the best of our knowledge, this has been the first report of IgAN urine proteomics profiling performed according to this protocol. As a result, we have defined several new proteins that are unique for patients with IgAN.

**Patients and Methods**

**Groups of patients**

The study included 30 patients with IgAN and 30 healthy age- and sex-matched volunteers serving as controls. Demographic and clinical data of both groups are presented in Supplementary material online, **Table S1**. Patients with biopsy-proven IgAN at different stages of chronic kidney disease (CKD) and older than 18 years were included. The inclusion criteria for the control group were as follows: age older than 18 years and absence of any kidney disease or other chronic diseases requiring treatment. The exclusion criteria for both groups included: active infection, history of malignancy, previous organ transplantation, or current pregnancy. To estimate GFR, we used the Chronic Kidney Disease Epidemiology Collaboration equations, which are the most accurate, have been evaluated in large diverse populations, and are applicable for clinical use. The study protocol was approved by the local ethics committee and informed consent was obtained from all participants. The study was performed in accordance with the Declaration of Helsinki.

**Urine collection**

Samples were collected from all individuals according to a uniform study protocol, following the recommendations on urine proteomic sample collection. The second- or third-morning midstream urine was collected to sterile urines containers 1 to 3 h after previous urination. The pH of each sample was stabilized at 7.2 by addition of 1/10th vol. of 1M HEPES pH 7.2 immediately after collection. Then, samples were vortexed for 2 min, centrifuged at 3000 × g at room temperature for 10 min to clear the debris, filtered (0.4-µm filter, Rottlabo-Spritzenfilter, Roth, Karlsruhe, Germany), and portioned into 1-ml aliquots that were stored at −80°C before further use.

**Sample filtration**

Membrane filters of the 10 kDa cut-off (Amicon Ultra-0.5, UFC501 096, Millipore, Billerica, United States) were washed twice with MilliQ (MQ) water prior to use. Urine was centrifuged through the membrane at 14,000 × g for 15 min. Next, 500 µl MQ was added to the retentate and centrifugation step was repeated. To recover the concentrated and desalted sample, the filter was placed upside down and centrifuged in a clean microcentrifuge tube for 2 min at 1000 × g. The protein concentration was measured by the Bradford method. Aliquots of samples were stored at −80°C.

**Sample preparation, study design, and mass spectrometry settings**

The study design is illustrated in Supplementary material online, **Figure S1**. Sample preparation, pooling, labeling, and MS measurements were performed as described elsewhere. Shortly, 30 IgAN samples were divided into 3 disease pooled samples (DPSs I, II, and III), and similarly, 30 control samples were divided into 3 control pooled samples (CPSs I, II, and III). Age and sex matching was preserved within the 3 pairs of pooled sample groups. All DPSs and CPSs were obtained in 2 technical replicates (marked A and B) each, making a set of 12 pooled samples to be compared after isobaric tags for relative and absolute quantitation (iTRAQ) labeling. As 4-plex iTRAQ was used, 2 technical replications of DPSs and CPSs were compared in 1 isoelectric focusing/liquid chromatography–mass spectrometry/mass spectrometry (IEF/LC-MS/MS) experiment. To analyze 12 samples, we conducted a set of 3 independent IEF/LC-MS/MS experiments. Aliquots with extracted peptides were stored at −80°C for the IEF/LC-MS/MS analysis.

**Mass spectrometry: qualitative MS/MS data processing**

The MS/MS data were pre-processed with Mascot Distiller (v. 2.3.2.0, Matrix Science, London, United Kingdom). Data search using the MASCOT search engine was conducted on the Swiss-Prot database with the taxonomy restricted to Homo sapiens (20,236 sequences) in a 3-step procedure described elsewhere to calculate MS and MS/MS measurement errors and to recalibrate the data for the repeated MASCOT.
The differences in protein levels (protein ratio) can be substantial; in some cases, the levels may be even 5-fold higher. Protein ratios were calculated as the median ratio of their peptide’s ratios. The statistical significance of a single protein ratio was assessed by an in-house program, Diffprot. Calculated P values were adjusted for multiple testing using a false discovery rate-controlling procedure, yielding protein ratio q values.

**Protein network analysis**  The proteins identified in our study were searched in the STRING database version 9.1 for protein–protein interactions.

**RESULTS**  Data on kidney function in both groups are presented in Supplementary material online, **TABLE S1**. Of 30 patients with IgAN, 15 were treated with angiotensin-converting enzyme inhibitors and 19 with immunosuppression, mainly oral steroids. The number of patients at different stages of CKD were as follows: 10 patients in stage 1, 6 in stage 2, 10 in stage 3, and 4 in stage 4.

A set of 30 IgAN samples was divided into 3 subsets, containing 10 samples each, which were then pooled into 3 DPSs: I, II, and III. Similarly, control set was divided into 3 CPSs: I, II, and III, retaining age and sex matching within the subsets. In addition, 2 technical replicates of each DPS or CPS were prepared, further denoted A or B to assess the intragroup technical variability.

As a result of qualitative analysis (peptide and protein identification) in each of the 3 IEF/LC-MS-MS/MS experiments, 761, 951, and 956 proteins were identified, respectively, each represented by 2 or more peptides (Supplementary material online, **TABLE S2**). One-peptide hits were not taken into account in subsequent quantitative analysis.

Qualitative results (protein lists) from 3 IEF/LC-MS-MS/MS experiments were combined, resulting in a dataset with all 1238 proteins identified by at least 2 peptides. Within this dataset, identified proteins were again grouped based on identical peptide sets and each group was treated as a single protein cluster in further processing. After quantitative analysis, the final combined protein list accepted for quantitation contained 1176 proteins. However, 627 of these proteins were common for all replicates of the experiment.

The statistical analysis of the quantitative results of the 3 IEF/LC-MS-MS experiments revealed 18 proteins that were differently populated (with P < 0.05) in the urine of patients with IgAN as compared with healthy controls. The differential protein list is presented in Supplementary material online, **TABLE S3**.

The differences in protein levels (protein ratio) can be substantial; in some cases, the levels may be even 5-fold higher. Among DPSs, 2 were downregulated and 16 were upregulated in patients with IgAN.

All proteins identified in our study were searched in the STRING database, which defines a metric called “confidence score” to describe interaction confidence. We fetched all interactions for our dataset, which had a medium confidence.

**FIGURE 1**  Results of qualitative analysis: a Venn diagram representing the number of proteins identified by 2 or more peptides in 3 biological replicates of the isobaric tags for relative and absolute quantitation experiment (3 isoelectric focusing gel strips); 627 proteins are common in all 3 experiments. Abbreviations: IgAN – IgA nephropathy.
score of 0.4 and higher. The resulting interactome had 13 nodes and 34 interactions (FIGURE 2).

**DISCUSSION**  The aim of the study was to evaluate urine proteomics of patients with IgAN. For the purpose of this experiment, patients with stable renal function, assessed by the serum creatinine concentration, GFR level, and daily proteinuria excretion, were selected and compared with healthy volunteers. We found specific proteins that were identified only in the IgAN cohort as compared with the control group. What differentiates our study from others is that we investigated also short proteins (≥2 peptides). Sometimes they are estimated to be produced in large amounts by proteolysis of large proteins. Therefore, their examination could be a novel way to establish IgAN biomarkers. The analysis of short peptides in the serum from patients with IgAN were previously reported. For decades, nephrologists have searched for diagnostic methods that would be repetitive, sufficiently sensitive, safe (especially compared with renal biopsy), and that would enable continuous monitoring and stratifying risk for progression of the disease, particularly in the ambulatory care. It would seem that nephrologists are in the privileged situation because the primary biological material that we usually assess—the urine—comes almost directly from the affected organ. Therefore, its skilful evaluation should allow us to establish a prompt and accurate diagnosis. Consequently, many people believe that proteomic studies of the urine have great potential to be used both in the diagnosis and evaluation of kidney disease activity, especially of glomerulonephritis.

We found increased urinary concentrations of complement components, coagulation factors, extracellular matrix, intracellular, transmembrane, and other proteins. Some of them have never been linked to IgAN before. Additionally, the results were consistent with urine proteomics of patients with renal failure in the course of autosomal dominant polycystic kidney disease (ADPKD), described elsewhere. Considering the pathophysiology of this disease, this is an important assessment because urinary proteins in ADPKD differ from those in glomerulopathies.

There is evidence that the complement system plays a critical role in the pathogenesis of IgAN. It is known that C3 glomerular deposition is detected in more than 90% of patients with IgAN, which indirectly proves that complement activation is involved in glomerular damage in IgAN. Moreover, dimeric and polymeric IgA have been found to activate the complement system in the glomeruli via alternative or lectin pathways, thus leading to glomerular damage. On the other hand, mesangial C3 deposition is an independent risk factor for IgAN progression. In any event, the activation of C3 and C3 convertase production is the key cause of histological damage induced following the formation of the membrane attack complex (C5b-9). Therefore, it would be important for the future studies of urine proteomics in IgAN to distinguish extrafrom intraglomerular C3 component. Also, an increased serum level of complement components for, e.g., factor H, properdin, and regulatory proteins, has been reported in IgAN. The concentrations of activated C3 were elevated in 30% of the patients with IgAN and correlated positively with hematuria.

One can argue that the administration of angiotensin-converting enzyme inhibitor, angiotensin II receptor antagonists, antplatelet drugs, or...
steroids, all frequently used by IgAN patients including those in our study, may affect the complement system. However, it was reported that none of the above affected the serum concentration of either fibrinogen or complement C3 fragments. Given the above, it seems that the simultaneous assessment of certain complement components, both in urine and serum (rather than in kidney biopsy), might be useful in the evaluation of IgAN. Moreover, 8 of 18 urinary proteins (47%) that were increased in our study are involved in complement activation and hemostasis, including α2-macroglobulin, ceruloplasmin, complement C3, complement C4A, haptoglobin, prothrombin, and antithrombin-III (ATIII). It is known that ATIII inhibits coagulation and inactivates the plasmin and complement system. All these facts link the complement activation pathways with platelet function and plasma coagulation factors. It appears that the stability of this system might be critical to the development and progression of IgAN.

In our opinion, urine proteomics might be an important tool, especially at the time of diagnosis and during exacerbations. In the first case, it may allow us to introduce an appropriate treatment, and in the second, it increases our chance of recognizing a relapse and prevent progression of IgAN. Currently, a practical approach in both situations involves estimation of daily proteinuria in combination with the serum creatinine concentration and the GFR change as a function of time. However, it is not surprising to see patients with daily proteinuria below 0.5 g but with high immune activity of the disease or advanced kidney fibrosis in renal histopathology. Therefore, a more sensitive methodology with a follow-up of IgAN patients is needed. This applies also to other glomerulopathies assessed by proteinuria.

Additionally, we found 6 other proteins linked to proteolysis and 4 to cell–cell communication. We demonstrated that the urine protein profile may be a useful tool to confirm IgAN in patients suspected of this glomerulonephritis. The limitation of our study was that we assessed proteins in the whole collected material. For this reason, we cannot indicate which protein might be a diagnostic marker of the disease activity and probably also of renal fibrosis due to IgAN. This resulted from the sensitivity of the methods, the fact that we looked for every protein with 2 peptides and more, as well as from limited financial resources. Further research is needed to address all those issues.

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TABLE S1  Demographic characteristics and renal function of the study and control groups

<table>
<thead>
<tr>
<th></th>
<th>IgAN group (n = 30)</th>
<th>Control group (n = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sex, male / female</td>
<td>15 / 15</td>
<td>15 / 15</td>
</tr>
<tr>
<td>age, y</td>
<td>39.8 (20–60)</td>
<td>39.3 (20–62)</td>
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<tr>
<td>body mass, kg</td>
<td>76.1 (46–135)</td>
<td>75.7 (45–105)</td>
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<tr>
<td>serum creatinine, μmol/l</td>
<td>94.5 (40.2–279.9)</td>
<td>120.1 (51.5–365.6)</td>
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<tr>
<td>GFR (CKD-EPI formula), ml/min</td>
<td>66.3 (17.5–127.6)</td>
<td>99.3 (66.6–132)</td>
</tr>
<tr>
<td>proteinuria, g/d</td>
<td>1.19 (0–3.9)</td>
<td>0</td>
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</tbody>
</table>

Data are presented as mean (range).

Abbreviations: CKD-EPI – Chronic Kidney Disease Epidemiology Collaboration, GFR – glomerular filtration rate, IgAN – IgA nephropathy

TABLE S2  Number of identified peptides and proteins in 3 replicates of the iTRAQ experiment on pooled samples

<table>
<thead>
<tr>
<th>Replicate (IEF gel strip)</th>
<th>Number of peptides (accepted PSMs)</th>
<th>Number of proteins (identified by ≥2 peptides)</th>
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<tr>
<td>1</td>
<td>6300</td>
<td>1468 (761)</td>
</tr>
<tr>
<td>2</td>
<td>7398</td>
<td>1726 (951)</td>
</tr>
<tr>
<td>3</td>
<td>7874</td>
<td>1811 (956)</td>
</tr>
<tr>
<td>Symbol of protein</td>
<td>Gene ID</td>
<td>Peptides</td>
</tr>
<tr>
<td>------------------</td>
<td>-----------</td>
<td>----------</td>
</tr>
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<td>AFM</td>
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<tr>
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<tr>
<td>C4A</td>
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<td>PSAP</td>
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<tr>
<td>SERPINA1</td>
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<tr>
<td>SERPINC1</td>
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<td>21</td>
</tr>
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ARTYKUŁ ORYGINALNY

Składowe dopełniacza, białka proteolityczne oraz uczestniczące w komunikacji między komórkami, stwierdzane w badaniu proteomicznym moczu są związane z nefropatią IgA

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1 Klínika Immunologie, Transplantologie i chorób Wewnętrznych, Instytut Transplantologie, Warszawski Uniwersytet Medyczny, Warszawa
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SŁOWA KLUCZOWE
afamina, fibronektyna, nefropatia IgA, proteomika, składowe dopełniacza

STRESZCZENIE

WPROWADZENIE
Nefropatia IgA (IgAN) jest najczęstszym kłębuszkowym zapaleniem nerek. Pierwszymi objawami IgAN są krwinkomocz lub krwiomocz, białkomocz, pogorszenie czynności nerek lub kombinacja powyższych objawów. Jedną z bardziej obiecujących metod diagnostycznych jest proteomika moczu.

CELE
Przeprowadzono badanie proteomiczne moczu pacjentów z IgAN oraz zdrowych ochotników dobranych pod względem płci i wieku. Aby zminimalizować ryzyko degradacji białek, zaproponowaliśmy nowy protokół zbierania i przygotowania moczu.

PACJENTI I METODY
W badaniu uczestniczyło 30 pacjentów z IgAN oraz 30 ochotników. 30 próbek pochodzących od pacjentów z IgAN oraz 30 próbek z grupy kontrolnej podzielono na 3 grupy (disease pooled samples – DPS I, II i III oraz control pooled samples – CPS I, II i III). Metodą zastosowaną do wykrycia białek większych niż 10 kDa była spektrometria mas sprzężona z chromatografią cieczową poprzedzona ogniskowaniem izoelektrycznym (IEF/LC-MS/MS).

WYNIKI
Na podstawie analizy jakościowej, w każdym z 3 eksperymentów IEF/LC-MS/MS zidentyfikowaliśmy odpowiednio 761, 951 i 956 białek. Połączenie tych wyników dało listę 1238 białek zidentyfikowanych przez co najmniej 2 peptydy. Analiza statystyczna wyników eksperymentów ilościowych wykazała 18 specyficznych dla IgAN białek, w porównaniu do grupy kontrolnej. W grupie pacjentów z IgAN stwierdziliśmy zwiększone stężenia w moczu składników dopełniacza, czynników krzepnięcia, macierzy pozakomórkowej, białek wewnątrzkomórkowych oraz przezbłonowych. Niektóre z nich nigdy wcześniej nie były opisane w moczu chorych na IgAN.

WYNIKI
Wykazaliśmy, że badanie proteomiczne moczu może być dobrym narzędziem do diagnostyki i monitorowania pacjentów z nefropatią IgA.

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