Proteomic analysis of urine from proteinuric patients shows a proteolitic activity directed against albumin

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Abstract

Background. Nephrotic syndrome is a condition that is clinically associated with poor outcome. In this study, we compared different techniques of urine sample preparation in order to develop a robust analytical protocol to define the differential urinary proteome of urinary abnormalities compared to nephrotic proteinuria.

Methods. We recruited 5 normal control subjects, 16 patients with urinary abnormalities and 16 patients with nephrotic syndrome. Proteins from normal urine were processed using three different protocols [acetone, ultrafiltration and trichloroacetic acid (TCA) precipitation], depletion of albumin and IgGs and then analysed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) gels and mass spectrometry.

Results. Comparing the three extraction methods by visual inspection of gels after 2D gel electrophoresis, the acetone precipitation and TCA methods yielded the best quality of protein extraction, while the acetone precipitation method was the most efficient. Furthermore, we tested three commercial kits for albumin and IgG depletion. We applied the optimized acetone extraction protocol to compare urinary samples from nephrotic patients (NP) to urinary samples obtained from patients presenting with urinary abnormalities (UAP). We observed a proteolytic activity directed against albumin. This observation was more prevalent in urinary samples from UAP than from NP. Within both groups, there was some inter-individual variability in the observed proteolytic activity. An increased concentration of alpha1 antitrypsin was also observed in urine of NP. We analysed albumin fragmentation by 1D and 2D western blots in the same samples skipping the albumin and IgG depletion steps to avoid the possible confound of albumin fragment removal. The analysis confirmed a stronger proteolytic activity in the nephrotic group.

Conclusions. The proteolytic activity against albumin and the anti-proteolytic activity of alpha1 antitrypsin are likely linked and could play an important role in the nephrotic process. If replicated in larger samples, this methodology may lead to a better understanding of the underlying pathophysiological process of nephrotic syndrome.

Keywords: albumin; kidney; nephrotic syndrome; proteomic; urine

Introduction

Nephrotic syndrome is a condition that is clinically associated with poor renal outcome and with severe extrarenal comorbidity such as coronary artery disease [1], susceptibility to infections [2] and thrombotic events [3]. Mild proteinuria and nephrotic proteinuria are not simply quantitative variations of the same process; rather they likely represent specific phenotypes due to different underlying conditions that produce variable clinical effects. Glomerular diseases presenting with nephrotic syndrome show characteristic podocyte and slit diaphragm alterations that are responsible for the massive proteinuria [4–6]. The slit diaphragm is a complex structure where many different proteins interact. Molecular alterations of the renal filtration structure may vary among patients with glomerular disease and can be used to develop new disease classifications. Over the next decade, these data may lead to improved disease nosology and prediction of which patients will respond to immunosuppressive therapies [7]. Based on this premise, we speculate that molecular alterations affecting the slit diaphragm, and, consequently the protein filtration barrier, may be represented by qualitative modifications of proteinuria among patients with different underlying conditions.
This study compares the urinary proteome of patients affected with urinary abnormalities (UAP) (not nephrotic proteinuria) or nephrotic syndrome to investigate the differential proteomic profile that may be involved in the proteomic process. Our hypothesis is that there will be differential proteomic expression between the two groups that may help to elucidate the underlying pathogenic causes of nephrotic proteinuria. The development of novel and specific biomarkers is pivotal in improving the clinical management of patients. The ease with which urine can be obtained makes it an ideal candidate sample to search for biomarkers [8]. Many different approaches and techniques have been explored for the expression profile of proteins in biological fluids, each one having their advantages and drawbacks [9–12]. In this study, we compared different techniques for urine sample preparation in order to develop a robust analytical protocol for urinary protein profiling by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry. We then applied our optimized protocol to analyse the differential protein expression in urine from patients presenting with nephrotic syndrome to that from patients presenting with urinary abnormalities.

Materials and methods

Patients

The study was approved by the local Research Ethics Boards. All subjects provided written informed consent to participate in accordance with the Declaration of Helsinki. We recruited five normal control subjects who did not participate in accordance with the Declaration of Helsinki. All subjects provided written informed consent to participate in accordance with the Declaration of Helsinki. The study was approved by the local Research Ethics Boards. All subjects provided written informed consent to participate in accordance with the Declaration of Helsinki. We selected 16 patients with nephrotic syndrome defined as proteinuria >3.5 g/24 h and albuminuria <2.5 g/dL. We excluded patients showing a reduction of glomerular filtration rate >30% in the last 2 weeks prior to urine collection.

Human urine sample preparation

About 30–50 mL of clean catch, first morning urine was collected in a sterile tube followed by the immediate addition of 1 mL of protease inhibitor cocktail (Sigma-Aldrich, St.Louis, MO, USA) to every 10 mL of urine. To remove insoluble materials and cellular debris, the samples were filtered through 3 MM Whatman filter paper and centrifuged at 2000 g, 4°C for 10 min. The supernatant was filtered through a syringe filter (0.45 μM pore size). The samples were stored at −80°C until analysed.

Extraction methods

(a) Acetone precipitation: an aliquot of urine (15 mL) was added to two volumes of ice-cold acetone (30 mL) and kept overnight at −20°C. The sample was centrifuged at 12 000 g, 4°C for 10 min. The supernatant was removed and the pellet was air-dried. The pellet was then re-suspended in 1 mL of sample buffer (buffer R: 7 M urea, 2 M thiourea, 2% CHAPS, 50 mM DTT, 0.2% ampholytes).

(b) Ultrafiltration: an aliquot of urine (15 mL) was placed into an Ultrafree-15 device (Millipore Corporation, Bedford, MA, USA) and centrifuged at 2000 g, 4°C until the volume of the concentrate reached ~500 μL.

(c) Trichloracetic Acid (TCA) precipitation: an aliquot of urine (15 mL) was added to 1.5 mL of 100% TCA and incubated overnight at 4°C. The mixture was centrifuged at 10 000 g, 4°C for 30 min. The supernatant was removed and the pellet washed with 90% ice-cold acetone. The sample was incubated for 15 min on ice and centrifuged as above for 15 min. The supernatant was removed and the pellet air-dried, and then re-suspended in buffer R as above.

Removal of albumin and IgGs

The protein concentrate was treated to remove albumin and/or IgG. Three different kits were compared: Proteoprep Blue Albumin Depletion kit (Sigma-Aldrich, St.Louis, MO, USA), Proteoprep Immunoaffinity Albumin and IgG Depletion kit (Sigma-Aldrich, St.Louis, MO, USA), and Aurum Affi-Gel Blue Mini kits (Bio-Rad Laboratories, Hercules, CA, USA). In each experiment, 4–6 mg of protein was used as a starting material.

Samples used for the monodimensional (1D) and two-dimensional (2D) western blot analyses of albumin did not undergo processing for removal of albumin and IgGs.

Protein concentration assay

The protein concentration was assessed with Bradford’s methods [13] and with Bradford Reagent (Sigma-Aldrich, St.Louis, MO, USA), employing BSA as a standard and stored at −80°C until analysed.

Two-dimensional polyacrylamide gel electrophoresis

Approximately, 150 μg of total protein, dissolved in buffer R with a drop of bromophenol blue, was loaded onto IPG 17 cm, pH 3–10, immobilized pH gradient (IPG) gel strips (Bio-Rad Laboratories, Hercules, CA, USA). The IPG strips were then passively rehydrated for 16 h, using protein isoelectric focusing (IEF) cell (Bio-Rad Laboratories, Hercules, CA, USA). The IEF condition was 300 V for 30 min followed by a step to 2000 V for 60 min, followed by linear ramping to 4000 V for 60 min and finally isoelectric focusing at 4000 V up to 15000 V/h. After IEF, the strips were equilibrated in the equilibration buffer I (6 M urea, 0.375 M Tris-HCl pH 8.8, 2% SDS, 20% glycerol, 2% DTT) for 10 min followed by the equilibration buffer II.
for 30 min, then washed twice for 15 min and stored in 1%
blocked by washing with 4% Tris-HCl and 2% acetic acid
sodium carbonate, 0.03% formalin and 0.2% sodium thio-
washed for 1 min. The gels were developed with 3% potas-
staining in the dark at room temperature for 1.5 h, then
10 min each. Pre-chilled 0.2% silver nitrate was used for
0.5 M potassium acetate, 30% ethanol and washed six times,
tings were done with ultrapure water. Gels were fixed in
incubated overnight at 37°C. Peptides were subsequently
mass spectrometry.

Silver stain
The gels were stained using the silver protocol. All wash-
ings were done with ultrapure water. Gels were fixed in
40% ethanol, 10% acetic acid for 2 h and washed for 20 min
twice with 40% ethanol. On the second day, the gels were
incubated for 45 min with 0.3% potassium tetrathionate,
twice with 40% ethanol. On the second day, the gels were
fixed in 10% SDS-PAGE gel, and electrophoresis was per-
dered using a Protean II xi electrophoresis cell (Bio-
Rad Laboratories, Hercules, CA, USA). The 2D-PAGE gels
were run at 50 mA and were stained with silver stain for
mass spectrometry.

Image analysis
The quality of the gels was defined by visual inspection
considering sharpness of spots and the absence of stripes.
Images of the stained gels were acquired using an EP-
SON Scanjet-5100 scanner, and imported into the image
analysis software programme, PDQuest Ver 7.3.1 (Bio-
Rad Laboratories, Hercules, CA, USA). Spots selected by an
intensity cut-off were detected and automatically counted by
spot recognition algorithms. These were then manually con-
firmed by inspection. The original image was normalized
according to the total density of the recognized spots, and
3D Gaussian spots were calculated. A matched standard
gel, obtained by merging the Gaussian images, was gen-
ervated. The protein spots from each set of gels were first
qualitatively described and then semiquantitatively anal-
yzed for statistical comparisons. To reflect the quantitative
variations between gel images, one gel was selected as the
reference gel and the amount of a protein spot of each
matched gel was calculated. Statistical analysis of the data
was performed using SPSS software (SPSS, Chicago, IL,
USA). All experimental results were compared by one-way
analysis of variance (ANOVA), and the data were expressed
as ratio between means. Group means were considered to
be significantly different at \( P \leq 0.05 \).

Liquid chromatography coupled to tandem mass
spectrometry (LC-MS/MS) peptide analysis
Protein spots were excised from the 2D-PAGE, washed
twice with ultrapure water, and then silver stain was re-
moved with 30 mM potassium ferricyanide/100 mM Na-
thiosulfate in the dark. For reduction and alkylation, the
spots were washed twice with 5 mM DTT/25 mM ammo-
nium bicarbonate, 50% acetonitrile and 1A and dried by
the vacuum concentration system (Eppendorf Concentrator
5301). Approximately, 5–20 \( \mu L \) of trypsin buffer (12.5 ng/
\( \mu L \) trypsin proteomic grade, Sigma-Aldrich, St.Louis, MO,
USA) was added to the dried residue and the samples were
incubated overnight at 37°C. Peptides were subsequently
extracted with 50% acetonitrile/5% formic acid and dried
again with a concentrator.

The digested and reduced proteins were injected into
a CapLC high pressure liquid chromatograph (HPLC, Waters and Micromass, Milford, MA, USA). The pep-
tides resolved by chromatographic analysis were analysed
by MS/MS analysis (Q-TOF Ultima Mass Spectrometer fitted with an Electrospray Ionization source, Waters and Micromass, Milford, MA, USA). Data and spec-
tra interpretation algorithms were managed by the software
ProteinLynx Global Server version 2.0 (Waters and Micromass, Milford, MA, USA).

The Mascot MS/MS programme (http://www.
matrixscience.com) was used for peptide sequence recogni-
tion. Search parameters allowed for carboxyamidomethylation of cysteine and one missed trypsin cleavage. Protein
identification was repeated at least once using spots from
different gels.

1D and 2D western blotting of albumin
1D western blot of acetone-precipitated samples was car-
ried out. In brief, 0.5 \( \mu g \) of each sample was loaded onto
a 7.5% SDS-PAGE gel. The gel was run at 38mA, and the
proteins electro-blotted on a nitrocellulose membrane for
2 h at 500 mA.

For 2D western blot analysis, the gel electrophoresis has
been carried out according to the same protocol previously
described for the 2D-PAGE. Subsequently, the blotting and
staining procedures are the same for both 1D and 2D west-
ern analyses as described below.

The membrane was blocked overnight in a blocking so-
lution (3% BSA and 0.15% Tween in 1 \( \times \) TBS), and then
incubated for 2 h with the primary antibody (polyclonal
rabbit anti-HSA, DakoCytomation, Glostrup, Denmark) di-
luted 1:40000 in the blocking solution. After washing the
membrane with 0.15% Tween in 1 \( \times \) TBS four times, 15 min
each, it was incubated with the secondary antibody (goat
anti-rabbit IgG, Santa Cruz Biotechnologies, Santa Cruz,
CA, USA) conjugated to horseradish peroxidase diluted 1:40000 in 0.15% Tween in 1 \( \times \) TBS for 2 h. Four further
cycles of 0.15% Tween in 1 \( \times \) TBS washing, 15 min each,
were applied. The signal was detected by the ECL Western
blotting Detection System (GE Healthcare, Little Chalfont,
England).

Alpha1-antitrypsin quantitative determination
Alpha1-antitrypsin (\( \alpha1 \)-AT) levels were determined from
human urine by immunonephelometry using specific anti-
tera (N antiserum to human alpha 1-antitrypsin, Siemens AG
Corporation, Munich, Germany). The assay was performed
on a BN™ System (Siemens AG Corporation, Munich,
Germany).
Table 1. Demographic and clinical characteristics of the two groups of patients

<table>
<thead>
<tr>
<th></th>
<th>Urinary abnormalities (16 patients)</th>
<th>Nephrotic syndrome (16 patients)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M:F</td>
<td>1.4:1</td>
<td>1.3:1</td>
</tr>
<tr>
<td>Age (years)</td>
<td>45 ± 23</td>
<td>51 ± 16</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>1.63 ± 1.13</td>
<td>1.15 ± 0.35</td>
</tr>
<tr>
<td>Azotaemia (mg/dL)</td>
<td>68 ± 62</td>
<td>43 ± 16</td>
</tr>
<tr>
<td>Proteinuria/24 h (g)</td>
<td>0.7 ± 0.5</td>
<td>7.1 ± 0.5</td>
</tr>
<tr>
<td>Albuminaemia (g/dL)</td>
<td>3.74 ± 0.45</td>
<td>2.13 ± 0.26</td>
</tr>
<tr>
<td>Systolic PA (mmHg)</td>
<td>124 ± 11</td>
<td>130 ± 18</td>
</tr>
<tr>
<td>Diastolic PA (mmHg)</td>
<td>76 ± 8</td>
<td>76 ± 9</td>
</tr>
<tr>
<td>Therapy (St/Cyt/ACEi)</td>
<td>14/12/11</td>
<td>14/14/14</td>
</tr>
</tbody>
</table>

Data are expressed as mean value ± SD and in the second line median value (25°–75° percentile) with the exception of sex that is expressed as a proportion and therapy as cumulative number of patients that assumed the specific treatment.

Table 2. Protein extraction strategies: acetone, ultrafiltration, TCA

<table>
<thead>
<tr>
<th>Method</th>
<th>Efficiency</th>
<th>Number of features</th>
<th>Quality of spots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>99%</td>
<td>228</td>
<td>Good</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>79%</td>
<td>170</td>
<td>Low</td>
</tr>
<tr>
<td>TCA</td>
<td>17%</td>
<td>199</td>
<td>Good</td>
</tr>
</tbody>
</table>

TCA: trichloroacetic acid.

We considered efficiency, numbers of features and quality of spots to evaluate the overall performance of the extraction method.

Results

Characteristics of the patients

Demographical and clinical data of patients are presented in Table 1. The underlying diagnoses of the nephrotic syndrome group were membranous glomerulonephritis (MGN) in 13 cases, MGN superimposed on diabetic nephropathy in 1 case, minimal change (MC) disease in 1 case and Alport’s syndrome in 1 case. The underlying diagnoses of the urinary abnormalities only group were IgA nephropathy (IgA-N) in seven cases, MGN in two cases, systemic lupus erythematosus (SLE) in two cases, Wegener’s granulomatosis in one case, chronic interstitial nephritis in one case, membranous proliferative glomerulonephritis (MPGN) in one case, myeloma kidney in one case and microscopic polyangiitis (micro-PAN) in one case (Table 5 online publication).

Patients with urinary abnormalities only present with a greater male:female ratio and are younger than nephrotic patients (NP). As expected, proteinuria is greater in NP, and milder in the urinary abnormalities only group. Creatinine is higher in the urinary abnormalities group.

Urinary protein extraction strategies

Using normal control subjects, we developed the best extraction protocol. We compared three different protein extraction strategies, as previously described, acetone, ultrafiltration and TCA [14]. We considered three parameters to evaluate the overall performance of the different extraction methods: efficiency, number of features and quality of spots; these parameters are summarized in Table 2. The efficiency of extraction was expressed as the ratio between protein concentration before and after the procedure. Using this definition, the highest efficiency procedure was acetone precipitation, followed by ultrafiltration, while TCA precipitation was relatively inefficient. For ultrafiltration, the efficiency was 79% while the quantity of protein lost in ultrafiltrate liquid was negligible (0.2%). We concluded that the missing proteins were probably adsorbed by the filter as previously reported [15].

Also, in normal subjects, we evaluated the number of spots obtained after 2D-PAGE. The extraction method that obtained the highest number of features is acetone precipitation (228 spots, Figure 1). The visual inspection of gels after 2D-PAGE suggested that the best quality was reached by the acetone and TCA methods. Figure 2 shows the number of spots for each extraction method. After spot matching across the three types of extraction methods, it is demonstrated that approximately all of the protein from the ultrafiltration extraction was present in the acetone- and TCA-extraction methods.

For further analysis, we used the acetone extraction method that, in our opinion, demonstrated the best overall...
Fig. 3. (A) 2D-PAGE silver stain gel from patients affected by nephrotic syndrome (pooled urinary samples). (B) From patients with urinary abnormalities (pooled urinary samples). The spots differentially expressed by the two groups of patients were extracted and processed by LC-MS/MS analysis.

performance, both in terms of efficiency and quality of spots.

**IgG and albumin depletion**

We evaluated three different commercial kits to reduce the amount of albumin and immunoglobulins in our samples. We applied these depletion steps to the urinary proteins previously extracted by the acetone method.

Based on the product insert, the Aurum Affi-Gel Blue Mini kits (Bio-Rad) are limited by their lack of ligand specificity. This was indeed confirmed in our analysis as the eluted fraction showed that not only albumin but also virtually any abundant spot in the original sample was bound and depleted by this method (Figure 7 online publication).

The Proteoprep Blue Albumin Depletion kit (Sigma-Aldrich, St. Louis, MO, USA) works on a similar principle with the difference being that the blue dye is coupled to protein G agarose. Although our analysis demonstrated increased efficiency of this method in immunoglobulin depletion, it also suffers from lack of specificity (Figure 7 online publication). The Proteoprep Immunoaffinity
Table 3. Spots obtained by the differential analysis of the two sets of gels (nephrotics versus urinary abnormalities)

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Experimental MW (kDa)/pI</th>
<th>Theoretical MW (kDa)/pI</th>
<th>Mowse score</th>
<th>Coverage</th>
<th>Accession no.</th>
<th>Protein name</th>
<th>Increased expression</th>
<th>Fold changes</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18/6.9</td>
<td>71.3/5.85</td>
<td>137</td>
<td>15%</td>
<td>P02768</td>
<td>Serum albumin</td>
<td>Nephrotic</td>
<td>31.82</td>
<td>0.021</td>
</tr>
<tr>
<td>2</td>
<td>18/7.1</td>
<td>71.3/5.85</td>
<td>149</td>
<td>19%</td>
<td>P02768</td>
<td>Serum albumin</td>
<td>Nephrotic</td>
<td>22.48</td>
<td>0.023</td>
</tr>
<tr>
<td>3</td>
<td>24/7</td>
<td>24.5/6.95</td>
<td>284</td>
<td>27%</td>
<td>CAA40949</td>
<td>Ig lambda light chain</td>
<td>Urinary abnormalities</td>
<td>0.31</td>
<td>0.032</td>
</tr>
<tr>
<td>4</td>
<td>48/4.9</td>
<td>52.5/3.7</td>
<td>751</td>
<td>38%</td>
<td>P01009</td>
<td>α1 antitrypsin</td>
<td>Nephrotic</td>
<td>69.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>5</td>
<td>58/6.5</td>
<td>71.3/5.85</td>
<td>470</td>
<td>27%</td>
<td>P02768</td>
<td>Serum albumin</td>
<td>Nephrotic</td>
<td>51.83</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>6</td>
<td>45/5.2</td>
<td>52/5.37</td>
<td>437</td>
<td>26%</td>
<td>P01009</td>
<td>α1 antitrypsin</td>
<td>Nephrotic</td>
<td>1.59</td>
<td>0.033</td>
</tr>
<tr>
<td>7</td>
<td>60/6.5</td>
<td>71.3/5.85</td>
<td>206</td>
<td>22%</td>
<td>P02768</td>
<td>Serum albumin</td>
<td>Nephrotic</td>
<td>1.46</td>
<td>0.047</td>
</tr>
<tr>
<td>8</td>
<td>47/5.5</td>
<td>71.3/5.85</td>
<td>65</td>
<td>7%</td>
<td>P02768</td>
<td>Serum albumin</td>
<td>Nephrotic</td>
<td>1.82</td>
<td>0.032</td>
</tr>
<tr>
<td>9</td>
<td>62/4.5</td>
<td>71.3/5.85</td>
<td>206</td>
<td>22%</td>
<td>P02768</td>
<td>Serum albumin</td>
<td>Nephrotic</td>
<td>64.07</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>10</td>
<td>62/4.5</td>
<td>71.3/5.85</td>
<td>65</td>
<td>7%</td>
<td>P02768</td>
<td>Serum albumin</td>
<td>Nephrotic</td>
<td>34.41</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>11</td>
<td>63/4.5</td>
<td>71.3/5.85</td>
<td>103</td>
<td>5%</td>
<td>P02768</td>
<td>Serum albumin</td>
<td>Nephrotic</td>
<td>35.78</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>12</td>
<td>57/4.5</td>
<td>71.3/5.85</td>
<td>38.4/6.45</td>
<td>63</td>
<td>8%</td>
<td>P02750</td>
<td>Leucine-rich α2-glycoprotein</td>
<td>Nephrotic</td>
<td>68.18</td>
</tr>
<tr>
<td>13</td>
<td>57/4.5</td>
<td>71.3/5.85</td>
<td>128</td>
<td>9%</td>
<td>P02768</td>
<td>Serum albumin</td>
<td>Nephrotic</td>
<td>57.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>14</td>
<td>57/4.5</td>
<td>71.3/5.85</td>
<td>38.4/6.45</td>
<td>66</td>
<td>8%</td>
<td>P02750</td>
<td>Leucine-rich α2-glycoprotein</td>
<td>Nephrotic</td>
<td>56.77</td>
</tr>
<tr>
<td>15</td>
<td>57/4.5</td>
<td>71.3/5.85</td>
<td>470</td>
<td>27%</td>
<td>P02768</td>
<td>Serum albumin</td>
<td>Nephrotic</td>
<td>58.48</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>16</td>
<td>57/6</td>
<td>71.3/5.85</td>
<td>211</td>
<td>15%</td>
<td>P02768</td>
<td>Serum albumin</td>
<td>Nephrotic</td>
<td>67.81</td>
<td>&lt;0.01</td>
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<tr>
<td>17</td>
<td>57/6.5</td>
<td>71.3/5.85</td>
<td>178</td>
<td>12%</td>
<td>P02768</td>
<td>Serum albumin</td>
<td>Nephrotic</td>
<td>67.81</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>18</td>
<td>60/6.5</td>
<td>71.3/5.85</td>
<td>115</td>
<td>11%</td>
<td>P02768</td>
<td>Serum albumin</td>
<td>Nephrotic</td>
<td>67.81</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>19</td>
<td>60/5.4</td>
<td>325/9.22</td>
<td>45</td>
<td>1%</td>
<td>Q53TG8</td>
<td>Hypothetical protein NEB</td>
<td>Nephrotic</td>
<td>47.67</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

The fold changes express the ratio nephrotic patients/urinary abnormalities patients.

Only spots with a significant P-value (P < 0.05) are listed.

Albumin and IgG Depletion kit (Sigma-Aldrich, St. Louis, MO, USA) is based on specific beaded small single-chain antibody ligands. Our analysis of the eluted fraction showed a specific binding of albumin and immunoglobulins, without binding of other not-specific proteins (Figure 7 online publication).

We considered this last kit as the most specific, and we used it in the subsequent analysis (with the exception of the 1D and 2D western blots of albumin that were performed skipping the albumin and IgG depletion steps).

Differential expression between patients with urinary abnormalities and NP

We compared the urine proteome of patients affected by urinary abnormalities with the urine proteome of patients of the nephrotic syndrome group. Urine proteins were extracted by acetone method and were depleted of albumin and immunoglobulins.

The analysis was performed using 2D-PAGE and pooled urinary samples of 12 patients from each of the comparison groups (Table 5 online publication for clinical details). The samples were mixed, and equal quantities of extracted proteins (150 µg total) were loaded onto gels (Figure 3). The analysis was repeated three times. The differential analysis of the two sets of gels (nephrotics versus urine abnormalities) showed 19 differentially expressed spots. These spots were excised and analysed by mass spectrometry. Three spots out of 19 were unidentifiable by mass spectrometry. The migrating features of all the spots, and the identifications of the remaining 16 spots are summarized in Table 3. Three of the spots (7, 13 and 14) obtained a double identification, one of which consisted of albumin. Twelve of the 16 spots were recognized as albumin fragments. The remaining proteins revealed by mass spectrometry analysis are Ig lambda light chain, hypothetical protein NEB, α1-AT, leucine-rich alpha2 glycoprotein. The last three recur each in two different spots. The Ig lambda light chain spot...
is more abundant in the urinary abnormalities only group, while all of the other spots are more highly expressed in the nephrotic syndrome group.

1D and 2D western blots of albumin fragments

We further characterized albumin fragmentation by 1D and 2D western blots. In this evaluation, we did not perform the albumin and IgG depletion steps. For the 1D western blot, we loaded individual samples from patients of the nephrotic group. We analysed 10 of the 16 NP (see Table 5 online publication for clinical details on the patients). The rate of fragmentation does not appear to be constant, that is, there are patients with low level (Figure 4: Patients b–f, h, l), intermediate levels (Patient a) and high level of albumin degradation (Patients g and i). There are no obvious
Table 4. Albumin fragments analysed from 2D-PAGE western blot obtained by the differential analysis of the two sets of pooled samples (nephrotics versus urinary abnormalities)

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>MW (kDa)</th>
<th>PI</th>
<th>Increased expression</th>
<th>Fold changes</th>
<th>P</th>
</tr>
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<tr>
<td>1</td>
<td>71.3</td>
<td>5.85</td>
<td>Nephrotic</td>
<td>23.12</td>
<td>NS</td>
</tr>
<tr>
<td>2</td>
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<td>6.09</td>
<td>Nephrotic</td>
<td>3.18</td>
<td>NS</td>
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<td>3</td>
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<td>5.86</td>
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<td>522.57</td>
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<td>4</td>
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<tr>
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<td>7</td>
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<td>6.31</td>
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<tr>
<td>8</td>
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<td>6.37</td>
<td>Urinary abnormalities</td>
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<td>NS</td>
</tr>
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<tr>
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<tr>
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</table>

Fig. 6. Levels of the α1-AT urinary creatinine ratio in the urinary abnormalities patients group and nephrotic syndrome patients group.

In an attempt to better characterize the nature of albumin fragmentation, we compared the protein fractions in the urinary abnormalities only group to those in the nephrotic syndrome group by 2D-PAGE western blot. We performed this analysis using the pooled urine as well as by individual patient analysis. The pooled analysis gave an average result, and we identified a variable pattern between the two groups (Figure 5A, B, E and Table 4). Furthermore, we applied this analysis to single patients homogeneous for histological diagnosis: four IgA-N subjects for the urinary abnormalities group and four MGN subjects for the nephrotic group. As previously described, using the 1D western blot, the 2D western blot demonstrated a large inter-individual variability in the rate and nature of fragmentation. This variability was present even among patients of the same diagnostic group (Figure 5C and D).

**Alpha1-antitripsin dosage**

Alpha 1-AT and some of its fragments were differentially present in the urine of patients analysed by 2D-PAGE. As such, we determined the concentration of this protein in the urine of each patient in both groups (16 patients with the nephrotic group and 16 from the urinary abnormalities group) employing an immunonephelometric method. Data are shown in Figure 6. This analysis confirmed a low concentration of α1-AT in the group with urine abnormalities only while the concentration in the nephrotic group was found to be significantly higher. Only two NP (one affected by MGN and the other by MGN superimposed to diabetic nephropathy) presented with levels <5 mg/dL.

**Discussion**

This study was divided into two main phases. The first phase involved the development of a robust analytical protocol for the proteomics of the urine by comparing several techniques of sample preparation. Based on our analysis, the acetone precipitation followed by albumin and immunoglobulin depletion based on a specific immunological method yielded the best result. In the second phase, we applied this protocol to compare the urine proteome of patients presenting with nephrotic syndrome to those presenting with mild urinary abnormalities only. We observed 19 protein spots that were differentially expressed. Sixteen of these spots obtained a database matching using mass spectrometry. All of the identified proteins are abundant serum proteins that reached the urine compartment at the glomerular level as a result of the reduced selectivity of the filtration barrier. Fifteen of these spots were present in our initial analysis in the NP and are either completely or nearly completely lacking in the urinary abnormalities group. Only one spot recognized as an Ig lambda light chain was more abundant in the urinary abnormalities group. This spot likely derives from the urine sample of the patient affected by myeloma kidney; this patient is affected by a light chain myeloma, and a free monoclonal lambda chain is present in his serum and urine. Most of the spots in the nephrotic group were recognized as fragments of albumin. It is noteworthy that intact albumin was present in the urine abnormalities group.
as well, but in the initial pooled analysis, fragmentation was not appreciable. This phenomenon suggested the presence of a proteolytic activity directed against albumin in the nephrotic group. However, this activity presumably could be directed even to other, less abundant protein targets. The protein responsible for this enzymatic activity was not identified among the differential spots in our experiments. This was expected given that a small amount of the presumed causative enzyme (or enzymes) can produce dramatic cleavage activity, while remaining occult in the so-called deep proteome. These low level but highly active proteins are not easily identified in a complex mixture such as urine samples even after the albumin and IgG depletion steps. Due to their presence, the removal of albumin was only partial in the nephrotic syndrome group while it was more efficient in the urine abnormalities only group. We cannot exclude the possibility that this phenomenon may have contributed to some of the divergence between the two groups, particularly in the presence of albumin fragments.

Our data suggest that on average, albumin fragmentation is more pronounced in the nephrotic group, but it is not a constant characteristic even within this group. By 1D western blot analysis, we concluded that there are different levels of albumin fragmentation and strong cleavage is actually present in a minority of patients (2 out of 10 in our cohort). This partially conflicts with the report by Giovanni Candi


cans et al. [16]. Indeed, they described a more homogeneous presence of fragmentation among the different nephrotic conditions. We could not find any strong association between rate of fragmentation and baseline clinical characteristics. However, because of our small sample size, we cannot definitively exclude this is a possible-confounding factor in our analysis. Furthermore, we analysed albumin fragmentation by two-dimensional western blot in the same pooled samples skipping the albumin and IgG depletion steps to avoid the possible confound of albumin fragment removal. The pooled analysis gave us an average result that confirmed a stronger proteolytic activity in the nephrotic group. However, albumin fragments analysed by the more sensitive technique of western blot without the confound of albumin removal are also present in the urinary abnormalities group but to a lesser extent. By image analysis, we have found that the migration pattern of the fragments in the urinary abnormalities group is significantly different compared to the nephrotic albumin fragments.

One of the most concerning issues in the urinary proteomics field is inter-individual variability. To overcome this limitation, we performed two-dimensional western blots in the urine extracts from single patients of two homogeneous groups: NP (four patients affected by MGN) versus patients with urinary abnormalities (four patients affected by IgA-N). Once again, this analysis confirmed a large inter-individual variability of the proteolytic activity among patients of both groups.

Whether this proteolytic phenomenon acts in plasma, in the kidney or in the urine collected in the bladder is not clear. An analogous proteolytic activity was attributed to a putative circulating factor responsible for the glomerular permeability alterations in focal segmental glomerulosclerosis (FSGS), a typical nephrotic condition [17]. Carraro et al. have observed that a serine protease isolated from the serum of FGS patients increases albumin permeability in isolated glomeruli. Furthermore, they demonstrated that only the serine protease inhibitors consistently blocked the activity of the permeability factor of the FSGS sera. In a previous report from the same group [18], 10 proteins showing permeability activity were purified from serum of patients affected by FSGS. Among these proteins, a serine protease, the mannan-binding lectin-associated serine protease, was identified. There is an interesting similarity between the findings of this report and our results. Our data also suggest that this proteolytic activity may be extended to a wide range of proteinuric conditions not strictly restricted to nephrotic proteinuria. In this scenario, a protease may be involved in the mechanisms of permeabilization of the glomerular barrier, and albumin fragmentation could be an epiphenomenon of its activity.

Alpha 1-AT found in three different spots is a well-known serine protease inhibitor. The abundant presence of α1-AT in the urine of NP may be associated with the hypothesized serine protease activity. α1-AT is a 52 kDa glycoprotein synthesized chiefly in the liver and to a lesser extent by macrophages and neutrophils. It is the main serine protease inhibitor for a broad range of proteases, and its main function is inhibition of neutrophil elastase. Alpha 1-AT is up-regulated during acute phase responses. Deficiency of this protein is related to lung and hepatic alterations, while there is not a clear relation between protein activity and expression of renal damage [19]. By immunonephelometric dosage, we ascertained a much higher presence of this protein in the nephrotic patient group compared to a relative absence in the urinary abnormalities group; only two patients of the nephrotic group showed low levels of α1-AT.

Proteomic analysis is a promising technology for the discovery of biomarkers. We applied this method to two groups of nephropathic patients in which we identified differentially expressed proteins. They have a plasma origin, and their abundance in NP probably reflects the well-known reduction of selectivity in nephrotic proteinuria. Interestingly, the most important finding in our report is not the identity of the proteins but rather their biochemical status. Our data suggest the presence of proteolytic activity against albumin; this effect is highly prevalent in the nephrotic group and is only minimally present in the patients affected by urinary abnormalities. The proteolytic activity is not simply related to the syndromic group of patients analysed, nor to the specific histological diagnosis. As more research becomes available, this line of work may overcome challenges associated with stratifying patients based on classical diagnostic classifications. It may also help to elucidate the underlying pathophysiologic causes of disease; some reports hypothesize a role of this proteolytic activity in the permeabilization of the glomerular barrier. The relation between proteolytic activity and the abundant presence of α1-AT must be addressed by further studies. Future work will be to better define the origin of this proteolytic activity and explore the possible implications for clinically relevant outcomes.

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Conflict of interest statement. None declared.

Supplementary data

Supplementary data is available online at http://ndt.oxfordjournals.org.

References


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