

# Proteomics of renal disorders: Urinary proteome analysis by two-dimensional gel electrophoresis and MALDI-TOF mass spectrometry

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The proteomes of urinary samples from patients with different renal conditions were analysed by two-dimensional electrophoresis and MALDI-TOF technology. Samples from three different renal conditions, namely kidney failure, nephrotic syndrome and microalbuminuria, were included in the analysis. Apart from the presence of albumin, the profiles of protein spots found in these urine samples were quite distinct. While kidney failure patients showed predominantly low molecular weight proteins, the nephrotic syndrome patients showed an abundance of relatively high molecular weight proteins clustering in the acidic range of the 2-D gels. Two different protein spots from kidney failure patients, four from nephrotic syndrome patients and three from micro-

albuminuria patients were identified by in-gel protease digestions and analysis of resulting peptides by MALDI-TOF. The proteins identified were albumin, alpha-1-antitrypsin, alpha-1-acid glycoprotein 2, Zn-alpha-2-glycoprotein and alpha-1-microglobulin. Among these, only one was common between the proteomes of renal failure and nephrotic syndrome patients. Among the limited proteins found in microalbuminuria patients, three were common with the proteome of nephrotic syndrome. Overall profiles were, however, quite different. Our study showed that urinary proteomes of different renal conditions were different and emphasized the potential of urinary proteome analysis to augment existing tools in the diagnosis of renal disorders.

HUMAN kidneys perform the important function of maintaining the internal milieu and composition of the blood. Nephron, the basic unit of the human kidney, is composed of two main regions: (1) A tuft of capillaries called glomeruli, which are involved in the filtration, and (2) the tubules that are mainly responsible for the reabsorption process. While glomerular filtration removes ions and small molecular weight proteins out of the blood stream, the process of tubular reabsorption prevents excess loss of ions, sugars and certain low molecular weight proteins. A careful balance of these two counteracting processes characterizes normal functioning of the kidney<sup>1</sup>.

As a result of the efficient filtration and reabsorption process in the nephron, urine of a normal person shows very small amount of protein. Large molecular weight proteins are effectively retained in the blood stream. Presence of the rich extracellular matrix (ECM) around the glomerular capillaries and the basement membrane of the Bowman's capsule provide a physical barrier for the passage of large molecular weight proteins through

the glomeruli. It is also believed that the anionic charge present on the ECM repels the negatively charged proteins, thereby minimizing their movement across the glomeruli. Relatively low molecular weight proteins that do escape through the basement membrane are subsequently reabsorbed by the epithelial cells lining the proximal tubules by receptor-mediated endocytosis. Any damage to the ECM around the glomeruli results in the release of proteins into the tubules and the appearance of serum proteins in the urine. Likewise, any perturbation of the epithelial cells of the proximal tubules can cause inefficient reabsorption of urinary proteins leading to the loss of proteins in the urine. These conditions are clinically referred to as proteinuria and are well-accepted indicators of glomerular or tubular damage to the kidneys<sup>1,2</sup>.

The routine urine analysis performed in medical laboratories involves quantitative measurement of protein in the urine, by the dipstick method. Depending on the amount of protein found in the urine, the disease is graded and classified into sub-categories. A relatively more advanced test that is often employed is the examination of microalbuminuria. This is a radioimmunoassay-based quantitation of albumin in the urine that

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routine tests are unable to detect. The test can diagnose early stages of proteinuria before the onset of overt proteinuria, nephrotic syndrome or renal failure. These examinations, however, fail to provide any information about the site of damage in the kidneys. The only recourse available to the nephrologist to arrive at a more definitive diagnosis is biopsy examination. It is therefore desirable to have a non-invasive analysis that can augment existing methods of urine analysis and provide more accurate information about the origin of proteinuria.

There are several reports in the literature describing the detection of specific proteins in the urine and linking them with specific disorders. The list of proteins identified in the urine in various disorders (Table 1) includes retinol-binding protein, albumin, alpha-1-microglobulin, beta-2-microglobulin, alpha-1-macroglobulin, annexin and N-acetyl glucosaminidase<sup>3-5</sup>. In some cases, the presence of specific proteins has been correlated with a particular clinical condition. The approach used in the detection of specific proteins in the urine has mostly been radioimmunoassay and ELISA (enzyme-linked immunosorbent assay) using specific antibodies to the protein under investigation<sup>5-8</sup>.

In this article we describe our efforts to explore the potential of two-dimensional electrophoresis approach for proteinuria analysis and diagnosis. The two-dimensional electrophoresis approach for urinary protein analysis has been described previously; however, clinical correlations were not attempted in these studies<sup>9</sup>. We have analysed two-dimensional electrophoresis profiles of urine samples from patients with clinically defined proteinuria. The samples included urinary proteins from patients with kidney failure, nephrotic syndrome and microalbuminuria. Distinct patterns of protein spots were observed in each case and the kinds of protein found were indicative of the predominant site of damage in the kidneys. While kidney failure patients showed mostly low molecular weight tubular markers, the nephrotic syndrome patients showed an abundance of relatively high molecular weight proteins. We identified two proteins found in kidney failure patients, four from nephrotic syndrome patients and three from microalbuminuria patients using in-gel protease digestions

and MALDI-TOF (matrix-assisted laser desorption ionization-time of flight) technology. These were previously reported urinary proteins—albumin, alpha-1-antitrypsin in renal failure patients and alpha-1-antitrypsin, Zn-alpha-2-glycoprotein, alpha-1-microglobulin, alpha-1-acid glycoprotein 2 in nephrotic syndrome patients. Among the limited number of proteins seen in microalbuminuria patients, Zn-alpha-2-glycoprotein, alpha-1-microglobulin and alpha-1-acid glycoprotein 2 were common with the nephrotic syndrome patients. The study describes our efforts towards developing specific urinary protein database for specific renal conditions.

## Materials and methods

### Clinical subjects

A total of fifty individuals diagnosed with various renal disorders were identified of which data for twenty-five are described here. Estimation of serum creatinine, glomerular filtration rate (GFR), urinary albumin and occurrence of proteinuria were the criteria used to categorize patients with renal failure, nephrotic syndrome and microalbuminuria. Renal failure patients were identified by serum creatinine (> 1.5 mg/dl), GFR (< 20 ml/min) and proteinuria (0.1–0.4 mg/ml), coincident with renal failure and were from Manipal Institute of Nephrology and Urology, Manipal Hospital, Bangalore. Nephrotic syndrome patients were from the Department of Pediatrics, St. John's Medical College Hospital, Bangalore and displayed high proteinuria (0.5–4.0 mg/ml, > 3 g/day), severe edema, urinary protein : creatinine ratio > 0.4 mg protein/mmol (3.5 mg/dl) and GFR (< 10 ml/min). Patients with urinary albumin levels falling in the range of 20–100 µg/min/l as estimated by radioimmunoassay were categorized as microalbuminuria and were from the Department of Endocrinology, M. S. Ramaiah Medical College Hospital, Bangalore. Overnight urine samples were collected from the patients and used for the investigation.

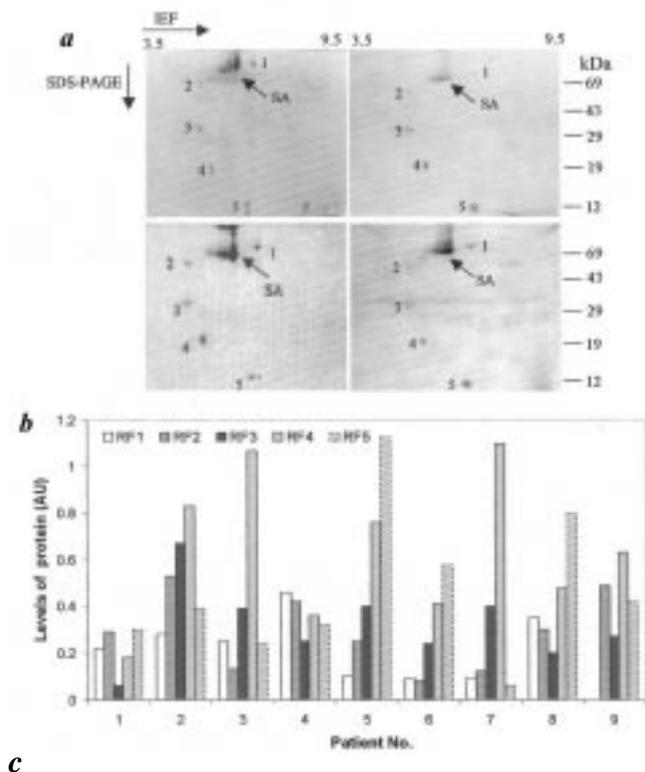
**Table 1.** Proteins identified in the urine in various disorders

Protein	Molecular weight (kDa)	Isoelectric point (pI)	Urinary marker of	Reference
N-acetyl-beta-D glucosaminidase	170	7.9	Tubular damage	5, 7
Immunoglobulin G	155	–	Glomerular damage	13
Transferrin	73	6.12	Glomerular damage	13
Serum albumin	69	5.7	Glomerular damage	13
Alpha-1-antitrypsin	46	5.2	Tubular damage	13
Alpha-1-acid glycoprotein	43	3.5	–	–
Zn-alpha-2-glycoprotein	38	5.3	Prostrate cancer	14, 15
Alpha-1-microglobulin	33	5.3	Tubular damage	5, 8, 16
Retinol-binding protein	21	5.4	Tubular damage	16, 17
Beta-2-microglobulin	11.7	6.07	Tubular damage	13, 16

### Sample preparation and two-dimensional electrophoresis

Five millilitres of human urine was collected and stored at 4°C. Proteins from 0.5 ml of urine were precipitated by adding 2 volumes of ice-cold acetone and incubating for 30 min at 4°C. The protein precipitate was pelleted by

centrifuging at 5000 g for 10 min. The pellet was dried, resuspended in 0.1 ml of PBS and protein concentration determined using Bradford's method<sup>10</sup>. Ten micrograms of protein from each urine sample was subjected to two-dimensional electrophoresis as described by O'Farrell<sup>11</sup> using ampholines of pI range 3.5–9.5 (Amersham-Pharmacia, Uppsala, Sweden). Following two-dimensional electrophoresis, the proteins were detected by Coomassie blue staining. The gels were scanned on a laser scanner and images were analysed using Melanie 2-D gel analysis software. The various protein spots were quantitated using Melanie and protein amounts represented as arbitrary units.



Patient no.	Levels of protein (AU)				
	RF1	RF2	RF3	RF4	RF5
1	0.22	0.29	0.06	0.18	<b>0.3</b>
2	0.28	0.53	0.67	<b>0.83</b>	0.39
3	0.25	0.13	0.39	<b>1.07</b>	0.24
4	<b>0.46</b>	0.42	0.25	0.36	0.32
5	0.1	0.25	0.4	0.76	<b>1.13</b>
6	0.09	0.08	0.24	0.41	<b>0.58</b>
7	0.09	0.12	0.4	<b>1.1</b>	0.06
8	0.35	0.3	0.2	0.48	<b>0.8</b>
9	0	0.49	0.27	<b>0.63</b>	0.42

**Figure 1.** Urinary two-dimensional electrophoresis profiles of renal failure patients. *a*, Urinary proteins from four different renal failure patients were subjected to two-dimensional electrophoresis as described in the text. Protein spots were visualized by Coomassie staining and labelled RF1 to RF5. Human serum albumin (SA) was identified based on its pI, molecular weight and MALDI-TOF analysis (see Figure 2 *a*); *b*, Quantitation of spots corresponding to RF1 to RF5 from at least nine patients using Melanie 2-D analysis software shown in the form of a bar graph; *c*, Relative amounts of spots RF1 to RF5 shown in (*b*) are represented as arbitrary units, with the peak values shown in bold.

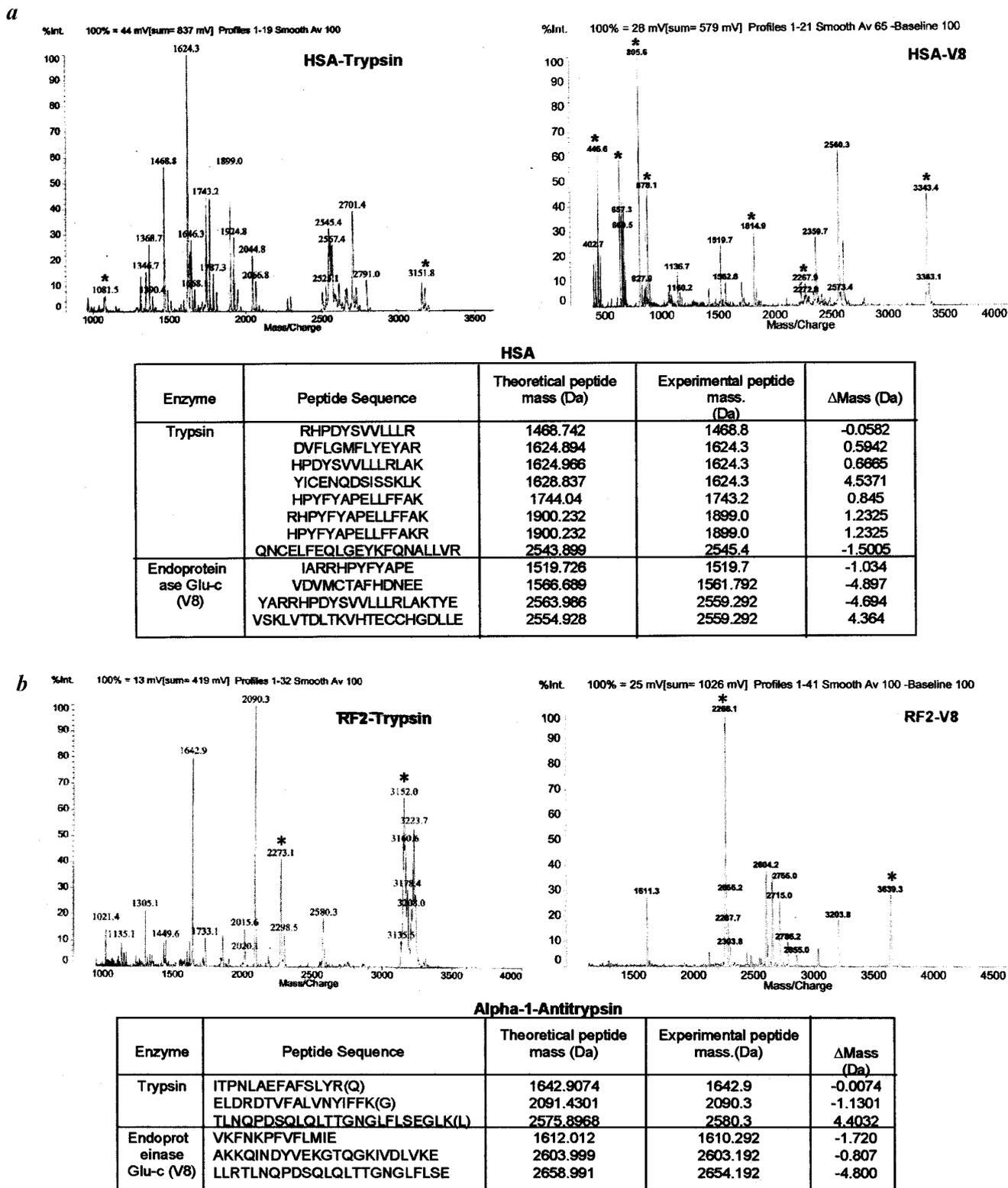
### In-gel proteolytic digestion and MALDI-TOF

For in-gel digestion, large-scale two-dimensional electrophoresis was performed as described by Celis<sup>12</sup>. Coomassie-stained spots of interest were cut out from at least three 2-D gels, pooled and cut further into small pieces ~ 2–3 mm in diameter. A blank gel piece with no protein spots was also separately processed as a control. The gel pieces were vortexed in 25 mM NH<sub>4</sub>HCO<sub>3</sub> in 50% acetonitrile 3–4 times. The gel pieces were then dried under vacuum, and incubated with 10 mM DTT in 25 mM NH<sub>4</sub>HCO<sub>3</sub> at 56°C for 1 h. Reduced cysteines were modified with 55 mM iodoacetamide in 25 mM NH<sub>4</sub>HCO<sub>3</sub> for 45 min at room temperature in the dark. The gel pieces were washed 3–4 times in 25 mM NH<sub>4</sub>HCO<sub>3</sub> in 50% acetonitrile and then completely dried. Trypsin (0.1 mg/ml) or endoproteinase Glu-C (V8 protease) (0.1 mg/ml) was added just enough to cover the gels and digestion carried out for 12 h at 37°C. The peptides were extracted from the gels with first 0.1 ml water and followed by two extractions with 0.5% TFA in 50% acetonitrile. The peptide solution was then concentrated in a speed-vac and used for MALDI-TOF analysis (Kratos Analytical Inc, Shimadzu Corp., Maryland, USA). The matrix used for MALDI-TOF was alpha-cyano cinnamic acid. The peptide masses obtained were used to identify the protein using at least three search programs, MS-FIT, Peptident and ProFound. Protein identification was based on best matches using molecular weight, pI and peptide masses as parameters. Peptide peaks arising from the matrix or from auto-proteolytic digestion of proteases were determined from the control and discounted from the search. The background peaks were marked with '\*' in the MALDI-TOF spectra.

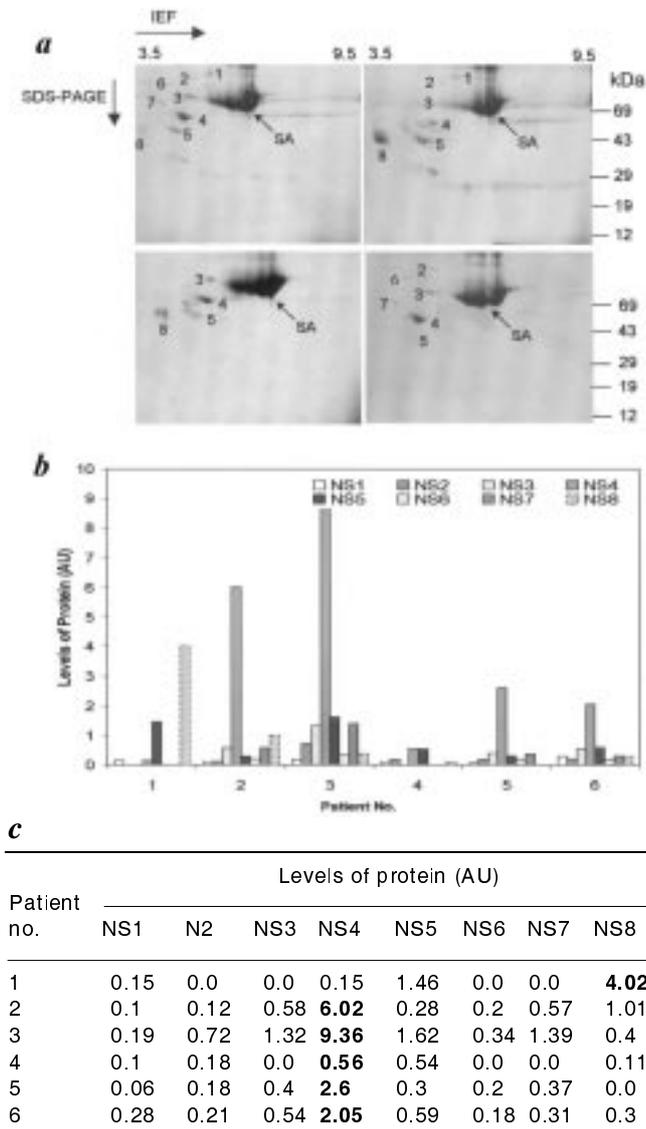
## Results

### Renal failure patients show the presence of albumin and other low molecular weight urinary proteins

To study the pattern of proteins excreted under different clinical conditions, we first chose to examine two-



**Figure 2.** MALDI-TOF analysis of urinary proteins from renal failure patients. *a*, Protein spots corresponding to SA (see Figure 1 *a*) were subjected to in-gel digestion with trypsin or endoproteinase Glu-C (V8) as described in the text. Tryptic (left panel) and V8 (right panel) peptide masses were obtained by MALDI-TOF analysis. (Bottom panel) Comparison of experimentally determined peptide masses of SA with theoretical peptide masses of human serum albumin (HSA); and *b*, MALDI-TOF profiles of proteolytic peptides from protein spots corresponding to RF2 (see Figure 1 *a*) were obtained as in (*a*). (Bottom panel) Comparison of experimentally determined peptide masses of RF2 with theoretical peptide masses of alpha-1-antitrypsin. Background peaks are marked with ‘\*’ in the MALDI-TOF spectra.



**Figure 3.** Urinary two-dimensional electrophoresis profiles of patients with nephrotic syndrome. *a*, Urinary proteins from four different nephrotic syndrome patients were subjected to two-dimensional electrophoresis as described in the text. Protein spots were visualized by Coomassie staining and labelled NS1 to NS8. Human serum albumin (SA) was identified based on its pI, molecular weight and MALDI-TOF analysis (see Figure 2 *a*); *b*, Quantitation of spots corresponding to NS1 to NS8 from at least six patients using Melanie 2-D analysis software shown in the form of a bar graph; *c*, Relative amounts of spots NS1 to NS8 shown in (*b*) represented as arbitrary units, with the peak values shown in bold.

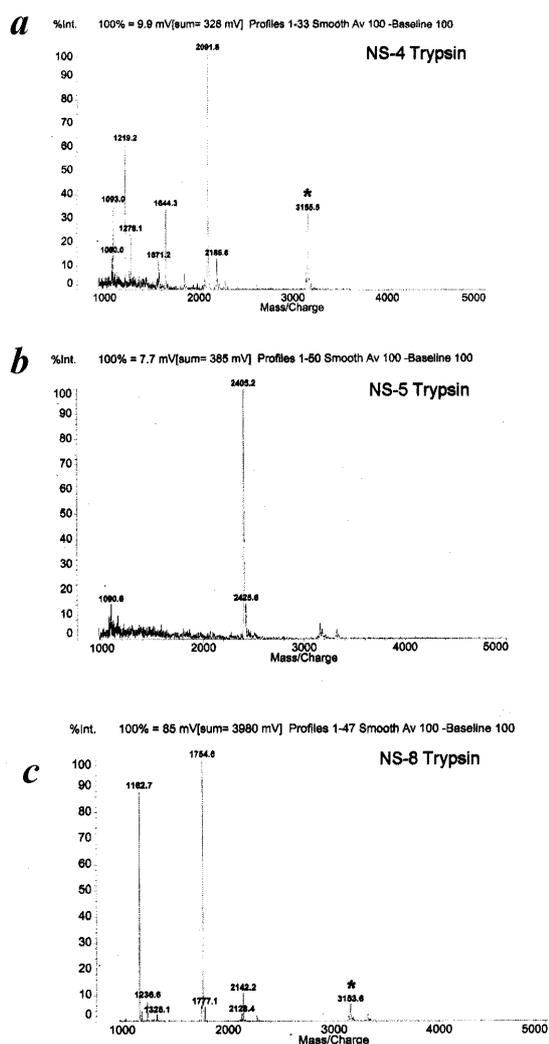
dimensional electrophoresis profiles of urinary proteins from kidney failure patients. About 5 ml of urine was collected from ten different patients on dialysis. The urine samples were acetone-precipitated and aliquots corresponding to 10  $\mu$ g protein were analysed by two-dimensional electrophoresis and Coomassie blue staining as described earlier. The two-dimensional electrophoresis profile of proteins seen in all the kidney failure

patients was quite similar. Figure 1 *a* shows examples of protein spots seen in four such patients. A large spot corresponding to albumin could be identified based on its known pI/molecular weight and also by MALDI-TOF analysis of its tryptic peptides (see below). In addition, five other spots of molecular weights either equal or smaller than albumin could also be seen. These were numbered as RF1 to RF5 as shown in the figure. The two-dimensional electrophoresis patterns of urinary proteins from all the renal failure patients were qualitatively similar, but the relative quantities of individual spots were different in different patients. Figure 1 *b* and *c* shows quantitation of spots seen in renal failure patients.

To identify the low molecular weight protein spots on the 2-D gels, we subjected different spots to in-gel digestions with trypsin or V8 protease and analysed the peptides by MALDI-TOF, as described earlier. The identity of the predicted albumin spot was confirmed by this approach and in addition we could identify spot RF2 as alpha-1-antitrypsin (Figure 2 *a* and *b*). Both are previously reported urinary proteins (see Table 1). The identity of spot 3, 4 and 5 is under investigation. The analysis indicated that urinary proteins from renal failure patients are characterized by the presence of relatively low molecular weight proteins, commonly used as markers of tubular injury.

### *Nephrotic syndrome patients show relatively high molecular weight urinary proteins*

Nephrotic syndrome is another example of renal abnormality characterized by a large amount of urinary protein excretion. Using an approach similar to that in Figure 1, we analysed the two-dimensional electrophoresis profiles of urinary proteins from ten different nephrotic syndrome patients. All the patients showed a fairly similar profile of protein spots. Figure 3 *a* shows examples of two-dimensional electrophoresis pattern seen in four nephrotic syndrome patients. Apart from the common presence of albumin, the pattern was quite distinct from that seen in renal failure patients (shown in Figure 1 *a*). Unlike renal failure patients, the urinary proteins of nephrotic syndrome patients mostly showed the presence of larger molecular weight proteins clustered in the acidic part of the 2-D gels. Eight distinct spots, in addition to the spot corresponding to albumin, could be easily identified. These are numbered as spots NS1 to NS8 in Figure 3. Relative quantitation of these spots in different nephrotic syndrome patients is shown in Figure 3 *b* and *c*. Despite the profiles being similar, there were quantitative differences in the spots among various patients. As shown in the quantitation, some spots such as NS2, NS3, NS6 and NS7 were absent in patient 1, while NS8 was undetectable in patient 5. It is



**Alpha-1-Antitrypsin**

Enzyme	Peptide Sequence	Theoretical peptide mass (Da)	Experimental peptide mass. (Da)	ΔMass (Da)
Trypsin	LYHSEFTVNFQDTEEAKK	2187.3	2186.8	0.5
	ELDRTVFALVNIYFFK	2091.4	2091.8	0.4
	ITPNLAEFQFSLYR	1642.9	1644.8	2.9
	DTVFALVNIYFFK	1571.2	1577.8	6.2
	GKWERPFEVK	1276.5	1278.1	1.6
	WERPFEVK	1091.2	1093.0	2.8
	FLENEDRR	1080.0	1079.1	0.9

**Zn Alpha-2-Glycoprotein**

Enzyme	Peptide Sequence	Theoretical peptide mass (Da)	Experimental peptide mass. (Da)	ΔMass (Da)
Trypsin	HVEDVPAFQALGSLNDLOFFR	2404.68	2405.2	0.52

**Alpha-1-acid Glycoprotein**

Enzyme	Peptide Sequence	Theoretical peptide mass (Da)	Experimental peptide mass. (Da)	ΔMass (Da)
Trypsin	YEGGREHVAHLLFLRDTK	2142.4	2141.8	0.6
	YEGGREHVAHLLFLR	1798.0	1798.4	0.4
	NWGLSFYADKPETTK	1757.9	1753.9	4.0
	WFYIASAFR	1161.4	1162.3	0.9

**Figure 4.** MALDI-TOF analysis of urinary proteins from nephrotic syndrome patients. *a*, Protein spots corresponding to NS4 (see Figure 3 *a*) were subjected to in-gel digestion with trypsin as described in the text. Tryptic peptide masses were obtained by MALDI-TOF analysis (left panel). (Right panel) Comparison of experimentally determined peptide masses of NS4 with theoretical peptide masses of alpha-1-antitrypsin; *b*, (Left panel) MALDI-TOF profiles of tryptic peptides from protein spots corresponding to NS5 (see Figure 3 *a*) were obtained as in (*a*). (Right panel) Comparison of experimentally determined peptide masses of RF2 with theoretical peptide masses of Zn-alpha-2-glycoprotein; and *c*, (Left panel) MALDI-TOF profiles of tryptic peptides from protein spots corresponding to NS8 (see Figure 3 *a*) were obtained as in (*a*). (Right panel) Comparison of experimentally determined peptide masses of NS8 with theoretical peptide masses of alpha-1-acid glycoprotein 2. Background peaks are marked with '\*' in the MALDI-TOF spectra.

likely that the levels of these spots were too low to be detected by our staining procedure. A subset of nephrotic syndrome patients also showed some low molecular weight protein spots that were common with the renal failure samples (not shown).

Selected spots from these gels were subjected to in-gel tryptic digestion and MALDI-TOF analysis. Based on MALDI-TOF analysis and comparison with the pI/molecular weights of known urinary proteins, we could identify spot NS4 to be alpha-1-antitrypsin (Figure 4 *a*), NS5 to be Zn-alpha-2-glycoprotein (Figure 4 *b*) and NS8 to be alpha-1-acid glycoprotein 2 (Figure 4 *c*).

*Microalbuminuria patients show urinary protein spots in addition to serum albumin*

Diabetic patients are predisposed to renal damage as a result of accumulation of advanced glycation end-products in the blood. These patients are advised to undergo microalbuminuria test for timely detection of traces of albumin in the urine, suggesting renal damage. We examined if the microalbuminuria-positive patients would show presence of urinary proteins other than albumin. We analysed urinary proteins from ten different patients tested positive for microalbuminuria by two-dimensional electrophoresis. Although the amount of

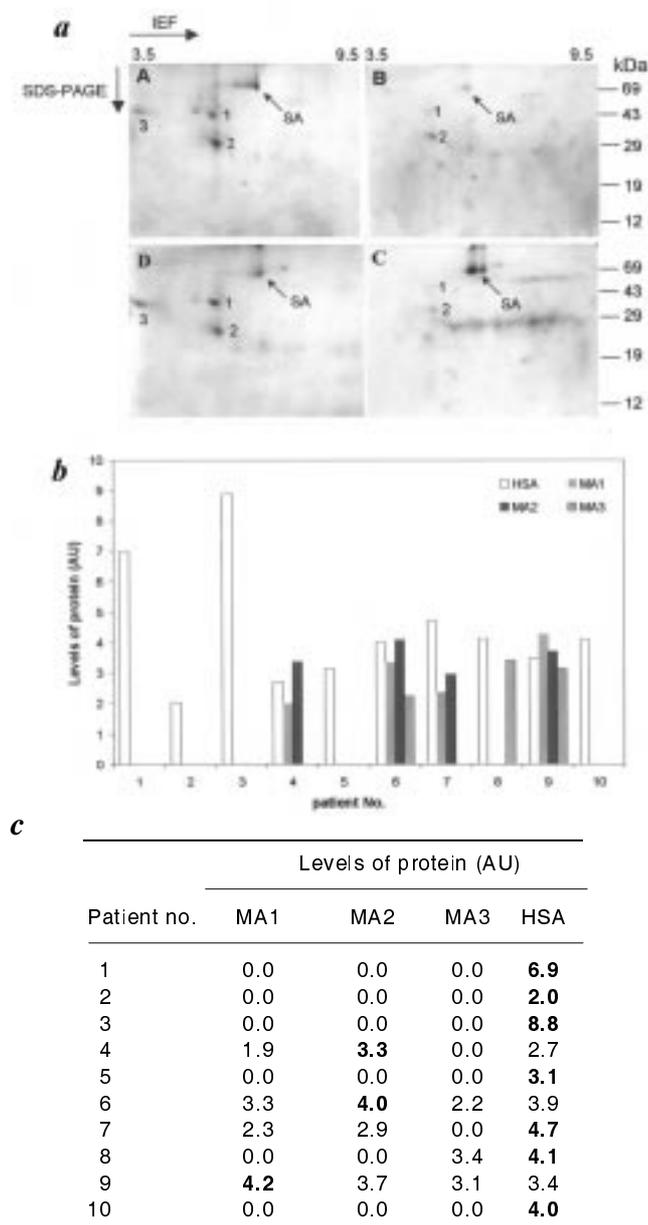
albumin in these samples was very low, the total protein was relatively high, indicating that proteins other than albumin were present (not shown). As shown in Figure 5 in addition to the presence of albumin, at least three other protein spots were obvious in four different patients (A to D). At least in patients A and D the spots

MA1 and MA2 are nearly as distinct as the albumin spot. As above we subjected spots MA1, MA2 and MA3 to in-gel tryptic digestion followed by MALDI-TOF analysis. The analysis of spectra (see Figure 6 *a-c*) and the total urinary protein database indicated that spot MA1 was Zn-alpha-2 glycoprotein, spot MA2 was alpha-1-microglobulin and MA3 was alpha-1-acid glycoprotein 2. These spots were the same as those of NS4, NS5 and NS8 seen in nephrotic syndrome patients.

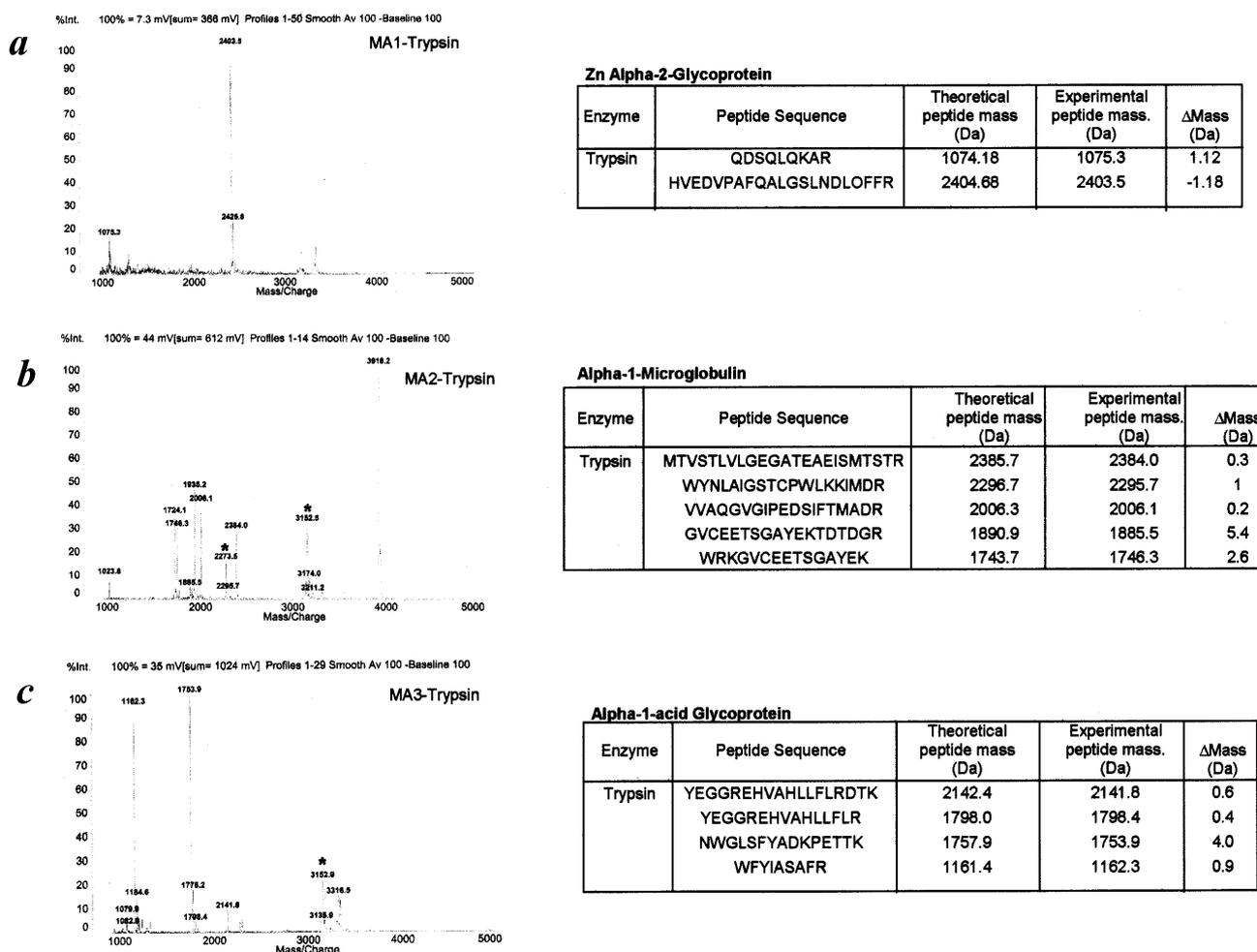
## Discussion

We have used two-dimensional electrophoresis approach to examine the kinds of urinary proteins excreted under different clinical conditions of proteinuria. Three groups of patients, namely renal failure, nephrotic syndrome and microalbuminuria were randomly selected for the study. Although the presence of albumin was common to all, the overall protein profiles of urinary samples from renal failure and nephrotic syndrome patients were quite distinct, showing only one common protein spot (Figure 7). In renal failure patients we found mainly low molecular weight proteins, while the nephrotic syndrome patients showed the presence of relatively high molecular weight proteins clustered in the acidic range (pI's 4 to 6) of the 2-D gels. Despite the low amounts of protein present in microalbuminuria patients, we could detect the presence of albumin and at least three other additional proteins in these patients. These three proteins were also found present in nephrotic syndrome patients.

Selected protein spots from renal failure and nephrotic syndrome patients were subjected to in-gel protease digestions and analysis using MALDI-TOF technology. Three different criteria were used for the identification of individual proteins. (1) The estimated pI and molecular weights were matched with the list of urinary proteins reported in the literature. (2) The mobilities of protein spots were compared with the urinary protein database reported by Celis<sup>12</sup>. (3) Final identification was done by analysis of peptide masses obtained by MALDI-TOF using various search engines. Among the proteins seen in renal failure patients we could identify albumin (HSA, 68 kDa, pI 5.8) and alpha-1-antitrypsin (RF2, 46 kDa, pI 5.2). In nephrotic syndrome patients we could identify alpha-1-antitrypsin (NS4, 46 kDa, pI 5.2), Zn-alpha-2 glycoprotein (NS5, 38 kDa, pI 5.3) and alpha-1-acid glycoprotein 2 (NS8, 43 kDa, pI 3.5). Among these, only alpha-1-antitrypsin was common to both renal failure (RF2) and nephrotic syndrome (NS4) patients. These proteins have been reported to be present in urine samples previously. In samples from microalbuminuria patients we could identify Zn-alpha-2-glycoprotein (MA1, 38 kDa, pI 5.3), alpha-1-microglobulin (MA2, 33 kDa, pI 5.3) and alpha-



**Figure 5.** Urinary two-dimensional electrophoresis profiles of patients with microalbuminuria. *a*, Urinary proteins from four different microalbuminuria patients were subjected to two dimensional electrophoresis as described in the text. Protein spots were visualized by Coomassie staining and labelled MA1 to MA3. Human serum albumin (SA) was identified based on its pI and molecular weight and MALDI-TOF analysis (see Figure 2 *a*). *b*, Quantitation of spots corresponding to MA1 to MA3 from at least ten patients using Melanie 2-D analysis software shown in the form of a bar graph; *c*, Relative amounts of spots MA1 to MA3 shown in (*b*) represented as arbitrary units, with the peak values shown in bold.



**Figure 6.** MALDI-TOF analysis of urinary proteins from microalbuminuria patients. *a*, Protein spots corresponding to MA1 (see Figure 5 *a*) were subjected to in-gel digestion with trypsin as described in the text. Tryptic peptide masses were obtained by MALDI-TOF analysis (left panel). (Right panel) Comparison of experimentally determined peptide masses of MA1 with theoretical peptide masses of Zn-alpha-2-glycoprotein; *b*, (Left panel) MALDI-TOF profiles of tryptic peptides from protein spots corresponding to MA2 (see Figure 5 *a*) were obtained as in (*a*). (Right panel) Comparison of experimentally determined peptide masses of MA2 with theoretical peptide masses of alpha-1-microglobulin; and *c*, (Left panel) MALDI-TOF profiles of tryptic peptides from protein spots corresponding to MA3 (see Figure 5 *a*) were obtained as in (*a*). (Right panel) Comparison of experimentally determined peptide masses of MA3 with theoretical peptide masses of alpha-1-acid glycoprotein 2. Background peaks are marked with ‘\*’ in the MALDI-TOF spectra.

1-acid glycoprotein 2 (MA3, 43 kDa, pI 3.5). As discussed above all the three proteins were also found to be present in nephrotic syndrome patients.

In the past, studies on urinary proteins have mainly relied on RIA or ELISA tests, which involve the use of specific antibodies and/or radiolabelled proteins of interest. The two-dimensional electrophoresis approach described here provides complete urinary protein profile in one step, without the need for specific antibodies to individual proteins. In addition, the approach has the potential to detect new urinary protein markers that have not been described previously. A similar approach to analyse urinary proteins was described recently by Spahr *et al.*<sup>9</sup>. These authors have described two-dimensional electrophoresis pattern of urinary protein mixture that is available commercially. They have also

elegantly demonstrated the possibility of identifying specific urinary proteins by LC-MS/MS, starting from tryptic digests of total urinary protein mixtures. The commercial source of urinary proteins used has precluded clinical correlations in this study.

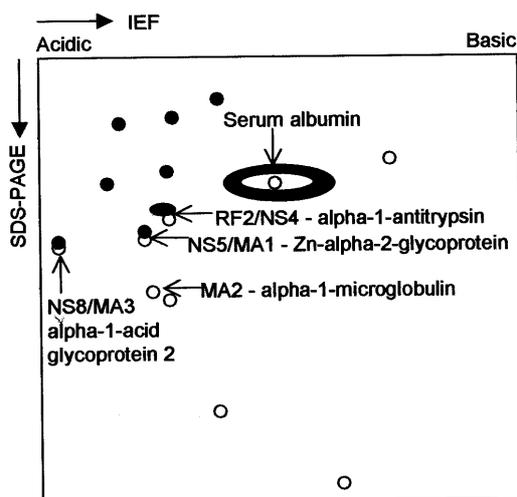
In our study we have used urine samples from patients with different renal conditions. Using two extreme examples of renal damage, our study shows that the profile of proteins obtained in different clinical conditions is quite different. The difference in the pattern of 2-D spots seen in renal failure and nephrotic syndrome patients is probably indicative of the different sites of damage. In renal failure the primary site of injury is likely to be the tubules, which are involved in the reabsorption of low molecular weight proteins that pass through the Bowman’s capsule. Accordingly, we found

the presence of tubular markers in these samples. The reason or the absence of high molecular weight proteins in kidney failure patients is not clear, but may have to do with the low glomerular filtration rates seen in these patients. In nephrotic syndrome patients, on the other hand, high molecular weight proteins were abundantly present. This is in agreement with the damage to the glomerular basement membrane resulting in the leakage of high molecular weight proteins. Furthermore, the abundance of proteins with acidic pI provided evidence for damage to the charge barrier at the basement membrane. Nephrotic syndrome is a term used to describe a collection of specific pathologies differing in both site and nature of glomerular damage such as focal segmental glomerulosclerosis (FSG), minimal change glomerulonephropathy, membranous glomerulonephropathy, mesangioproliferative glomerulonephropathy, etc. Currently the only method available to distinguish among these is renal biopsy examination. The quantitative differences in the spots in different nephrotic syndrome patients that we observed in our study may reflect differences in underlying pathologies. Further analysis may allow us to ascribe specific profiles, both qualitative and quantitative, within nephrotic syndrome to one or the other of the subgroups of the disease.

A small group of patients with nephrotic syndrome also showed the presence of low molecular weight protein spots (in addition to the high molecular weight proteins) characteristically seen in renal failure cases (not shown). Similarly, a small number of microalbuminuria patients also showed similar low molecular weight tubular markers in their urinary proteins (not shown). Such 'mixed profiles' are probably suggestive of early

stages of tubular damage without an overt renal failure in these patients. These examples highlight the potential of two-dimensional electrophoresis analysis in timely prediction of tubular damage. The approach may be of particular value in the diagnosis of diabetic nephropathy, wherein the clinical signs range from 'microalbuminuria' to 'macroalbuminuria without renal failure' to 'full blown nephrotic syndrome with renal failure'.

The sample study presented here suggests that two-dimensional electrophoresis analysis of urinary proteins can support existing tools available to study proteinuria in more accurately defining the stage of renal injury. Distinct protein patterns observed under different renal conditions emphasize the need to develop specific urinary protein database for specific renal disorders. The approach may make it possible to use protein markers to define and categorize renal pathology. A systematic study of specific protein markers in various types of nephrotic syndrome and their correlation with the biopsy findings may allow us to use this approach as a surrogate marker for the renal biopsy findings. The potential of this method to serve as a non-invasive method of diagnosis, ultimately replacing renal biopsy as the principle method of diagnosis of renal disorders remains to be explored.



**Figure 7.** Renal failure and nephrotic syndrome patients show distinct proteomes. Schematic representation of protein spots found in renal failure, nephrotic syndrome and microalbuminuria patients. Spots are colour coded. Red shows profile for renal failure patients, green for nephrotic syndrome patients and clear spots denote profile seen in microalbuminuria patients. Overlapping spots with different colour codes indicate identical protein found in two different conditions. Proteins identified by MALDI-TOF analysis are labelled.

- Guyton, A. C. and Hall, J. E., *Textbook of Medical Physiology*, 1996, 9th edn, pp. 153–421.
- Mogensen, C. E., *J. Diabetic Compl.*, 1994, **8**, 135–136.
- Mogensen, C. E., *Lancet*, 1995, **346**, 1080–1084.
- Airolidi, G. and Campanini, M., *Recent Prog. Med.*, 1993, **84**, 210–224.
- Guder, W. G. and Hoffman, W., *Clin. Nephrol.*, 1992, **38**, S3–S7.
- Tolkoff-Rubin, N. E., Rubin, N. H. and Bonventre, J. V., *Clin. Lab. Med.*, 1988, **8**, 507–526.
- Price, R. G., *Clin. Nephrol.*, 1992, **38**, S14–S29.
- Weber, M. H. and Verwiebe, R., *Eur. J. Clin. Chem. Clin. Biochem.*, 1992, **30**, 683–691.
- Spahr, S. S. *et al.*, *Proteomics*, 2001, **1**, 93–107.
- Bradford, M. M., *Anal. Biochem.*, 1976, **72**, 248–254.
- O'Farrell, P. M., *J. Biol. Chem.*, 1975, **250**, 4807–4821.
- Celis, J. E., *Cell Biology: A Laboratory Handbook*, 1998, 2nd edn, vol. 4, pp. 375–385.
- Steinhoff, J., Feddersen, A., Wood, W. G., Hoyer, J. and Sack, K., *Clin. Nephrol.*, 1991, **35**, 255–262.
- Celis, J. E., Wolf, H. and Ostergaard, M., *Electrophoresis*, 2000, **21**, 2115–2121.
- Rasmussen, H. H., Orntoft, T. F., Wolf, H. and Celis, J. E., *J. Urol.*, 1996, **155**, 2113–2119.
- Tomlinson, P. A., Dalton, R. N., Hartley, B., Haycock, G. B. and Chantler, C., *Pediatr. Nephrol.*, 1997, **11**, 285–290.
- Mastroianni Kirsztain, G., *Nephron*, 2000, **86**, 109–114.

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