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Proteomic profiling of nephrotic syndrome in serum using magnetic bead based sample fractionation & MALDI-TOF MS

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***Background & objectives*: At present, the diagnosis of nephrotic syndrome (NS) requires a renal biopsy which is an invasive procedure. We undertook this pilot study to develop an alternative method and potential new biomarkers for diagnosis, and validated a set of well-integrated tools called ClinProt to investigate serum petidome in NS patients.**

***Methods*: The fasting blood samples from 49 patients diagnosed with NS by renal biopsy, including 17 mesangial proliferative glomerulonephritis (MsPGN), 12 minimal change nephrotic syndrome (MCNS), 10 focal segmental glomerulosclerosis (FSGS) and 10 membranous nephropathy (MN), were collected and screened to describe their variability of the serum peptidome. The results in NS group were compared with those in 10 control healthy individuals. Specimens were purified with magnetic beads-based weak cation exchange chromatography and analyzed in a MALDI-TOF MS.**

***Results*: The results showed 43, 61, 45 and 19 differential peptide peaks in MsPGN, MCNS, MN and FSGS groups, respectively. A Genetic Algorithm was used to set up the classification models. Cross validation of healthy controls from MsPGN, MCNS, MN and FSGS was 96.18, 100, 98.53 and 94.12 per cent, respectively. The recognition capabilities were 100 per cent.**

***Interpretation & conclusions*: Our results showed that proteomic analysis of serum with MALDI-TOF MS is a fast and reproducible approach, which may give an early idea of the pathology of nephrotic syndrome.**

Key words ClinPro Tools - magnetic bead - MALDI-TOF MS - nephrotic syndrome - serum peptidome

Nephrotic syndrome (NS) is characterized by massive proteinuria and requires successful early detection along with adequate treatment. Long term use of steroids or other immunosuppressant drugs carry severe toxicities and other risks. Percutaneous renal biopsy is currently the only means of making a definite diagnosis. Biopsies are invasive, inconvenient, and can result in complications such as haemorrhage, infection, pneumothorax, hypotension, bleeding, gross hematuria, adjacent organ injury or decrease in haemoglobin

requiring a transfusion[1-3]. Thus, it is important to develop a noninvasive alternative to biopsy which would decrease the likelihood of complications.

Proteomics is a new, exciting and largely unexplored area in NS. Preliminary studies have shown that this technique may provide a novel noninvasive means of diagnosing NS, and it may have additional value as a prognostic tool[4]. Serum proteome analysis has the potential to facilitate disease diagnosis and therapeutic monitoring, because serum is more easily accessible and widely collected sample, which contains >10,000 different proteins and peptides[5-7]. These proteins and peptides are from almost every tissue and cell, and the changes in their quantity and quality are specific not only to the tissues affected but also to the disease process itself. Advances in mass spectrometry (MS) now permit the display of hundreds of small- to medium sized peptides using only microliters of serum[8,9].

We evaluated matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI-TOF MS) in combination with magnetic bead-based sample cleanup and ClinPro Tools to generate and analyze protein profiles from serum samples collected from NS patients and healthy individuals. The aim of this pilot study was to obtain the serum peptide fingerprint in NS patients and assess the utility of peptide profiling in a small sample population to identify potential biomarkers of NS, and to launch preliminary work for better understanding of the pathogenesis and early diagnosis of NS from an integrated perspective of proteomics.

Material & Methods

Seventeen adult patients with mesangial proliferative glomerulonephritis (MsPGN), 12 with minimal change nephrotic syndrome (MCNS), 10 with focal segmental glomerulosclerosis (FSGS) and 10 with membranous nephropathy (MN) were enrolled in the study. These patients were enrolled consecutively from the inpatient section in the Department of Nephrology of 181th Hospital, Guilin, P.R. China from November 2009 to April 2010. All the patients had undergone a renal biopsy for an exact histological diagnosis. Diagnostic criteria for NS were massive proteinuria (>3.5 g/day) and hypoalbuminaemia (<30 g/l). The duration of NS in the patients before collection of the blood sample ranged from 1 to 3 years. Age- and race- matched healthy controls (n=10) were recruited through in hospital boards. Demographic and clinical data are given in Table I.

Informed oral consent was obtained from all patients and the Medical Ethics Committee of which hospital 181th Hospital approved the study protocol. Blood samples were collected before starting treatment. The samples were collected in a 5 ml Serum Separator Vacutainer Tube and centrifuged at 249.75 *g* for 5 min. The serum samples were distributed into 0.5 ml aliquots and stored at -80^{o}C until the experiment.

Peptidome separation: Magnetic beads-based weak cation exchange chromatography (MB-WCX) (Bruker Daltonic, Germany) was used for peptidome separation of samples following the standard protocol by the manufacturer[10]. Ten µl of MB-WCX binding solution and 10 µl of WCX-beads were combined in a 0.5 ml microfuge tube after thorough vortexing both reagents, 5 µl serum sample was added and mixed by pipetting up and down. Microfuge tubes were then placed in a magnetic bead separator (MBS) and agitated back and forth three times. The beads were collected on the wall of the tubes in the MBS 1 min later. The supernatant was removed carefully by using a pipette, and 100 µl MB-WCX wash buffer was added to tubes, which

Table I. Demographic and clinical data in the various nephrotic syndrome (NS) groups

Disease group	Female/male ratio	Age (yr)	Cr (µmol/l)	BUN (m/mol)	ALB (g/l)	Pro (g/24 h)
MsPGN	6/11	27.822±8.957 (17 to 52)	93.583±8.771 (82.61 to 112.65)	4.732±0.858 (3.47 5.86)	23.488±4.281 (14.37 to 28.68)	3.73±2.71 (0.35 to 9.59)
MCNS	3/9	19.665±2.753 (16 to 54)	95.864±12.527 (80.94 to 115.58)	5.062±0.894 (4.11 to 5.98)	21.469±6.323 (14.08 to 28.39)	4.07±3.61 (0.92 to 9.91)
FSGS	2/8	47.751±14.963 (22 to 59)	105.516±11.233 (88.86 to 113.31)	4.433±0.521 (3.85 to 5.11)	22.272±3.713 (18.37 to 26.54)	3.72±2.98 (1.21 to 7.82)
MN	1/9	26.504±10.208 (15 to 53)	99.279±14.958 (83.15 to 116.42)	4.246±0.717 (3.56 to 5.25)	18.044±4.561 (12.55 to 23.69)	3.99±3.27 (0.24 to 6.18)

MsPGN, mesangial proliferative glomerulonephritis; MCNS, minimal change nephrotic syndrome; FSGS, focal segmental glomerulosclerosis; MN, membranous nephropathy.; Figures in parentheses denote range; Cr, creatinine; BUN, blood urea nitrogen; ALB, albumin; Pro, protein

were agitated back and forth in the MBS ten times. The beads were collected on the wall of the tubes, and supernatant was removed carefully by using a pipette. After two washes, 5 µl of MB-WCX elution buffer was added to disperse beads in tubes by pipetting up and down. The beads were collected on wall of tubes for 2 min and the clear supernatant was transferred into fresh tubes. MB-WCX stabilization solution (4 µl) was added to the eluate, mixing intensively by pipetting up and down. The eluate was then ready for spotting onto MALDI-TOF MS targets and measurement or stored at -20°C.

MALDI-TOF MS peptide analysis: The serum peptide profiles were analyzed with an Autoflex MALDI-TOF/TOF MS (Bruker Daltonik, Germany) equipped with a gridless ion source, operating in linear mode; a delayed-extraction (DE) electronics, a high-resolution timed ion selector (TIS), a 2-GHz digitizer and a 7-S/N ratio. Ions formed with a N2 pulse laser beam (337 nm) were accelerated to 25 KV. With this spectra serum preparation peptide/protein peaks in the m/z range of 1,000-10,000 Da were measured. Each spectrum was the sum spectrum of 800 laser shots, delivered in 16 sets of 50 shots (at 50-Hz frequency) to each of 16 different locations on the surface of the spot. Spectra were collected automatically using the flexAnalysis software (Bruker Daltonik, Germany) for fuzzy controlled adjustment of critical instrument settings to generate raw data of optimized quality.

Statistical analysis: The ClinPro Tools software 2.2 (Bruker Daltonik, Germany) was used for analysis of all serum sample data derived either from patients and normal controls. Data analysis began with raw data pretreatment, including baseline subtraction of spectra, normalization of a set of spectra, internal peak alignment using prominent peaks, and a peak picking procedure. The pretreated data were then used for visualization and statistical analysis in ClinPro Tools. Statistically significant different quantities of peptides were determined by means of Welch's *t*-tests. Class prediction model was set up by Genetic Algorithm (GA). The GA works on a population, which consists of a multitude of peak combinations. During selection, the fittest peak combinations are chosen and the less capable are abandoned. The cross-over combines randomly selected pairs of peak combinations to produce child peak combinations, which replace their parent peak combination. Finally, a small amount of peak combinations is modified randomly during mutation. This is done to keep genetic diversity and to prevent a premature convergence to a local optimum. To determine the accuracy of the class prediction, a cross-validation was implemented by the ClinPro Tools software 2.2.

Results

Serum protein profiles purified by WCX: The MB-WCX kept the regularity within the group, but it showed significant difference between spectra after purification.

Over 300 peptides were detected in serum samples from each group and there were distinct differences in multiple peptide clusters between the groups. The resulting multiple spectrum profiles were analyzed and compared to obtain characteristically disease-related peptides. GA class prediction was applied to identify differential peptides between MsPGN and normal controls, and 43 peptides were significant. Three peptide peaks were highly elevated (*m/z* 5917.49, 5902.94, 4978.20) and two peptide peaks were highly degraded (*m/z* 4053.72, 2660.15). Between MCNS and normal controls, 61 peptides were significant. Four peptide peaks were highly elevated (*m/z* 5902.73, 5917.41, 2952.03, 5335.65) and three peptide peaks were highly degraded (*m/z* 4053.60, 4977.86, 2660.06). Between MN and normal controls, 45 peptides were significant. Three peptide peaks were highly elevated (*m/z* 6628.49, 6430.59, 6642.88) and four peptide peaks were highly degraded (*m/z* 4053.45, 3934.54, 4209.40, 2659.95). Between FSGS and normal controls, 19 peptides were significant. One peptide peaks were highly elevated (*m/z* 1896.09) and four peptide peaks were highly degraded (*m/z* 7760.88, 9281.40, 4053.43, 2659.94) (Table II).

Similarly, GA class prediction was used to identify differential peptides between each disease group. Between MsPGN and MCNS group, 59 peptides were significant. Three peptide peaks were highly elevated (*m/z* 4963.38, 4053.97, 4209.87) and three peptide peaks were highly degraded (*m/z* 2952.28, 5903.18, 5917.75). Between MsPGN and MN group, 32 peptides were significant. Four peptide peaks were highly elevated (*m/z* 4209.80, 4090.88, 9282.45, 4053.87) and three peptide peaks were highly degraded (*m/z* 6629.17, 6431.29, 6643.35). Between MsPGN and FSGS group, 17 peptides were significant. Three peptide peaks were highly elevated (*m/z* 9282.45, 4643.86, 4978.40) and two peptide peaks were highly degraded (*m/z* 6643.84, 6629.18). Between MCNS and MN group, 35 peptides were significant. Two peptide peaks were highly

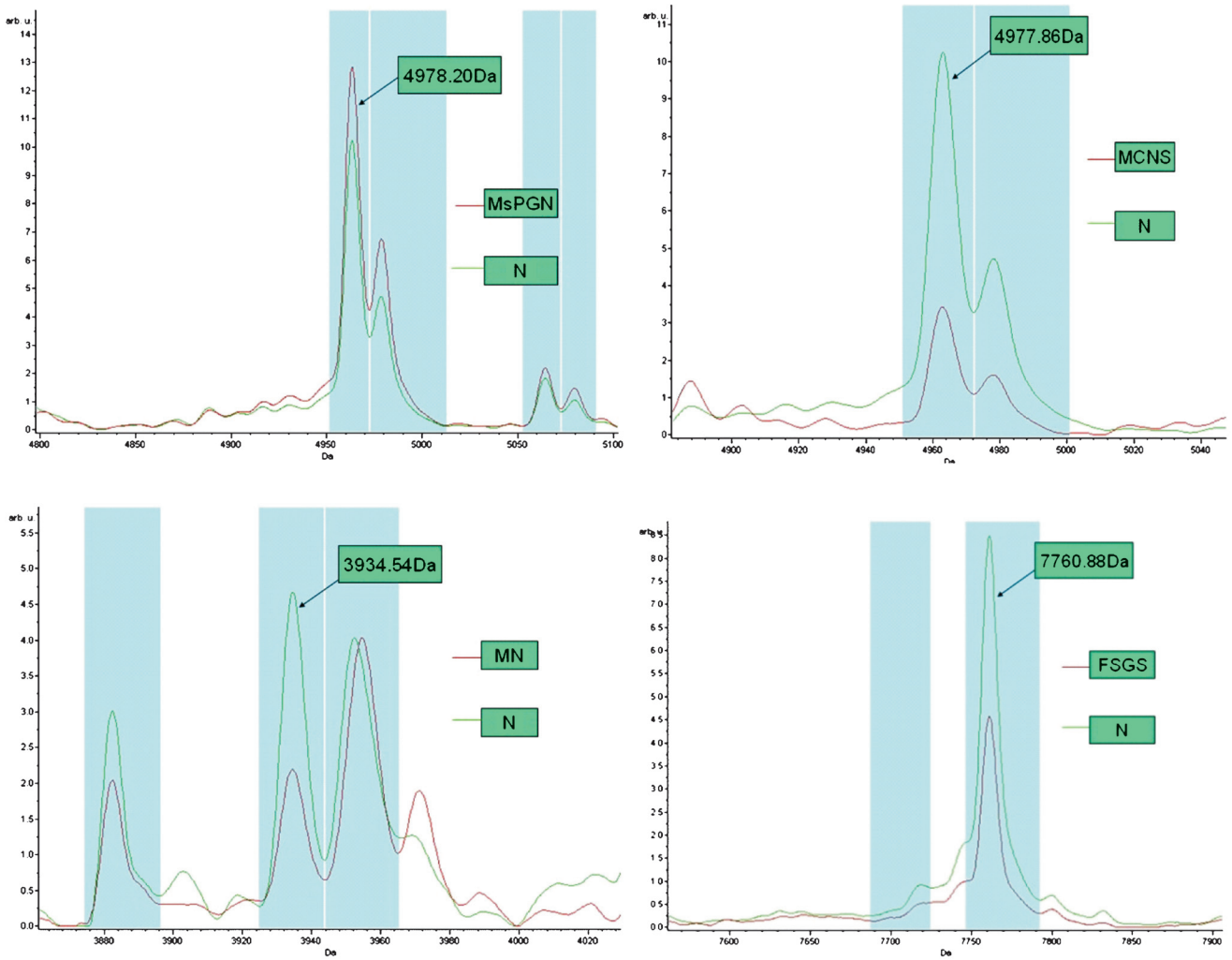

Fig. Representative spectra view of peptide peaks at the mass of 4978.20, 3934.54, 4977.86 and 7760.88 between normal controls and MsPGN, MCNS, MN, FSGS groups.

elevated (*m/z* 5917.31, 5902.89) and two peptide peaks were highly degraded (*m/z* 8686.78, 7761.55). Between MCNS and FSGS group, 32 peptides were significant. Two peptide peaks were highly elevated (*m/z* 5902.84, 5917.42) and one peptide peaks were highly degraded (*m/z* 4053.78). Between MN and FSGS group, 26 peptides were significant. Two peptide peaks were highly elevated (*m/z* 6430.67, 6628.50) and two peptide peaks were highly degraded (*m/z* 2104.41, 4053.55) (Table II; Fig.).

The raw data were analyzed using ClinPro Tools software 2.2, and GA processing was used to classify sample and establish diagnostic model. With the established model, the patients who experienced MsPGN, MCNS, MN and FSGS could be distinguished from normal controls with a cross validation of 96.18, 100, 98.53 and 94.12 per cent, respectively. The recognition capabilities were 100 per cent. MsPGN could be distinguished from MCNS, MN, FSGS with a cross validation of 96.08, 98.65, 90.76 per cent and with a recognition capability of 100, 100, 96.88 per cent, respectively. MCNS could be distinguished from MN and FSGS with a cross validation of 89.21 and 97.28 per cent, respectively. The recognition capabilities were 100 per cent. MN could be distinguished from FSGS groups with a cross validation of 100 per cent and with a recognition capability of 100 per cent.

Discussion

Compared to genomic approaches, proteomic analysis has the advantage of visualizing co- and post-translational modifications of proteins, possibly of relevance for biologic function. Alternative approaches

Table II. The significant differential peptide peaks between NS groups and Normal control groups using genetic algorithm

Groups	Mass	Dave	PTTA	Ave1	Ave2	StDev1	StDev2	CV1	CV2
MsPGN vs N	4053.72	7.99	<0.001	15.28	23.41	7.61	6.35	39.78	27.29
	5917.49	3.33	<0.001	12.79	9.47	6.01	2.76	36.92	29.12
	5902.94	5.45	0.005	24.41	18.96	11.56	5.51	37.36	29.07
	4978.20	2.12	0.014	9.39	5.28	4.68	2.98	43.33	36.53
	2660.15	8.64	0.024	19.48	28.12	9.92	19.32	50.93	58.73
MCNS vs N	4053.60	16.04	<0.001	7.23	23.28	4.08	6.35	46.42	27.29
	4977.86	3.11	<0.001	2.16	11.28	1.59	2.98	53.48	36.53
	5902.73	26.19	<0.001	45.15	18.96	20.65	5.51	45.74	29.07
	5917.41	14.76	<0.001	24.22	9.47	12.19	2.76	50.33	29.12
	2952.03	8.73	<0.001	14.95	6.21	7.36	1.37	49.24	22.06
	5335.65	7.74	<0.001	15.03	7.29	8.49	2.60	46.47	35.75
	2660.06	10.82	<0.001	17.31	28.12	7.29	19.32	42.18	58.73
MN vs N	4053.45	14.25	<0.001	9.03	23.28	4.96	6.35	54.92	27.29
	3934.54	2.78	<0.001	2.72	13.49	1.46	1.17	53.65	21.21
	4209.40	16.39	0.002	35.48	51.88	13.71	13.75	38.64	26.51
	6628.49	10.43	0.009	16.83	6.41	11.06	6.12	35.70	55.52
	6430.59	6.02	0.009	8.35	2.33	6.51	1.99	48.24	55.08
	6642.88	5.22	0.009	9.31	4.10	5.82	3.35	42.56	51.66
	2659.95	12.11	0.021	16.01	28.12	12.95	19.32	40.84	58.73
FSGS vs N	7760.88	4.06	<0.001	5.19	15.25	1.59	1.90	30.70	20.52
	9281.40	10.45	<0.001	14.55	25.01	5.87	6.99	40.38	27.94
	4053.43	9.54	<0.001	13.74	23.28	5.68	6.35	41.38	27.29
	1896.09	1.32	0.005	5.03	1.71	1.31	0.41	43.36	23.88
	2659.94	10.05	0.031	18.06	28.12	8.07	19.32	44.67	58.73
MsPGN vs MCNS	4963.38	9.33	<0.001	13.03	3.70	8.85	3.20	27.90	46.53
	4053.97	7.75	<0.001	14.62	6.87	7.24	3.97	29.51	37.77
	2952.28	6.89	<0.001	6.59	13.49	2.96	6.63	44.95	49.13
	5903.18	19.75	<0.001	23.12	42.88	10.96	19.61	47.42	45.74
	5917.75	10.92	<0.001	12.15	23.07	5.77	11.50	47.5	49.85
	4209.87	14.66	0.041	53.52	38.84	22.93	28.06	42.86	52.25
MsPGN vs MN	4209.80	19.69	<0.001	53.54	33.81	22.93	13.11	42.86	38.79
	4090.88	5.41	<0.001	15.79	10.39	6.67	3.48	42.27	33.44
	9282.45	7.62	<0.001	23.51	15.89	5.62	5.58	23.91	35.12
	4053.87	6.06	0.001	14.62	8.55	7.24	4.67	49.51	54.62
	6629.17	11.23	0.004	4.88	16.11	3.24	10.66	56.25	56.18
	6431.29	6.08	0.006	1.86	7.94	1.29	6.23	49.16	58.5
	6643.35	5.52	0.006	3.41	8.93	1.83	5.64	43.75	53.16
MsPGN vs FSGS	9282.45	9.66	<0.001	23.51	13.85	5.62	5.68	23.91	40.98
	4643.86	3.57	0.002	12.91	9.34	2.62	2.86	20.30	30.60
	6643.84	2.18	0.020	3.41	5.59	1.83	2.43	53.75	43.53
	6629.18	3.28	0.043	4.88	8.16	3.24	4.31	56.25	42.78
	4978.40	2.82	0.043	6.96	4.14	4.47	3.46	44.22	53.68
MCNS vs MN	5917.31	11.77	0.007	23.07	11.30	11.50	7.56	49.85	56.94
	5902.89	17.56	0.027	42.88	25.31	19.61	17.39	45.74	58.70
	8686.78	1.05	0.034	0.85	2.90	0.73	1.35	55.82	40.91
	7761.55	2.16	0.034	4.62	6.78	2.57	2.43	55.66	35.86
MCNS vs FSGS	5902.84	22.77	<0.001	42.88	20.11	19.61	10.15	45.74	50.47
	5917.42	12.18	<0.001	23.07	10.90	11.50	5.04	49.85	46.28
	4053.78	6.23	0.003	6.87	13.18	3.97	5.46	57.77	41.66
MN vs FSGS	2104.41	3.96	0.021	4.41	8.37	1.97	4.19	44.63	50.09
	6430.67	5.25	0.027	7.94	2.69	6.23	0.98	58.50	36.52
	6628.50	7.95	0.039	16.11	8.16	10.66	4.31	56.18	52.78
	4053.55	4.54	0.049	8.55	13.18	4.67	5.46	54.62	41.66

Mass, *m/z* value; Dave, Difference between the maximal and the minimal average peak area/intensity of all classes; PTTA: *P* value of *t*- test; Ave, Peak area/intensity average of class; StDev, Standard deviation of the peak area/intensity average of class; CV, Coefficient of variation in % of class; "1", The former class of groups; "2", The latter class of groups; N, normal individuals. Other abbreviations are as given in Table I

of measuring polypeptides are time-consuming for routine use, such as the classical method of comparing data from two-dimensional electrophoresis (2DE), subsequent isolation of the proteins from the gel and analysis by MS[11]. Another method, the surface-enhanced laser desorption and ionization (SELDI)[12-14], have several disadvantages, such as the low resolution and the loss of most proteins and peptides[15].

MALDI is a soft ionization technique used in mass spectrometry, allowing the analysis of biomolecules such as proteins, peptides, sugars, and large organic molecules. It is closely similar in character to electrospray ionization both in the relative softness and the ions produced. TOF-MS is ideally suited type to the MALDI. It has large mass range, which can reach a resolving power m/Δm of well above 20'000 full width at half maximum (FWHM). As a powerful tool for surveying complex patterns of biologically informative molecules, MALDI-TOF MS protein profiling has been applied in proteomics biomarker research[16-18] and become a promising tool in nephrology research[19-22].

In our study, serum samples were divided in five groups, including MsPGN, MCNS, FSGS, MN and normal controls. Differential peptide peaks were compared between the groups. These may be involved in specific immunity response or related to some risk factors, and may become potential biomarkers in clinical diagnosis. In our study valuable differential peptides were identified; potential biomarkers for NS diagnosis were obtained. With established diagnostic model, NS could be effectively separated from controls, which demonstrated that GA processing would be an effective method to set up diagnostic model with high sensitivity and specificity. The established model may act as alternative means to clinical diagnosis of NS. The next step would be to isolate and identify the unique biomarkers for the diagnosis of NS. A study with large sample is needed to generate more objective and conclusive results. One limitation in this pilot study was the small population. With a large number of cases, different stages of NS would be observed, different impact factors would be considered, more objective evaluation would be made for ClinProt technology applied in NS.

In conclusion, proteomic technology was applied to analyze the serum profiles of NS patients. This preliminary study indicated that proteomic analysis of serum with MALDI-TOF MS is a fast and reproducible approach, which may give an early idea of the pathology of nephrotic syndrome. Complete characterization would still require kidney biopsy until further advances in the field of proteomics is made.

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