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Proteomics and
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Characterization of Diabetic Nephropathy by Urinary Proteomic Analysis: Identification of a Processed Ubiquitin Form as a Differentially Excreted Protein in Diabetic Nephropathy Patients

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Background: **Identification of markers for prediction of the clinical course of diabetic nephropathy remains a major challenge in disease management. We established a proteomics approach for identification of diabetic nephropathy-related biomarkers in urine.**

Methods: **We used SELDI-TOF mass spectrometry and SAX2 protein arrays to compare protein profiles from urine of 4 defined patient groups. Samples from patients with type 2 diabetes (DM; n = 45) without nephropathy and without microalbuminuria (DM-WNP), patients with DM with macro- or microalbuminuria (DM-NP; n = 38), patients with proteinuria due to nondiabetic renal disease (n = 34), and healthy controls (n = 45) were analyzed. Anionic exchange, reversed-phase fractionation, gel electrophoresis, and mass spectrometry were used to isolate and identify proteins with high discriminatory power.**

Results: **A protein with *m/z* 6188 (P <0.0000004) was strongly released in the urine of healthy controls, patients with proteinuria due to nondiabetic disease, and DM-WNP in contrast to DM-NP patients. An *m/z* 14 766 protein (P <0.00008) was selectively excreted in the urine of DM-NP patients, whereas the protein with *m/z* 11 774 (P <0.000004) was significantly excreted by patients with proteinuria and DM-NP. The *m/z* 11 774 and *m/z* 14 766 mass peaks were identified as β_2-microglobulin and UbA52, a ubiquitin ribosomal fusion protein, respectively. The protein with *m/z* 6188 was identified as a processed form of ubiquitin.**

Conclusion: **The release of high amounts of UbA52 in urine of DM-NP patients could serve as a diagnostic marker, whereas the lack of the short form of ubiquitin raises interesting questions about the pathophysiology.**

The number of patients with end-stage renal disease is increasing worldwide. This increase is due mainly to the rapidly increasing number of patients with diabetic nephropathy. Diabetes is, after arterial hypertension, the 2nd most common disease in industrialized countries. According to American Diabetes Association criteria, the prevalence of diabetes is estimated to be approximately 7.9% *(1)*. In particular, incidence and prevalence of type 2 diabetes (DM)[4] have increased, with a disproportionate increase in prevalence *(2)*. This increase is attributable mainly to decreased cardiovascular mortality in patients with DM *(3)*. Thus, a higher percentage of patients with diabetes live long enough to develop other complications of the disease, including retinopathy and nephropathy. The incidence of nephropathy in type 1 diabetes patients is 20%–40% after a disease duration of 15–30 years *(4)*, and 10%–50% of DM patients will develop renal involvement. Diabetes patients with renal disease have a poor prognosis. Compared with dialysis patients without

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[4] Nonstandard abbreviations: DM, type 2 diabetes; DM-NP, DM with macro- or microalbuminuria; DM-WNP, DM without nephropathy and without microalbuminuria; WDM-NP, macroalbuminuria due to nondiabetic disease; MS, mass spectrometry; β2-M, β_2-microglobulin.

diabetes, diabetes patients have a 22% increased 1-year mortality and a 15% increased 5-year mortality *(5)*. Estimated dialysis treatment costs for a diabetes patient are approximately $51 000 per year compared with $39 000 for patients without diabetes *(5)*.

Risk factors for the development of diabetic nephropathy include genetic predisposition, poor glycemic control, arterial hypertension, and smoking. In type 1 diabetes, microalbuminuria is the best predictor of subsequent development of nephropathy [defined as persistent macroalbuminuria (>300 mg/24 h)], with approximately 50% of patients with microalbuminuria progressing to overt nephropathy. Conversely, microalbuminuria has less predictive value in DM because these patients are older and their microalbuminuria is often induced by arterial hypertension or heart failure *(6)*. Thus additional markers are urgently needed to identify diabetes patients at the highest risk for developing diabetic nephropathy. Proteomics offers a promising new alternative for detection of useful biomarkers *(7–9)*. In this study we aimed at establishing a robust procedure for the identification and characterization of proteins differentially excreted in the urine of diabetic patients with micro- and/or macroalbuminuria.

Materials and Methods

STUDY POPULATION AND SAMPLES

Four patient groups were rigidly defined on the basis of clinical course, microalbuminuria, and serum creatinine concentration. The groups included patients with DM with micro- or macroalbuminuria (DM-NP; n = 38), patients with DM without micro- or macroalbuminuria (DM-WNP; n = 45), patients with macroalbuminuria due to nondiabetic disease (WDM-NP; n = 34), and healthy controls (n = 45; Table 1).

HANDLING OF URINE SAMPLES FOR PROTEOMICS ANALYSIS

Clinical sample procurement and analysis as well as data management of this study were approved by the local Institutional Ethics Review Committee of the University Hospital of the Georg-August-University, Goettingen, Germany. All patients had given their informed consent before the study.

For all of our proteomics experiments, midstream urine was used because of its limited protease activity. Urine was collected in 100-mL tubes supplied with protease inhibitors in the form of the Complete™ protease 1 tablet from Roche. To decrease the protein degradation effect, 2 protease inhibitor tablets were dissolved in 1 mL 100 mmol/L phosphate buffer and the dissolved protease cocktail was added to each 100-mL tube before collection of urine. After collection of urine, tubes were reversed immediately and gently to mix the protease inhibitors with the urine proteins. Subsequently, urine samples were centrifuged for 45 min at 3000*g* and 4 °C. After elimination of the precipitates, the supernatant was divided into 10-mL aliquots and used immediately or stored at −80 °C until use.

LABORATORY METHODS

For each collected urine sample, we used 10 mL to measure clinical chemical variables, including urine creatinine, protein, and albumin concentrations, by use of routine methods at the Institute of Clinical Chemistry or the laboratory of the Department of Nephrology and Rheumatology, Georg-August-University, Goettingen, Germany.

PROTEIN PROFILING OF URINE SAMPLES USING PROTEIN-CHIP ARRAYS

Microalbuminuria measurement was used for standardization of urine sample handling. All samples were adjusted to the same concentration of albumin (20 mg/L) before starting the profiling experiments. In the case of urine control samples, a concentration step using a 1000-Da cutoff filter (Amicon) was necessary to attain the required albumin concentration. The samples were subsequently denatured for 15 min at 4 °C with 8 mol/L urea in 40 mmol/L Tris/HCl, pH 7.2, supplemented with protease inhibitors. The protein profiling was performed as described in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue9.

This study followed criteria from published guidelines for peptide and protein identification *(10)*.

PROTEIN PRECIPITATION AND ESTIMATION

Before the sample fractionation and enrichment of protein markers, total protein precipitation was performed. Five different urine aliquots per case were pooled together and 20 mL from the pooled urine was concentrated to 2 mL with a column 1000-Da cutoff filter (Amicon). Subsequently protein precipitation was carried out by addition of 3 volumes of ice-cold acetone containing 200 g trichlo-

Table 1. Characteristics of patients whose urine was sampled for the study.

	DM-NP patients (n = 38)	DM-WNP patients (n = 45)	WDM-NP patients (n = 34)	Controls
Age, years	65 (11)	60 (11)	57 (20)	40 (15)
Male/female	25/13	28/17	20/14	26/19
Albuminuria, (mg/24 h)	689 (341)	6 (3)	1320 (317)	<30
HbA1c	9.4 (0.55)	8.6 (0.37)	Not tested	4.9
Retinopathy	17	13	0	0
Creatinine clearance, (mL/min)	117 (12)	103 (16)	Not tested	112

roacetic acid/L. The protein concentrations were measured according to the Bradford method *(11)*, using BSA as a calibrator.

HYDROPHOBIC AND ANIONIC EXCHANGE FRACTIONATIONS

Hydrophobic and anionic exchange fractionations were carried out as described in the online Data Supplement. For analytical preparation, sample fractions of interest were separated by SDS-PAGE before in-gel digestion and mass spectrometry (MS) analysis. Gels were fixed and stained with a modified silver stain as previously described *(12)*.

PASSIVE ELUTION AND MOLECULAR WEIGHT CONTROL

To control the molecular weight of the identified proteins, the gel bands with the proteins were cut and processed for passive elution as described previously *(13)*.

IN-GEL DIGESTION AND PEPTIDE SEQUENCE ANALYSIS

In-gel digestion and peptide extraction were carried out as described previously *(14)*. Subsequently the extracted peptides were subjected to peptide sequence analysis. The samples were dissolved in 1 g/L formic acid and processed as described previously *(14)*. Processed data were searched against the MSDB and Swiss-Prot databases through the Mascot search engine with a peptide mass tolerance of 50 parts per million and fragment tolerance of 100 millimass units. Protein identifications with at least 2 peptides sequenced were considered significant.

SELDI-TOF MS IMMUNOCAPTURING OF UBIQUITIN

The SELDI immunoassay was performed according to Tolson et al. *(15)* with urine from controls and DM-NP and DM-WNP patients. Spectra were externally calibrated with bovine ubiquitin (*m/z* 8564.8) and bovine cytochrome C (*m/z* 12 230.9).

WESTERN BLOT ANALYSIS

We performed verification of the proteomic analysis data by Western blot analysis according to the methods described by Towbin et al. *(16)*. Mouse antiubiquitin monoclonal antibody (Sigma-Aldrich) and horseradish peroxidase–linked sheep antimouse antibody (Amersham Biosciences) were used as primary and secondary antibodies, respectively.

UBIQUITIN DEGRADATION ASSAY

Urine samples were collected from healthy controls and from DM-NP patients with or without addition of protease inhibitors. We incubated 100 μL of each urine sample with added ubiquitin (1 μmol/L) for different times at room temperature. Subsequently, the urine samples were prepared for the ProteinChip array as described above, and the ubiquitin degradation in urine was monitored by sample profiling on SAX2 protein arrays.

STATISTICAL ANALYSIS

The protein profiles obtained from the different samples were normalized to the control group. The differences in the peak intensity for the various clusters were quantified using the nonparametric Mann–Whitney *U*-test implemented in the Biomarker Wizard software. The peaks differentially expressed between DM-NP and the other patient groups were used for classification analysis with Biomarker Patterns™ software (Ciphergen) as previously described *(17)*.

Results

REPRODUCIBILITY OF THE PROTEIN PATTERN

The peak detection and data preprocessing of the proteins retained on the different protein arrays were carried out on a PBS-II mass reader. Acquisition of the highest mass was set to 150 kDa, with an optimization range from 5 to 20 kDa. A mass accuracy of 0.1% was achieved by external calibration. The reproducibility of the protein pattern was strongly dependent on the sample collection procedures. We optimized a urine-handling protocol that allows fast collection and processing of urine, leading to almost total inhibition of protein degradation and good reproducibility of the data. The mass spectra of samples from the same urine specimen were identical in the quality and quantity of protein/peptide peaks. Using the Biomarker Wizard Software (Ciphergen), we generated 52 peaks or common peaks (signal-to-noise ratio >5) from the identified peaks. Most of the peaks were detected between *m/z* 2000 and *m/z* 20 000, and these were considered the most useful for protein profiling. For 8 protein peaks present in all different urine samples, measured in 8 independent experiments, we determined the mean CVs of reliable peaks in urine control obtained from different samples and laser power settings. The CVs of the peak intensities for the 8 peaks ranged from 12% to 32% and did not differ statistically between the different sample and laser settings.

PROTEIN EXCRETION PATTERNS IN NORMAL AND PATHOLOGICAL URINES

Urine samples from 45 DM-WNP patients, 38 DM-NP patients, 34 WDM-NP patients, and 45 healthy controls were analyzed on SAX2 ProteinChip arrays. From all protein peaks detected, 15 had low *P* values in different statistical tests ($P < 0.001$, Student *t*-test; see Table 1 in the online Data Supplement), indicating strong statistical significance of differential protein release in urine. Special focus was on proteins differentiating the DM-NP patients from the rest of the sample groups. The analysis revealed striking excretion patterns in a subset of DM-NP urine samples, exemplified as spectral views in Fig. 1, for a set of prominent peaks with *m/z* 6188 (Fig. 1A), 11 774, and 14 766 (Fig. 1B).

A heat map from DM-NP and DM-WNP samples was generated (see Fig. 1 in the online Data Supplement), and the 3 mentioned peaks are indicated on the map. These peaks clearly differentiated the 2 sample groups. The *m/z*

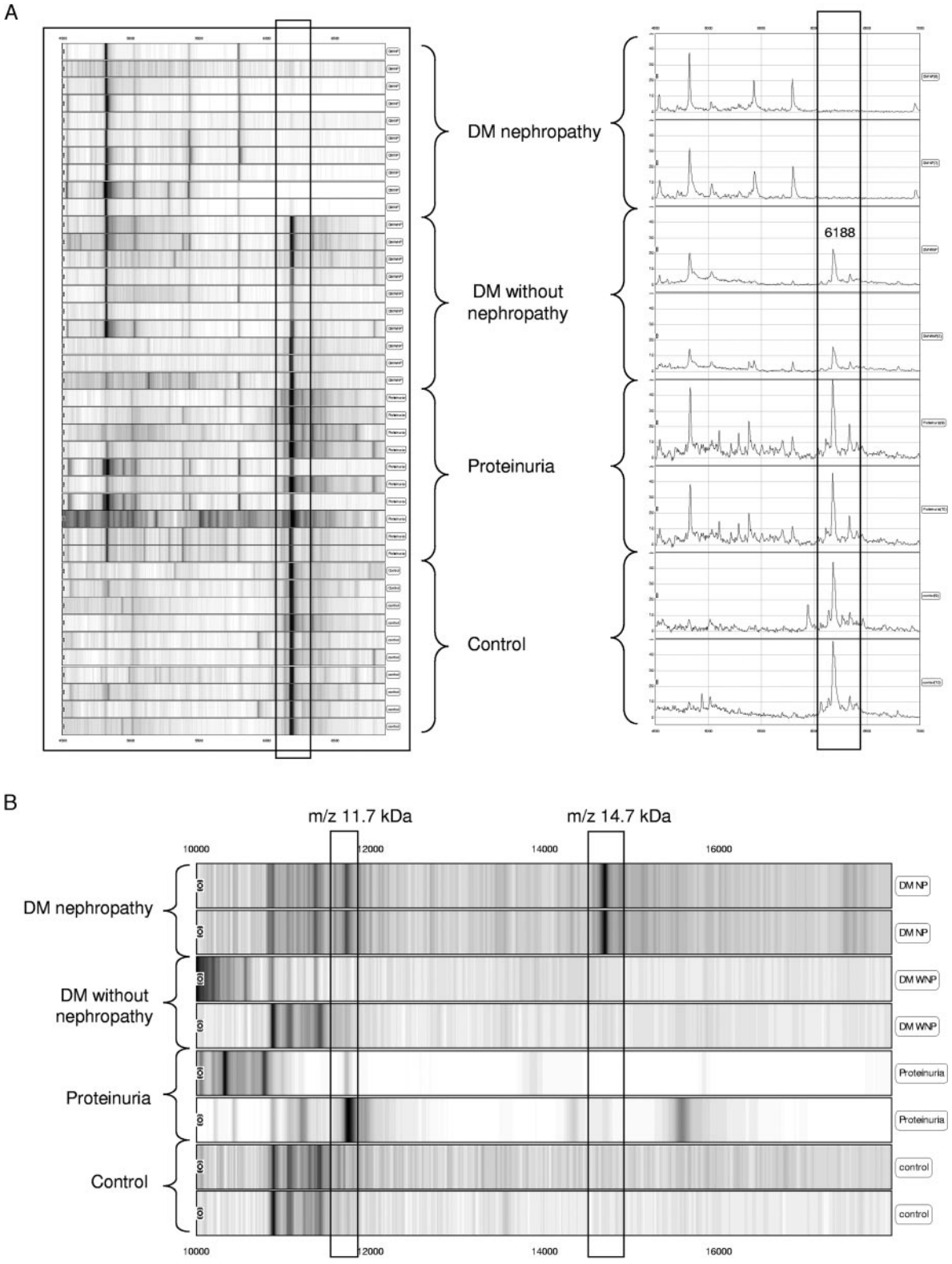


Fig. 1. Representative SELDI-TOF MS protein pattern from urine of the different investigated groups.

(*A*), the low molecular weight range between 4500 and 7000 Da in 10 urine samples from each studied group (10 from DM-NP, 10 from DM-WNP, and 10 from WDM-NP patients and 10 from healthy controls) illustrated in gel view in the *left panel*. The *mass peak* with *m/z* 6188 differentiating DM-NP from the other groups is *highlighted* with the *box*. The *right panel* indicates the mass spectral view from the same mass peak. (*B*), gel view from the middle molecular weight range, 10 000–18 000. *Boxes* indicate the peak masses *m/z* 11 774 and 14 766 found to be differentially excreted in DM-NP patient urines.

6188 was downregulated in DM-NP samples compared with DM-WNP, whereas the proteins with *m/z* 11 774 and 14 766 showed a strong negative correlation to the smaller protein with *m/z* 6188. Quantitatively, the mean normalized intensities of the peak with *m/z* 6188 were severalfold lower in DM-NP compared with other groups (Fig. 2A). Conversely, the *m/z* 14 766 intensities were higher in DM-NP patients and lower in all other groups (Fig. 2C). The peak *m/z* 11 774 was clearly higher in DM-NP and WDM-NP patients than the other groups (Fig. 2B). With few exceptions (6 samples), none of the DM-NP samples showed significant excretion of the protein with *m/z* 6188 in urine. The diagnostic value for these 3 peaks was demonstrated in highly significant ROC plots showing areas under the curves well above 0.9 (see Fig. 2 in the online Data Supplement).

We created a classification tree from the training set to differentiate DM-NP and DM-WNP groups. The selected classification tree used 4 splitters with distinct masses of *m/z* 14 766, 6188, 11 774, and 8602 and classified the cases into 5 terminal nodes (see Fig. 3 in the online Data Supplement). The error rate of the generated classification tree was estimated through a process of cross-validation.

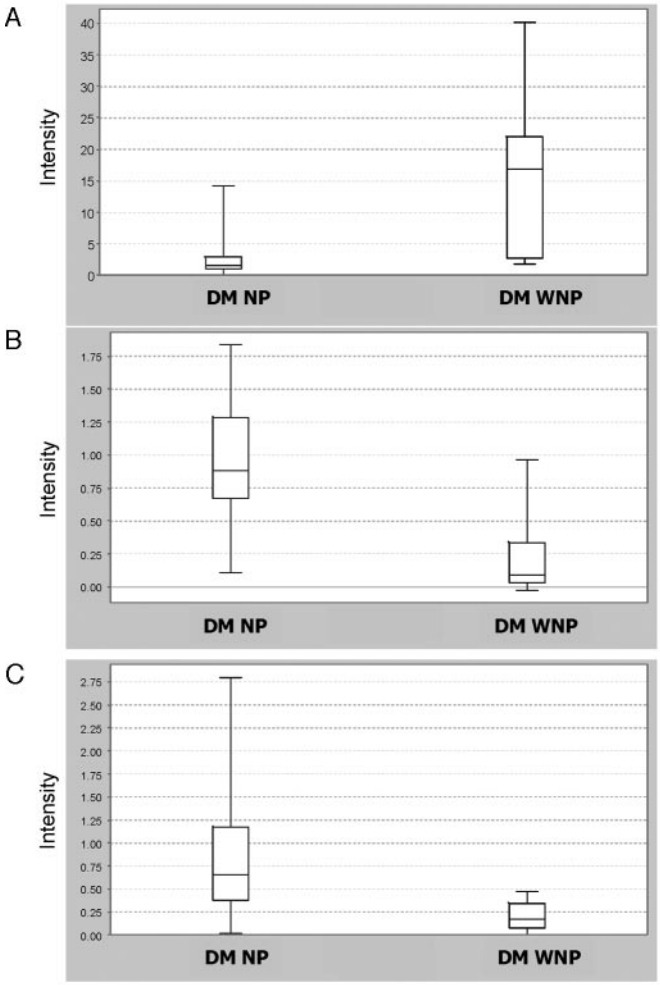


Fig. 2. Differential excretion of the 3 mass peaks (*m/z* 6188, 11 774, and 14 766) in urine from DM-NP and DM-WNP patients.

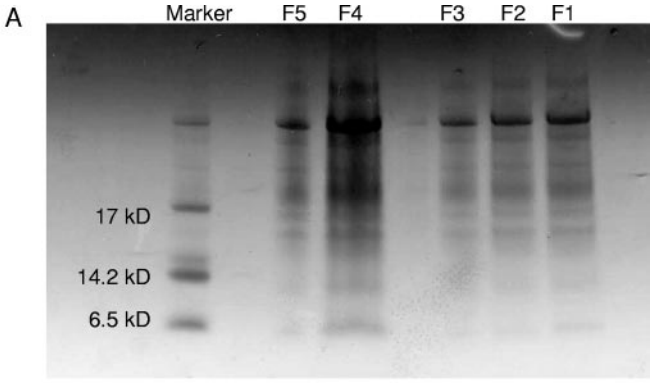
Box plots of relative intensities of *m/z* 6188 (*A*), *m/z* 11 774 (*B*), and *m/z* 14 766 (*C*) as detected by SELDI-TOF analysis of 38 DM-NP and 45 DM-WNP urine samples. The *lines* represent the median value within each group. The *boxes* represent range of variation statistics, and the *error bars* indicate the minimum and maximum values.

PURIFICATION AND IDENTIFICATION OF DIFFERENTIALLY EXPRESSED PROTEINS

To purify and identify differentially excreted proteins from pathological and healthy urine, samples were selected in which the protein peaks of interest were present in high abundance. These samples were fractionated by anion-exchange and reversed-phase chromatography as described in *Materials and Methods.* After anion-exchange fractionation, the fraction containing large amounts of the protein of interest, e.g., F4 for *m/z* 6188 (Fig. 3A), was used for the 2nd fractionation. The reversed-phase eluates showed high amounts of the protein *m/z* 6188 in the fraction with 20% acetonitrile in elution buffer (Fig. 3B). This fraction was used for passive elution from the gel and molecular weight control of the protein. Subsequently the rest of the eluate served for in-gel digestion and MS analysis to identify the protein (Fig. 4A). Both MALDI-TOF MS and electrospray ionization–MS/MS analysis allowed the identification of the *m/z* 6188 as ubiquitin, *m/z* 11 774 as β_2-microglobulin (β-2M), and *m/z* 14 766 as ubiquitin and ribosomal protein L40. The MALDI-TOF MS–derived peptide fingerprint for ubiquitin (*m/z* 6188) is shown in Fig. 4A. The ubiquitin has a theoretical molecular weight of 8565 Da, the ubiquitin identified in urine has a mass of 6188 Da. The identification of the ubiquitin was based on MS/MS sequencing of selected tryptic peptides; 3 peptides/protein could be sequenced. The fragmentation of the doubly charged tryptic peaks of *m/z* 534.35, *m/z* 894.49, and *m/z* 541.32 from the 6188-Da ubiquitin showed a complete y- and b-ion series corresponding to the sequences ESTLHLVLR, TITLEVEPSDTIENVK, and TLSDYNIQK, respectively (Fig. 4B). In addition, the high Mascot score (up to 91) and extensive sequence coverage (up to 53%) demonstrated a high level of confidence in the protein identification. The summary of the MS/MS sequencing analysis of all 3 proteins is given in Table 2 in the online Data Supplement.

VALIDATION OF DIFFERENTIAL UBIQUITIN EXCRETION BY WESTERN BLOT AND SELDI-TOF MS IMMUNOCAPTURING

To validate the differential excretion of *m/z* 6188 ubiquitin in the urine of DM-NP patients compared with the other groups, we performed Western blotting on pooled samples from each group. In the case of DM-NP, patients with almost no *m/z* 6188 peak were chosen for the analysis. Ubiquitin was highly excreted in the urine of the DM-WNP patients, healthy controls, and WDM-NP patients (see Fig. 4A in the online Data Supplement). DM-NP patients released nondetectable amounts of *m/z* 6188 ubiquitin (see Fig. 4A in the online Data Supplement).



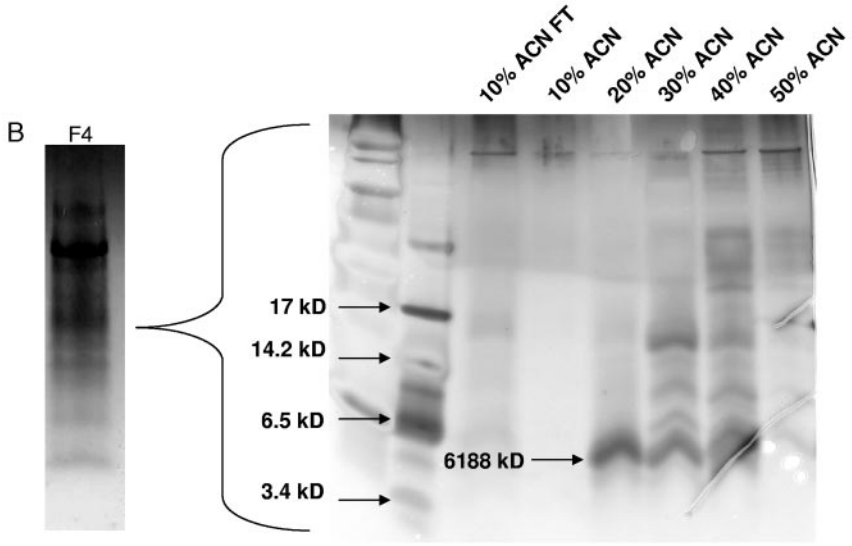


Fig. 3. Workflow of protein fractionation and purification of the protein markers.

The purification and identification of the *m/z* 6188 is given as an example. (*A*), *SDS gel* from the anionic exchange fractionation. *F1–5* indicate the different pH fractions eluted from the column. (*B*), *SDS gel* from the reversed-phase fractionation. *F4* from the anionic exchange fractionation, containing the highest amount of the mass peak *m/z* 6188, was taken for the fractionation. The eluates from the different ACN concentrations are visible on the *SDS gel*. The fraction from the 20% ACN shows the highest purity of the protein *m/z* 6188. *ACN*, acetonitrile; *FT*, flow-through; *kD*, kilodalton.

Similar results were found when using SELDI-TOF MS immunocapturing of ubiquitin. In this case a very small amount of *m/z* 8565 ubiquitin was detected in the urine of DM-NP patients but almost no *m/z* 6188 ubiquitin (see Fig. 4B in the online Data Supplement).

UBIQUITIN DEGRADATION ASSAY

To investigate whether 6188-Da ubiquitin is a degradation product or a splice form of normal ubiquitin, we performed a ubiquitin degradation assay as described in *Materials and Methods.* In control urine without protease inhibitors, ubiquitin was degraded within 10 min and the peak with *m/z* 6188 increased in intensity (Fig. 5A). Conversely, urine spectra from DM-NP patients showed almost no degradation of ubiquitin even after 1 h incubation at room temperature (Fig. 5B). Addition of protease inhibitors blocked ubiquitin degradation in control urine (Fig. 5C).

Discussion

Definite diagnosis of diabetic nephropathy is currently based on renal biopsy findings. In most cases, however, the diagnosis can be reliably made in patients with macroalbuminuria in the presence of diabetic retinopathy. Microalbuminuria is often used as a prognostic marker in type 1 diabetes, because approximately 50% of type 1 diabetes patients with microalbuminuria will eventually develop diabetic nephropathy. Conversely, microalbuminuria is of much less value as a marker in DM because it has a variety of causes, including hypertension. Thus, additional markers are needed to identify patient groups with a high risk of developing overt diabetic nephropathy. We used a proteome urine analysis approach to identify such markers.

Proteinuria is routinely evaluated in the urine by use of 3 different collection methods: random spot collection, mostly for albumin-to-creatinine ratio; 24-h urine collections; and timed collections *(18)*. For our urine proteome analysis, midstream urine was collected in the morning after the 1st morning void because similar protocols were used with success for proteome urine analysis *(19, 20)*. Proteome-wide searches for biomarkers of disease processes have recently been shown to be useful in discovering biomarkers for the diagnosis of different diseases *(21–23)*. Concerning diabetic nephropathy, proteomics have recently been applied with success to analyze kidney tissues from rodent models *(24, 25)*, but the larger amounts of material needed seem to be a limiting factor

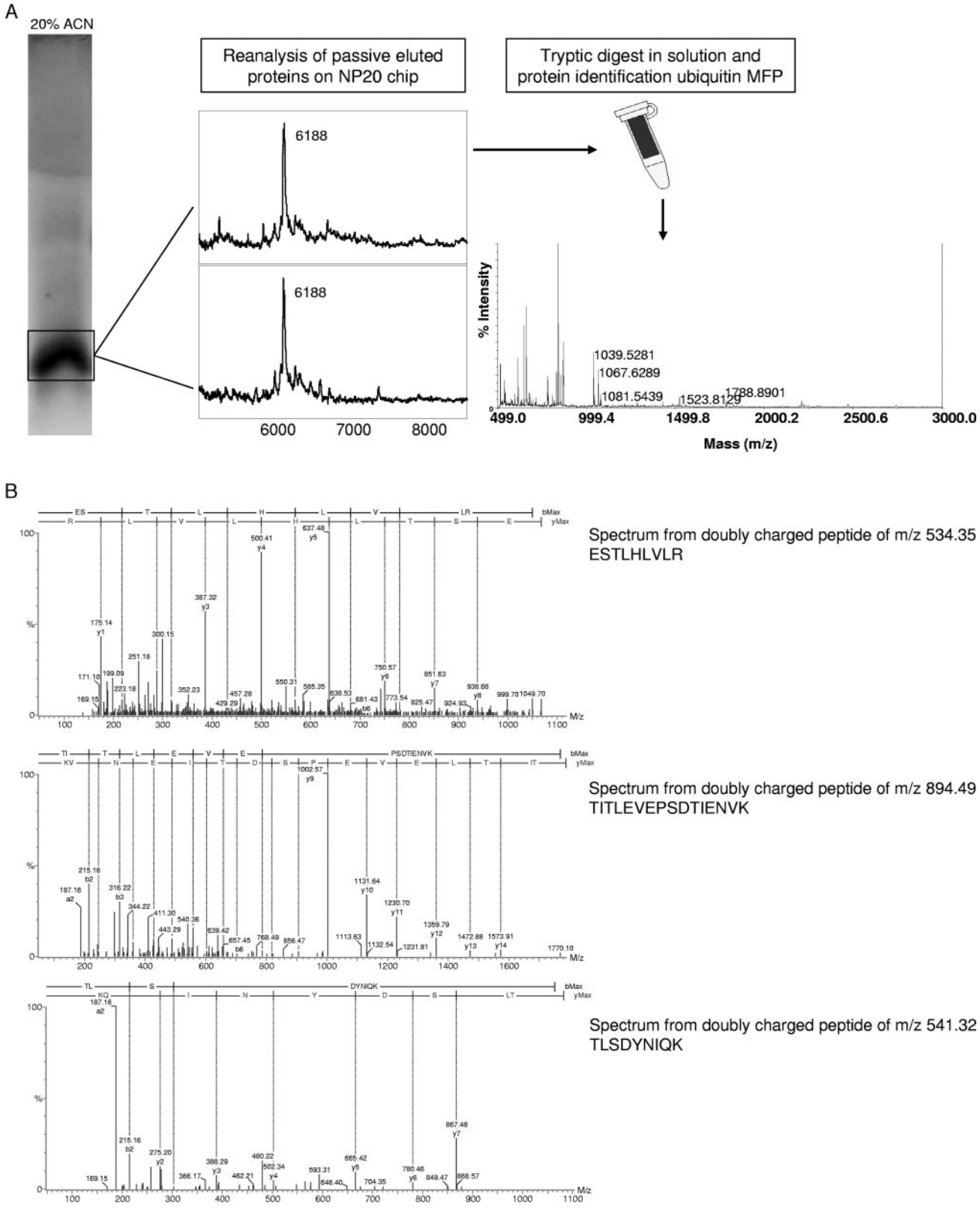


Fig. 4. Identification of proteins by MS.

(*A*), the *protein band* in the *box* was excised from the gel and submitted to passive elution before tryptic digest and protein identification. The proteins were analyzed on normal phase (*NP20*) array by SELDI-TOF MS. The *peaks* showing masses of *m/z* 6188 were identical to the protein peaks of interest in SELDI-TOF MS. The tryptic digest from peak *m/z* 6188 generated the MFP identifying the protein as ubiquitin. The peptide mass sequencing confirmed the MFP results. (*B*), representative product ion mass spectrum from the doubly charged tryptic precursor peaks of *m/z* 534.35, 894.49, and 541.32 from the 6188-Da ubiquitin showed a complete y- and b-ion series corresponding to the sequences ESTLHLVLR, TITLEVEPSDTIENVK, and TLSDYNIQK, respectively. *MFP*, mass fingerprint.

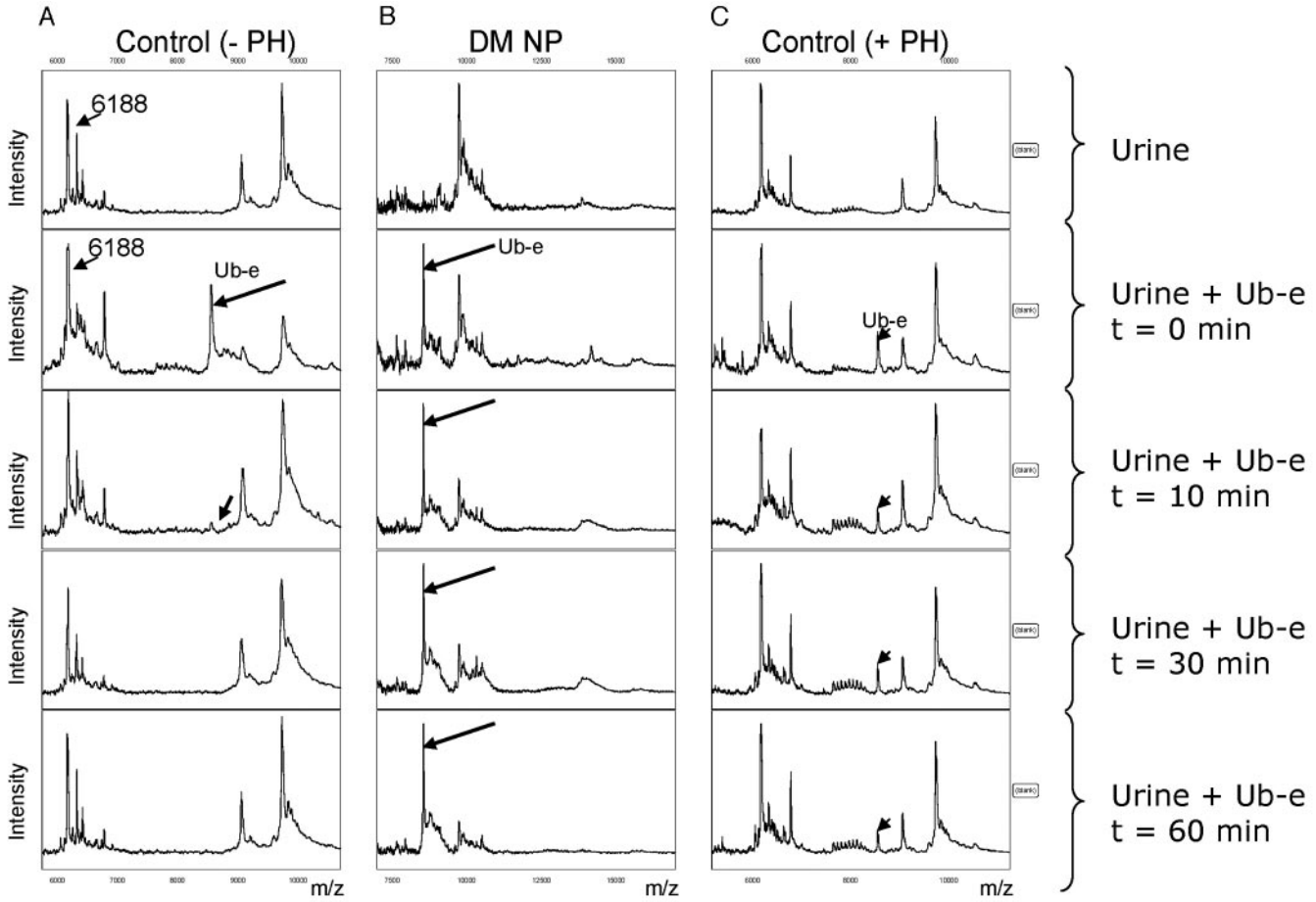


Fig. 5. Ubiquitin degradation assay.

Urine samples from healthy controls and DM-NP patients were collected with and without protease inhibitors. Subsequently the samples were supplied with exogenous ubiquitin and incubated for different time periods at room temperature. (*A*), control urine without protease inhibitors. Exogenous ubiquitin showed strong degradation within the first 10 min. (*B*), the DM-NP urine spectra showed almost no degradation of exogenous ubiquitin even after 1 h incubation. (*C*), protease inhibitors impeded exogenous ubiquitin degradation in control urine. *Ub-e*, exogenous ubiquitin.

for applications in human biopsies *(26)*. The noninvasive and easy sampling of urine makes it very attractive for proteomic analyses. Urinary proteomics has been successfully applied to the study of DM and diabetic nephropathy *(27, 28)*. We combined proteomic techniques with immunological validation methods to determine whether urine can be used for the identification of a marker specific for diabetic nephropathy. The analysis methods of the protein patterns can be critical for the identification of valid markers. Earlier studies performed with SELDI-TOF MS and fluids used different methods to compare protein profiles between different clinical outcomes. Visual inspection has the advantage of high stringency in the detection of differences in the protein patterns *(29)*, and bioinformatics facilitates the detection of small but significant differences within patterns *(21, 30, 31)*. In our pattern analysis, we combined both methods to get a maximum outcome with high stringency. Three different proteins were identified as markers for differentiating urine from DM-NP, DM-WNP, and WDM-NP patients. As expected, β2-M was found in larger amounts in the urine of DM-NP and WDM-NP patients compared with controls and DM-WNP patients. Schardijn et al. *(32)* and Schardijn and Statius van Eps *(33)* demonstrated a strong correlation between the β2-M concentration in serum and the glomerular filtration rate. Alteration in β2-M concentration has been observed in patients with different diseases, including diabetic nephropathy *(23, 33, 34)*. Our results confirm the alteration of β2-M concentration in urine of patients with DM-NP and in patients with proteinuria due to nondiabetic renal disease.

UbA52 is a ubiquitin fusion protein (128 amino acids) made of 60S ribosomal protein (52 amino acids) attached to a ubiquitin peptide (76 amino acids) *(35)*. In the kidney, the UbA52 was exclusively located in renal tubules and its expression in mouse kidney was found to be proportional to the glucose concentrations in blood. Gene expression analyses displayed an overexpression of the UbA52 protein in kidney of diabetic newborn mice *(36)*. UbA52 expression is regulated by various types of injuries, such as oxidative and carbonyl stress, which are important factors in the pathophysiology of diabetic nephropathy

and apoptosis *(36, 37)*. Selective expression of UbA52 in the renal tubules suggests that the ubiquitin-proteasome proteolytic system is indeed operative in this compartment of the kidney and might play an important role in diabetic nephropathy. Our data support the hypothesis that the increased expression of UbA52 in tubular cells of DM-NP patients combined with acute tubular injury and cell apoptosis results in alteration of UbA52 concentration in urine of diabetic nephropathy patients.

In addition to UbA52, another ubiquitin form was found to be released in smaller amounts in urine of DM-NP patients than in the 3 other study groups. The ubiquitin 6188 Da is smaller than the known ubiquitin 8565 Da. MS analysis of the tryptic digest from the 6188-Da protein showed 53% sequence coverage of the 8565-Da protein. The sequenced peptides are from the middle part of the protein (see Fig. 5 in the online Data Supplement). Neither mass fingerprinting nor peptide sequencing analysis could deliver any sequence coverage from the N- and C-terminal part of the protein. We therefore hypothesize that the identified shorter form of ubiquitin lacks the N- and C-terminal amino acids, unlike regular ubiquitin. Our ubiquitin degradation assay showed strong degradation of ubiquitin in the urine of healthy individuals without protease inhibitors, whereas in the urine of DM-NP patients, ubiquitin remained intact. These data support the presence in control urine of a protease that is responsible for ubiquitin degradation; this protease is missing or is present in low amounts in DM-NP urine. Because of the degradation of short-lived and abnormal proteins, ubiquitination and the ubiquitin-proteasome pathway control many processes, including proteolysis and intracellular trafficking *(35, 38)*. Ubiquitin-specific proteases are likely to play a central role in the regulation of all processes in which ubiquitin is involved *(39, 40)*. The protease responsible for the degradation of ubiquitin to ubiquitin 6188 Da is still not known, and the role of the short form of ubiquitin in the protein degradation system remains a matter for further investigation.

In summary, in this pilot study we identified 3 different proteins that were differentially excreted in the urine of diabetic nephropathy patients compared with the other groups. A processed form of ubiquitin with *m/z* 6188 was missed in the urine of diabetic nephropathy patients. This ubiquitin form could be used as a prognosis marker for DM-NP. Quantification of this protein during the progressive disease course in the DM-NP patients will give interesting information on the development of the diseases and serve as a good marker for prognosis. Moreover, the ubiquitin degradation assays confirmed the potential role of a urinary protease whose absence was specific for diabetic nephropathy. The identification of this protease, and longitudinal studies in larger patient groups, will determine the usefulness of the short form of ubiquitin as a marker for predicting the clinical course and the potential role of the protease in the pathophysiology of diabetic renal involvement.

UbA52 was highly expressed in tissue of DM-NP mice *(36)*. In our study, we found an alteration of UbA52 concentrations in urine of DM-NP patients. The presence of such an indicator of tissue injury in urine may result in clinical applications for diagnosis of DM-NP. Further studies in larger patient groups will determine the usefulness of this urine protein as a diagnosis marker.

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References

1. Mokdad AH, Ford ES, Bowman BA, Dietz WH, Vinicor F, Bales VS, et al. Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001. JAMA 2003;289:76–9.
2. Janka HU. Increased cardiovascular morbidity and mortality in diabetes mellitus: identification of the high risk patient. Diabetes Res Clin Pract 1996;30(Suppl):85–8.
3. Ritz E, Dikow R. Hypertension and antihypertensive treatment of diabetic nephropathy. Nat Clin Pract Nephrol 2006;2:562–7.
4. Fioretto P, Bruseghin M, Berto I, Gallina P, Manzato E, Mussap M. Renal protection in diabetes: role of glycemic control. J Am Soc Nephrol 2006;17:S86–9.
5. Remuzzi G, Schieppati A, Ruggenenti P. Clinical practice. Nephropathy in patients with type 2 diabetes. N Engl J Med 2002;346: 1145–51.
6. Mykkanen L, Haffner SM, Kuusisto J, Pyorala K, Laakso M. Microalbuminuria precedes the development of NIDDM. Diabetes 1994;43:552–7.
7. Dihazi H. Clinical proteomics: an insight into the urinary proteome: interview with Dr. Hassan Dihazi. Expert Rev Proteomics 2006;3: 481–2.
8. Dihazi H, Muller GA. Urinary proteomics: a tool to discover biomarkers of kidney diseases. Expert Rev Proteomics 2007;4: 39–50.
9. Muller GA, Muller CA, Dihazi H. Clinical proteomics: on the long way from bench to bedside? Nephrol Dial Transplant 2007;22: 1297–300.
10. Wilkins MR, Appel RD, Van Eyk JE, Chung MC, Gorg A, Hecker M, et al. Guidelines for the next 10 years of proteomics. Proteomics 2006;6:4–8.
11. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
12. Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 1996;68:850–8.
13. Dihazi H, Müller C, Asif A, Flad T, Elmaouhoub A, Müller G. Whole cell profiling and identification of galectin-1 as a potential marker of renal cell carcinoma. Proteomics Clinical Applications 2007;1: 200–14.

14. Dihazi H, Asif AR, Agarwal NK, Doncheva Y, Muller GA. Proteomic analysis of cellular response to osmotic stress in thick ascending limb of Henle's loop (TALH) cells. Mol Cell Proteomics 2005;4: 1445–58.

15. Tolson JP, Flad T, Gnau V, Dihazi H, Hennenlotter J, Beck A, et al. Differential detection of S100A8 in transitional cell carcinoma of the bladder by pair wise tissue proteomic and immunohistochemical analysis. Proteomics 2006;6:697–708.

16. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications: 1979. Biotechnology 1992;24: 145–9.

17. de Bont JM, den Boer ML, Reddingius RE, Jansen J, Passier M, van Schaik RH, et al. Identification of apolipoprotein A-II in cerebrospinal fluid of pediatric brain tumor patients by protein expression profiling. Clin Chem 2006;52:1501–9.

18. Molitch ME, DeFronzo RA, Franz MJ, Keane WF, Mogensen CE, Parving HH. Diabetic nephropathy. Diabetes Care 2003;26(Suppl 1):S94–8.

19. Decramer S, Wittke S, Mischak H, Zurbig P, Walden M, Bouissou F, et al. Predicting the clinical outcome of congenital unilateral ureteropelvic junction obstruction in newborn by urinary proteome analysis. Nat Med 2006;12:398–400.

20. Schaub S, Wilkins J, Weiler T, Sangster K, Rush D, Nickerson P. Urine protein profiling with surface-enhanced laser-desorption/ ionization time-of-flight mass spectrometry. Kidney Int 2004;65: 323–32.

21. Clarke W, Silverman BC, Zhang Z, Chan DW, Klein AS, Molmenti EP. Characterization of renal allograft rejection by urinary proteomic analysis. Ann Surg 2003;237:660–4.

22. Li J, Zhang Z, Rosenzweig J, Wang YY, Chan DW. Proteomics and bioinformatics approaches for identification of serum biomarkers to detect breast cancer. Clin Chem 2002;48:1296–304.

23. Schaub S, Wilkins JA, Antonovici M, Krokhin O, Weiler T, Rush D, et al. Proteomic-based identification of cleaved urinary beta2-microglobulin as a potential marker for acute tubular injury in renal allografts. Am J Transplant 2005;5:729–38.

24. Thongboonkerd V, Barati MT, McLeish KR, Pierce WM, Epstein PN, Klein JB. Proteomics and diabetic nephropathy. Contrib Nephrol 2004;141:142–54.

25. Thongboonkerd V, Malasit P. Renal and urinary proteomics: current applications and challenges. Proteomics 2005;5:1033–42.

26. Susztak K, Bottinger EP. Diabetic nephropathy: a frontier for personalized medicine. J Am Soc Nephrol 2006;17:361–7.

27. Hong CY, Hughes K, Chia KS, Ng V, Ling SL. Urinary alpha1-microglobulin as a marker of nephropathy in type 2 diabetic Asian subjects in Singapore. Diabetes Care 2003;26:338–42.

28. Rossing K, Mischak H, Parving HH, Christensen PK, Walden M, Hillmann M, et al. Impact of diabetic nephropathy and angiotensin II receptor blockade on urinary polypeptide patterns. Kidney Int 2005;68:193–205.

29. Schaub S, Rush D, Wilkins J, Gibson IW, Weiler T, Sangster K, et al. Proteomic-based detection of urine proteins associated with acute renal allograft rejection. J Am Soc Nephrol 2004;15:219–27.

30. O'Riordan E, Orlova TN, Mei JJ, Butt K, Chander PM, Rahman S, et al. Bioinformatic analysis of the urine proteome of acute allograft rejection. J Am Soc Nephrol 2004;15:3240–8.

31. Petricoin EF, Ardekani AM, Hitt BA, Levine PJ, Fusaro VA, Steinberg SM, et al. Use of proteomic patterns in serum to identify ovarian cancer. Lancet 2002;359:572–7.

32. Schardijn G, Statius van Eps LW, Swaak AJ, Kager JC, Persijn JP. Urinary beta 2 microglobulin in upper and lower urinary-tract infections. Lancet 1979;1:805–7.

33. Schardijn GH, Statius van Eps LW. Beta 2-microglobulin: its significance in the evaluation of renal function. Kidney Int 1987; 32:635–41.

34. Hong CY, Chia KS. Markers of diabetic nephropathy. J Diabetes Complications 1998;12:43–60.

35. Hershko A, Ciechanover A. The ubiquitin system. Annu Rev Biochem 1998;67:425–79.

36. Sun L, Pan X, Wada J, Haas CS, Wuthrich RP, Danesh FR, et al. Isolation and functional analysis of mouse UbA52 gene and its relevance to diabetic nephropathy. J Biol Chem 2002;277: 29953–62.

37. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature 2001;414:813–20.

38. Glickman MH, Ciechanover A. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev 2002;82:373–428.

39. Wilkinson KD. Ubiquitination and deubiquitination: targeting of proteins for degradation by the proteasome. Semin Cell Dev Biol 2000;11:141–8.

40. Wing SS. Deubiquitinating enzymes: the importance of driving in reverse along the ubiquitin-proteasome pathway. Int J Biochem Cell Biol 2003;35:590–605.