Urine Proteome Analysis May Allow Noninvasive Differential Diagnosis of Diabetic Nephropathy

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OBJECTIVE — Chronic renal insufficiency and/or proteinuria in type 2 diabetes may stem from chronic renal diseases (CKD) other than classic diabetic nephropathy in more than one-third of patients. We interrogated urine proteomic profiles generated by surface-enhanced laser desorption/ionization-time of flight/mass spectrometry with the aim of isolating a set of biomarkers able to reliably identify biopsy-proven diabetic nephropathy and to establish a stringent correlation with the different patterns of renal injury.

RESEARCH DESIGN AND METHODS — Ten micrograms of urine proteins from 190 subjects (20 healthy subjects, 20 normoalbuminuric, and 18 microalbuminuric diabetic patients and 132 patients with biopsy-proven nephropathy: 65 diabetic nephropathy, 10 diabetic with nondiabetic CKD [nd-CKD], and 57 nondiabetic with CKD) were run using a CM10 ProteinChip array and analyzed by supervised learning methods (Classification and Regression Tree analysis).

RESULTS — The classification model correctly identified 75% of patients with normoalbuminuria, 87.5% of those with microalbuminuria, and 87.5% of those with diabetic nephropathy when applied to a blinded testing set. Most importantly, it was able to reliably differentiate diabetic nephropathy from nd-CKD in both diabetic and nondiabetic patients. Among the best predictors of the classification model, we identified and validated two proteins, ubiquitin and β_2-microglobulin.

CONCLUSIONS — Our data suggest the presence of a specific urine proteomic signature able to reliably identify type 2 diabetic patients with diabetic glomerulosclerosis.

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Diabetic nephropathy is one of the most relevant long-term complications of diabetes in terms of morbidity and mortality and currently accounts for up to 50% of patients requiring renal replacement therapy in the western world, although its prevalence varies largely among countries (1). By extension from type 1 diabetes, microalbuminuria is usually viewed as the earliest putative diagnostic sign of diabetic renal damage also in type 2 diabetes. Indeed, microalbuminuria grossly correlates with the complex histopathological picture of glomerular and tubular damage (2), thus probably representing a nonspecific indicator of ongoing renal injury. Moreover, micro- and macroalbuminuria can stem from chronic nonspecific changes related to vascular damage (arterio-arteriolosclerosis and ischemic glomerular lesions) as well as nondiabetic glomerular diseases, which may occur either alone or together with diabetic glomerulosclerosis (3). To date, an accurate diagnosis of histological damage in diabetes can only be achieved by renal biopsy, which points to the need for easier and noninvasive tools to help define kidney damage and, possibly, drive therapeutic options.

Over the last few years, proteomics, a novel science focused on analyzing global protein content of a biological sample, has been applied to the search for novel biomarkers of diabetic and nondiabetic chronic kidney disease (CKD) (4–7). Very recently, proteomic-based strategies to discover urine or serum biomarkers of diabetic nephropathy have been extensively reviewed (8). Among the available proteomic approaches, the so-called profiling methods are gaining remarkable success as promising tools for the identification of new putative biomarkers of diabetic nephropathy (9–12).

In this study, we implemented supervised statistical methods [Classification and Regression Tree (CART) analysis] for the analysis of urine protein patterns generated by surface-enhanced laser desorption/ionization (SELDI)-time of flight (TOF)/mass spectrometry (MS) to evaluate their ability to distinguish biopsy-proven diabetic nephropathy from other forms of CKD in both nondiabetic and diabetic patients.

RESEARCH DESIGN AND METHODS — We first recruited a group of 65 type 2 diabetic patients with a steady decline in glomerular filtration rate (GFR) and various degrees of urine albumin excretion rate, with biopsy-proven diabetic nephropathy and without any histological evidence of concomitant nondiabetic glomerular or vascular disease.

Next, we enrolled a second group of 10 patients with diabetes and CKD other than diabetic nephropathy (5 with membranous glomerular nephropathy, 2 with IgA nephropathy, 2 with focal segmental glomerular sclerosis, and 1 with atheroembolic disease). Of note, patients with diabetic nephropathy came from three independent Divisions of Nephrology: University of Foggia ($n = 32$), Sant'Orsola Malpighi Hospital, University of Bologna ($n = 5$), and University of Modena and Reggio Emilia ($n = 28$). Next, we recruited 38 diabetic patients without any deterioration of estimated GFR (eGFR), 20 who were normoalbuminuric (NAD group) and 18 who were microalbuminuric (MICRO group). Finally, we enrolled 57 nondiabetic patients with CKD (nd-CKD), namely IgA nephropathy ($n = 20$), membranous nephropathy ($n = 24$) and benign nephroangiosclerosis ($n = 13$). Twenty healthy subjects were recruited as control group. The clinical and laboratory features of all the subjects studied are reported in supplementary Table A1 (available in an online appendix at http://care.diabetesjournals.org/cgi/content/full/dc10-0345/DC1).

eGFR was calculated using the Modified Diet in Renal Disease six-variable formula. Most patients examined showed fair blood pressure and glycemic control at the time of urine collection. In addition, most of them, with the exception of patients in NAD group, were taking ACE inhibitors and/or angiotensin II receptor antagonists as a part of their antihypertension treatment.

The study was approved by the local ethics committee, and informed written consent was obtained from all participating subjects. All investigations were conducted according to the principles expressed in the Declaration of Helsinki.

Renal biopsy examination
Paraffin-embedded renal specimens were used for conventional histological staining (hematoxylin-eosin, periodic acid-Schiff, silver methenamine, and Masson's trichrome). Immunofluorescence microscopy was performed on cryostat sections with the use of antisera against IgG, IgM, IgA, C3, C4, C1q, and fibrinogen. Diabetic nephropathy was diagnosed in the presence of nodular or diffuse glomerulosclerosis, glomerular hypertrophy, mesangial (diffuse or nodular) widening, glomerular capillary wall thickening, evidence of exudative lesions or fibrin caps (i.e., hyaline material heaped up on the inner side of the glomerular basement membrane), and the presence of microaneurysms of glomerular capillaries (13). Patients with concomitant evidence of nondiabetic glomerular disease or vascular disease were excluded from the analysis.

Urine collection and management
Urine samples were collected in the morning, after overnight fasting, and tested for standard parameters (including pH, glucose, blood content, and specific gravity) using Multistix reactive stripes (Bayer Diagnostics, Munich, Germany). Then they were centrifuged to remove cell debris, divided into aliquots with the addition of protease inhibitors, and stored at −80°C until analysis.

SELDI profiling
Urine samples were concentrated by 3-kDa cutoff Amicon filter devices (Millipore, Billerica, MA), and 10 μg of urine proteins, diluted (2:3, v/v) with a denaturing buffer solution (9 mol/l urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate [CHAPS], and 100 mmol/l dithiothreitol), were analyzed in duplicate using a CM10 ProteinChip (Bio-Rad, Hercules, CA) array, whose chemical surface binds proteins by cationic exchange. All experiments were performed according to the manufacturer's instructions. At the end of each experiment, the chips were read by a ProteinChip Reader (PCS-4000 Enterprise version; Bio-Rad) and, after acquisition, the spectra were analyzed by ProteinChip DataManager 3.5 software (Bio-Rad). Before each analysis, the software was externally calibrated by using a protein standard kit (ProteinChip OQ kit; Bio-Rad), and all of the spectra were normalized by means of total ion current. The analysis was performed in a range of 3,000 to 30,000 m/z, considering as real peaks those having a signal-to-noise and valley depth ratio >4. The reproducibility of the SELDI analysis was assessed by running one urine sample in quadruplicate and measuring the percent coefficient of variation in the number and intensity of mass peaks. According to our previously published data (14) and the manufacturer's instruction, percent coefficient of variation values for the number and intensity of peaks were about 8 and 23%, respectively (data not shown).

CART analysis
Urine samples were divided into a training set and a testing set to construct and validate the classification tree, respectively. The intensity (microamperes) of all the mass peaks in the training set was transferred to Biomarker Pattern Software (Bio-Rad) that identified a set of mass peaks whose intensity allowed us to classify each sample of the training set with the least error. The independent testing set was then scored using the classification tree to evaluate the classification power on a blinded dataset. The sensitivity was defined as the probability of predicting diabetic nephropathy, and the specificity was defined as the probability of predicting nd-CKD.

Multivariate analysis
A logistic regression model was used to determine factors significantly related to the urinary proteomic signature. Significance <0.05 for simple logistic analysis was required for independent variables to be entered into a multivariate logistic model.

Protein identification and validation
Two proteins of about 11,700 and 8,589 m/z, chosen among the most prominent predictors included in the classification tree, were isolated, identified by tandem mass spectrometry, and validated by enzyme-linked immunosorbent assay (ELISA) (β_2-microglobulin [β2MG]) and immunoprecipitation (ubiquitin), respectively (for details of methods, see supplementary data, available in an online appendix).

RESULTS

Differential diagnosis between diabetic nephropathy and nd-CKD
We first tested the ability of Biomarker Pattern Software to differentiate diabetic nephropathy from nd-CKD (Fig. 1A). Initially, urine samples from 54 patients with diabetic nephropathy and 57 nondiabetic patients with nd-CKD were distributed into the training set (31 diabetic nephropathy and 41 nd-CKD) and the testing set (23 diabetic nephropathy and 16 nd-CKD). To minimize the influence of preanalytical biases on the classification approach, urine samples of patients with diabetic nephropathy collected in three distinct nephrology units were evenly distributed in both the training and the testing set. The best classification tree created in the training set was then applied to an
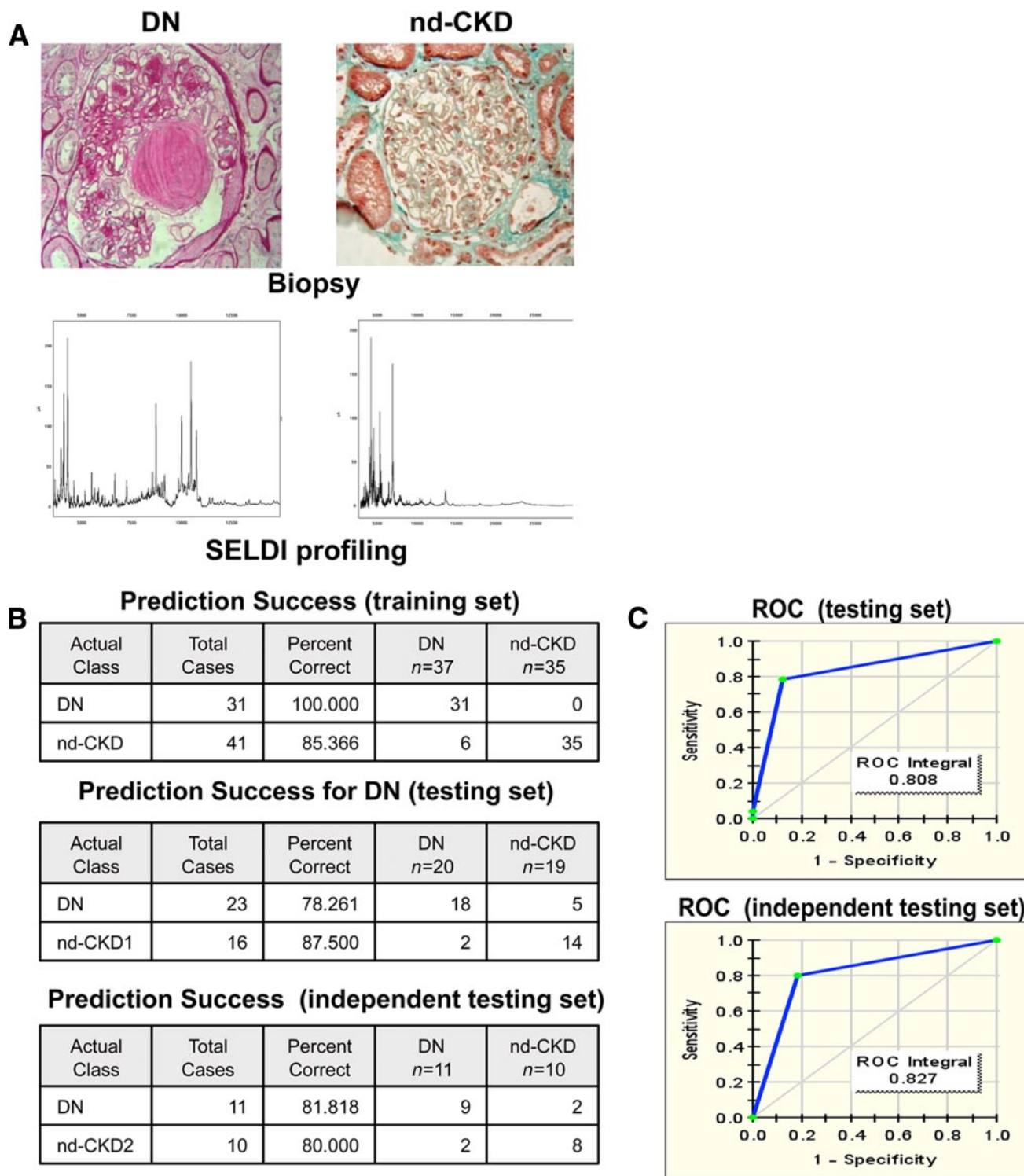
Figure 1—*Classification and regression tree analysis of diabetic nephropathy and nd-CKD. A: Histological picture of one patient with diabetic nephropathy and one patient with nd-CKD and their respective SELDI urine protein profiles. B: Prediction success of CART analysis on the training set (upper table) and on the testing set with nondiabetic (intermediate table) and diabetic (lower table) patients with nd-CKD. C: ROC analysis of the ability of the proteomic signature to identify diabetic nephropathy. DN, biopsy-proven diabetic nephropathy; nd-CKD1, nondiabetic patients with nondiabetic chronic kidney disease; nd-CKD2, diabetic patients with nondiabetic chronic kidney disease. (A high-quality digital representation of this figure is available in the online issue.)*

independent blinded set (testing set) of urine samples to validate its discriminatory power. Diabetic nephropathy and nd-CKD were correctly classified with 78.2 and 87.5% sensitivity and specificity, respectively (Fig. 1B). Eighteen mass peaks (8,586, 13,593, 13,687, 8,515, 8,665, 11,724, 8,423, 13,902, 13,780, 19,335, 6,320, 13,422, 4,115, 4,049,
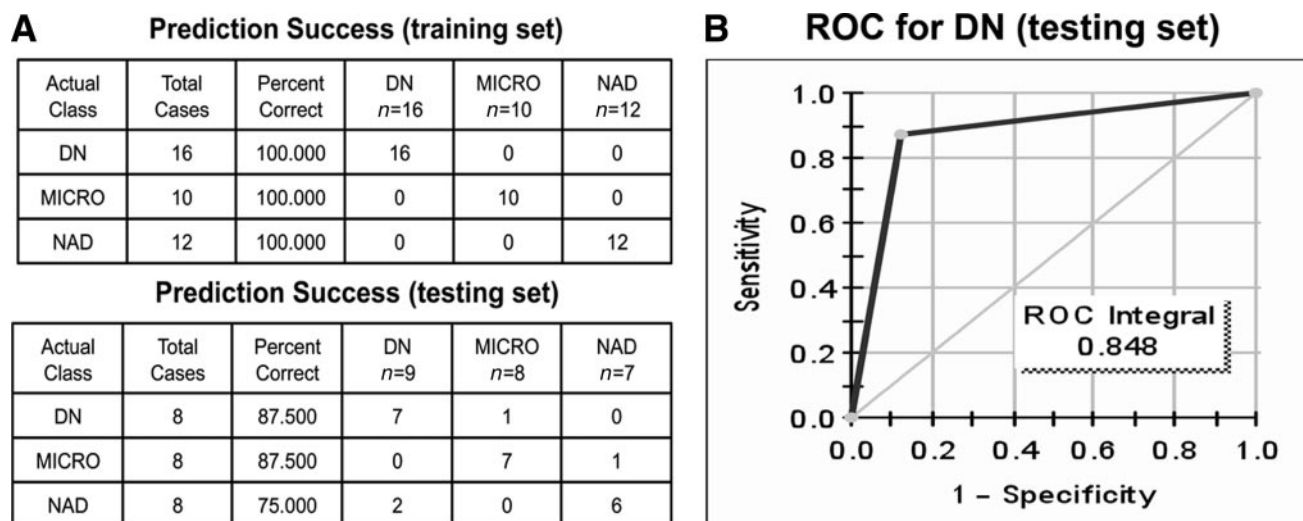
A Prediction Success (training set)

Actual Class	Total Cases	Percent Correct	DN n=16	MICRO n=10	NAD n=12
DN	16	100.000	16	0	0
MICRO	10	100.000	0	10	0
NAD	12	100.000	0	0	12

Prediction Success (testing set)

Actual Class	Total Cases	Percent Correct	DN n=9	MICRO n=8	NAD n=7
DN	8	87.500	7	1	0
MICRO	8	87.500	0	7	1
NAD	8	75.000	2	0	6

B ROC for DN (testing set)

ROC Integral 0.848

Figure 2—*CART analysis of diabetic patients with normoalbuminuria, microalbuminuria, and diabetic nephropathy. A: Prediction success of the CART analysis for the training set (upper table), after 10-fold cross-validation and the independent testing set (lower table). B: ROC analysis of the ability of the proteomic signature to identify diabetic nephropathy. DN, biopsy-proven diabetic nephropathy; MICRO, microalbuminuric diabetic patients; NAD, normoalbuminuric diabetic patients.*

4,024, 4,308, 4,370, and 3,086 *m/z*) were identified as the best predictors for the diagnosis of diabetic nephropathy. Moreover, receiver operator characteristic (ROC) analysis showed 80% accuracy in the diagnosis of diabetic nephropathy (Fig. 1*C*). Next, we were interested in examining the discriminatory power of the classification tree toward diabetic patients with CKD other than diabetic nephropathy. To this purpose, the diabetic nephropathy urine signature was scored on a second independent testing set including 11 newly recruited patients with diabetic nephropathy and 10 diabetic patients with nd-CKD. The diabetic nephropathy classification tree correctly distinguished diabetic nephropathy from nd-CKD with 80% specificity, as further confirmed by ROC analysis (Fig. 1*B* and *C*). The accuracy of the proteomic signature to discriminate diabetic nephropathy from nd-CKD was thus largely independent from the presence or the absence of diabetes in patients with nd-CKD.

To further explore the relationship between proteomic signature and histological diagnosis, we applied logistic regression analysis, selecting a wide range of clinical, laboratory, and demographic variables (age, sex, duration of diabetes, blood pressure levels, eGFR, daily proteinuria, use of renin-angiotensin system [RAS] blockers, and smoking) and setting the proteomic signature as the dependent nominal variable (absent/present). Simple logistic analysis showed that only smoking was significantly associated with

the diabetic nephropathy signature. When smoking and histological diagnosis were entered into a multiple logistic model, only the latter independent variable retained the same coefficient, indicating the lack of a significant confounding effect of smoking (not shown).

Setup of a classification tree to discriminate normoalbuminuria, microalbuminuria, and biopsy-proven diabetic nephropathy

We examined the ability of CART analysis to discriminate among diabetic patients with normoalbuminuria, microalbuminuria, and diabetic nephropathy. Thirty-eight patients from all classes (16 with diabetic nephropathy, 10 with microalbuminuria, and 12 with normoalbuminuria) were used to constitute the training sample group, whereas the remaining 8 samples of each group were used as a testing set. We chose to limit the analysis to 24 randomly selected patients with diabetic nephropathy of 65 patients because a higher prevalence of diabetic nephropathy in the training set and mainly in the testing set could influence the results of the analysis. The intensity of all the protein peaks and the presence or absence of diabetic retinopathy were used to construct the classificatory model. Among the differently expressed mass peaks, those of 10,533, 7,919, 8,185, 9,072, 9,135, 3,396, 8,982, 22,735, 8,847, 22,245, 17,084, and 16,710 *m/z* were identified as the best

predictors to set up the discriminatory tree. The cross-validation of the model on the training set showed 100% correct classification for all groups. When the classification tree was applied to the blinded testing set, seven of eight patients (87.5%) of the MICRO and diabetic nephropathy groups and six of eight patients (75%) of the NAD group were correctly classified (Fig. 2*A*). The ROC curve for diabetic nephropathy showed a diagnostic power of 84% for the current analysis (Fig. 2*B*).

Because many variables (sex, duration of diabetes, blood pressure levels, eGFR, daily proteinuria, use of RAS blockers, and smoking) differed among groups, we examined their impact on proteomic profile, set as a dependent polytomous variable, by logistic analysis. Nonetheless, none of the independent variables reached a significance <0.05 at simple logistic analysis.

Protein identification

We examined and identified two mass peaks (~11,700 and 8,589 *m/z*) among the most prominent predictors included in the classification tree for diabetic nephropathy.

β2MG

The ~11,700 *m/z* peak was identified as β_2-microglobulin by matrix-assisted laser desorption ionization-TOF/tandem mass spectrometry (supplementary Figure A1, available in an online appendix). To explore the relationship between β2MG uri-
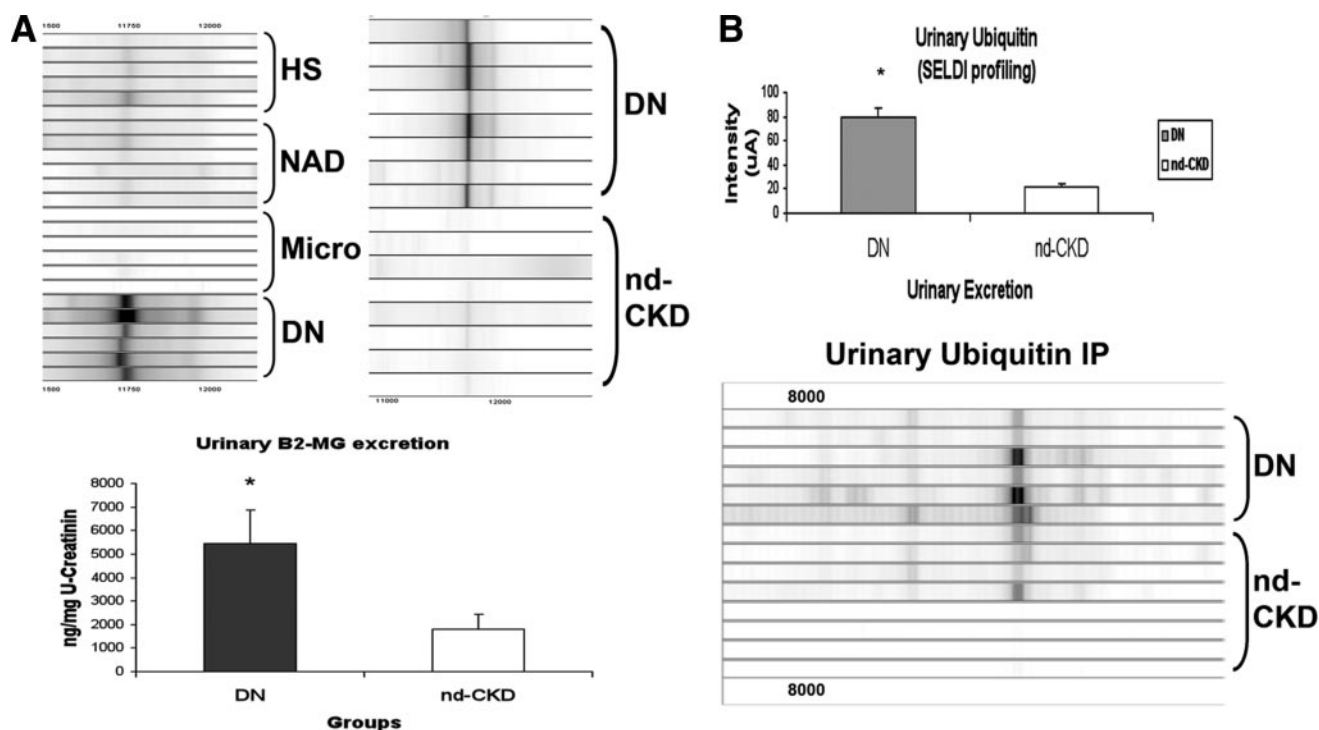
Figure 3—*Validation of β2MG and ubiquitin differential excretion. A, top: Representative SELDI spectra (gel view) showing β2MG excretion in patients with diabetic nephropathy compared with that in healthy subjects and patients with normoalbuminuria, microalbuminuria (left), and nd-CKD (right). Bottom: β2-MG urine (U) excretion as measured by ELISA (mean ± SEM) in patients with diabetic nephropathy compared with nd-CKD. B, top: Ubiquitin urine excretion as measured by SELDI analysis on the whole urine profile (mean ± SEM) in patients with diabetic nephropathy compared with nd-CKD. Bottom: SELDI profiling of urine ubiquitin immunoprecipitated by a specific monoclonal antibody (ubiquitin IP) and run on a CM10 ProteinChip array. Representative SELDI spectra (gel view) from six patients with diabetic nephropathy and eight patients with nd-CKD are shown. *P < 0.05. DN, biopsy-proven diabetic nephropathy; MICRO, microalbuminuric diabetic patients; HS, healthy subjects; NAD, normoalbuminuric diabetic patients.*

nary excretion and diabetic nephropathy, we compared β2MG levels in the urine of diabetic patients with and without diabetic nephropathy (Fig. 3*A,* top panel) by SELDI profiling and measured β2MG urinary excretion in patients with diabetic nephropathy and nd-CKD by ELISA (Fig. 3*A,* bottom panel). β2MG urine excretion was considerably higher in diabetic patients with diabetic nephropathy compared with that for the NAD or MICRO groups or for patients with nd-CKD.

Ubiquitin
The 8,589 *m/z* peak was provisionally identified as ubiquitin on the basis of its molecular weight and of the available literature on putative biomarkers of diabetic nephropathy. To confirm the identification, a recombinant ubiquitin standard was loaded on a blank spot of the CM10 ProteinChip array, and its mass and shape were compared with those of the 8,589 *m/z* peak in the protein profiles of the patients (supplementary Figure A2, available in an online appendix). Further, we immunoprecipitated ubiquitin from the

urine of six patients with diabetic nephropathy and eight patients with nd-CKD, and each immunoprecipitate was then analyzed by a CM10 ProteinChip array to confirm the increased excretion of ubiquitin in urine samples from patients with diabetic nephropathy (Fig. 3*B*).

CONCLUSIONS — The data reported in the present work suggest that the analysis of urine protein profiles of type 2 diabetic patients, as generated by SELDI-TOF/MS with a CM10 ProteinChip, can be considered a reliable method to identify patients with diabetic nephropathy among diabetic patients and, more importantly, to distinguish biopsy-proven diabetic nephropathy from nd-CKD in both diabetic and nondiabetic patients.

Currently, microalbuminuria is the only noninvasive marker of early diabetic nephropathy. However, microalbuminuria may instead reflect the existence of endothelial damage, in the absence of specific renal lesions, or, on the contrary, be associated with advanced renal pathological changes. Furthermore, clinically

overt nephropathy and/or albuminuria in diabetic patients does not necessarily imply the presence of diabetic glomerulosclerosis. In the largest histological study (393 renal biopsies) performed to date, other glomerulonephritides superimposed on diabetic glomerulosclerosis or glomerulonephritides without the presence of diabetic nephropathy occurred in up to 57% of patients (3). Moreover, patients with type 2 diabetes can progress to a significant degree of renal impairment even if they remain normoalbuminuric, and this occurrence may reflect renal parenchymal diseases other than classic diabetic glomerulosclerosis (15). Diabetic patients with nd-CKD have a different rate of GFR decline (16,17) and, more importantly, may be amenable to specific treatments. At present, however, there is not a strong predictor to differentiate diabetic nephropathy from nd-CKD by clinical or biochemical data. Given this background, novel biomarkers for earlier diagnosis of diabetes-related renal damage as well as for the proper identification

of diabetic glomerulosclerosis are crucially required.

Over the last several years, only a few studies have adopted proteomic strategies focused on identifying one or more urine biomarkers that would allow either the early detection of diabetic nephropathy or its discrimination from other nd-CKD or the identification of normoalbuminuric type 2 diabetic patients prone to develop diabetic nephropathy (10–12,18,19). All of them, however, identified diabetic nephropathy exclusively on a clinical basis (i.e., the presence of macroalbuminuria with or without a decline of eGFR), which may potentially lead to a misclassification in more than one-third of patients in the absence of histological verification (3). Moreover, most studies failed to apply supervised learning algorithms to validate the proposed signatures.

The main aim of the present study was to identify biopsy-proven diabetic nephropathy by urine proteomic fingerprint. We therefore started by comparing the urine proteome of 54 biopsy-proven diabetic nephropathy with that of 57 nondiabetic patients with proteinuria and CKD. CART analysis of the blinded testing set revealed 78.2 and 87.5% sensitivity and specificity, respectively, thus indicating that the urine proteome contains a set of key information useful to accurately distinguish biopsy-proven diabetic nephropathy from nd-CKD. Moreover, multivariate analysis allowed us to rule out the confounding effect of a number of potentially relevant covariates (see RESULTS). To corroborate the discriminatory power of the classification tree, we tested the urinary fingerprint of biopsy-proven diabetic nephropathy in a further group of 21 diabetic patients, 11 with diabetic nephropathy and 10 with nd-CKD. CART analysis identified patients with diabetic nephropathy with 80% accuracy, thus demonstrating the ability of the urine proteomic fingerprint to discriminate diabetic nephropathy from nd-CKD, both in the presence and in the absence of diabetes. However, we are aware that the small size of the dataset we explored limits the possibility of drawing any final conclusions.

We then applied SELDI protein profiling to the study of the urine proteome of the whole population of diabetic patients and analyzed urine profiles by both unsupervised and supervised learning methods. When SELDI spectra were analyzed by unsupervised methods, a list of mass peaks showed a significantly different ex-

pression among groups (data not shown), but none of these putative biomarkers was, by itself, sensitive and specific enough to allow a reliable discrimination among classes. To overcome the limitations of the univariate analysis, we analyzed SELDI spectra by supervised learning methods, which screened all mass peaks to build up an optimal classification tree. The classification tree correctly identified 87.5% of patients with microalbuminuria and biopsy-proven diabetic nephropathy and 75% of patients with normoalbuminuria in the blinded test set. The inclusion of a retinopathy score (present/absent) in the analysis did not improve the accuracy of the classification model (data not shown). Finally, by logistic analysis we ruled out the possibility that the urinary proteomic signature would be significantly influenced by a number of variables (sex, blood pressure levels, degree of deterioration of renal function, smoking, and RAS blocker therapy) differently distributed among groups.

We must recognize that albuminuria per se could not properly reflect the type and the degree of renal damage, and this fact might help explain some apparent misclassification within the groups lacking histological examination. If so, proteomic "signature" might unmask the existence of a clinically silent renal injury. Indeed, all patients with normoalbuminuria were checked again after a median of 1.8 years, but none of them had progressed toward microalbuminuria nor did they show a deterioration in eGFR. For these reasons, we were unable to correlate the initial misclassification of the two patients with normoalbuminuria with the progression of renal disease. We may infer that a longer follow-up is required to ascertain whether some of the protein peaks in the normoalbuminuria signature depicted here would serve as early predictive biomarkers of diabetic glomerulosclerosis in normoalbuminuric diabetes (11). Interestingly, all biopsy-proven diabetic nephropathy showed a conserved proteomic pattern, independent from the individual rate of urine albumin excretion, which supports the assumption that the albumin excretion rate does not necessarily reflect the type and degree of renal damage.

The identification of some of the proteins in the proteomic signature of diabetic nephropathy might provide insight into the mechanisms underlying the disease, besides serving as candidate

biomarkers to diagnose diabetic nephropathy. Therefore, two proteins ~8,589 and 11,700 *m/z*, chosen among the most prominent predictors included in the classification tree, were isolated and identified as ubiquitin and β2MG, respectively. Their increased excretion in the urine of patients with diabetic nephropathy compared with that in diabetic patients without diabetic nephropathy and with nd-CKD was confirmed by immunoprecipitation (ubiquitin) or ELISA (β2MG) and was found to be independent of the severity of daily proteinuria.

Our findings are consistent, at least in part, with those reported by Dihazi et al. (12). These authors found a significant excretion of β2MG in proteinuric diabetic patients and a selective excretion of ubiquitin ribosomal fusion protein in micro- and macroalbuminuric diabetic patients, who instead released very low amounts of the truncated form of ubiquitin. These partial discrepancies may be ascribed to the use of protease inhibitors in our study, which possibly prevented ubiquitin-specific degradation (12). Regardless, deranged excretion of ubiquitin is a novel and potentially interesting biomarker of diabetic nephropathy. At variance, increased levels of urine β2MG, a recognized marker of renal tubular damage, have long been described in diabetic patients with micro- and macrovascular complications and mainly in those with associated hypertension (20), as well as in diabetic patients with micro- or macroalbuminuria (12), but studies comparing β2MG release in patients with diabetic nephropathy versus those with nd-CKD are currently lacking.

In summary, we report that the standardization of urine analysis by SELDI-TOF/MS (14,21) and the elaboration of its complex datasets by means of supervised statistical methods allowed us to generate a robust multiparametric panel of mass peaks that was able to reliably discriminate biopsy-proven diabetic nephropathy from nd-CKD in both diabetic and nondiabetic patients. These findings, if confirmed in larger cohorts of diabetic patients, encourage the use of supervised learning approaches for the analysis of urine proteomic profiles to achieve a noninvasive differential diagnosis of renal lesions in diabetic patients, whereas the appraisal of their possible predictive power demands longitudinal studies. Finally, the selective release of high amounts of ubiquitin and β2MG in the

urine of patients with diabetic nephropathy may suggest a role as candidate biomarkers and possible involvement in the pathophysiology of the disease.

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