URINE PROTEOME ANALYSIS MAY ALLOW NON-INVASIVE DIFFERENTIAL DIAGNOSIS OF DIABETIC NEPHROPATHY

Running title: Diagnosis of Diabetic Nephropathy by urine proteomics

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Submitted 21 February 2010 and accepted 22 July 2010.

Additional information for this article can be found in an online appendix at http://care.diabetesjournals.org

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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 (DN) in over one third of cases. We interrogated urine proteomic profiles generated by SELDI-TOF/MS with the aim to isolate a set of biomarkers able to reliably identify biopsy-proven DN and to establish a stringent correlation with the different patterns of renal injury.

Research design and methods: Ten μg urine proteins from 190 subjects [20 healthy subjects (HS), 20 normoalbuminuric (NAD) and 18 microalbuminuric (MICRO) diabetic patients, and 132 patients with biopsy-proven nephropathy (65 DN, 10 diabetics with non-diabetic CKD (nd-CKD) and 57 non-diabetic patients with CKD)] were run by CM10 ProteinChip array and analysed by supervised learning methods (CART analysis).

Results: The classification model correctly identified 75% NAD, 87.5% MICRO and 87.5% DN when applied to a blinded testing set. Most importantly, it was able to reliably differentiate DN from nd-CKD in both diabetic and non-diabetic patients. Among the best predictors of the classification model, we identified and validated 2 proteins, ubiquitin and β2-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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analysis of urine protein patterns generated by SELDI/TOF/MS to evaluate their ability to distinguish biopsy-proven DN from other forms of CKD in both non-diabetic and diabetic patients (nd-CKD).

RESEARCH DESIGN AND METHODS

Patients: We first recruited a group of 65 type 2 diabetic patients with a steady decline in GFR and variable degrees of urine albumin excretion rate, with biopsy-proven DN and without any histological evidence of concomitant non-diabetic glomerular or vascular disease; secondly, we enrolled a second group of 10 patients with diabetes and CKD other than DN (5 Membranous GN, 2 IgA Nephropathy, 2 FSGS and 1 ateroembolic disease). Noteworthy, DN patients came from three independent Divisions of Nephrology: University of Foggia (n.=32), Sant’Orsola Malphighi Hospital, University of Bologna (n.=5), and University of Modena and Reggio Emilia (n.=28).

Afterwards, we recruited 38 diabetic patients without any deterioration of estimated GFR, 20 being normoalbuminuric (NAD Group) and 18 microalbuminuric (MICRO Group). Finally, we enrolled 57 non-diabetic patients with CKD (nd-CKD), namely IgA Nephropathy (N=20), Membranous Nephropathy (N=24) and benign Nephroangiosclerosis (N=13).

Twenty healthy subjects (HS) were recruited as controls. The clinical and laboratory features of all the subjects studied are reported in Table A 1.

Estimated GFR (eGFR) was calculated using the Modified Diet in Renal Disease six-variable formula.

Most patients examined showed a fair blood pressure and glycemic control at the time of urine collection. 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 anti-hypertensive treatment.

The study was approved by the local Ethical Committee and an 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 histologic 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. DN 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 non-diabetic 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 (pH, glucose, blood content, specific gravity, etc.) 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 cut-off Amicon® filter devices (Millipore, Billerica, MA, USA) and 10 µg urine protein, diluted (2:3, v/v) with a denaturing buffer solution (9 M Urea, 2% CHAPS and 100 mM DTT), were analysed in duplicate by CM10 ProteinChip (BIORAD), whose chemical surface binds proteins by cationic exchange. All experiments were
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performed according to manufacturer’s instructions. At the end of each experiment, the chips were read by ProteinChip Reader (PCS-4000 Enterprise version- BIORAD) and, after acquisition, the spectra were analysed by Protein chip DataManager™ 3.5 software (BIORAD, Hercules, CA, USA). Prior to each analysis, the software was externally calibrated by using a protein standard kit ( ProteinChip OQ kit- BIORAD) and all the spectra were normalized by means of Total Ion Current (TIC). The analysis was performed in a range of 3,000-30,000 m/z, considering as real peaks those having Signal/Noise (S/N) and valley depth ratio greater than 4. The reproducibility of the SELDI analysis was assessed by running one urine sample in quadruplicate and measuring the % CV in the number and intensity of mass peaks. According to our previously published data [14] and manufacturer’s instruction, % CV for the number and intensity of peaks was about 8 % and 23%, respectively (data not shown).

Classification and Regression Tree (CART) analysis: Urine samples were divided into a training set and a testing set to construct and validate the classification Tree, respectively. The intensity (µA) of all the mass peaks in the training set was transferred to Biomarker Pattern Software (BPS® -BIORAD) that identified a set of mass peaks whose intensity allowed 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 data set. The sensitivity was defined as the probability of predicting DN, while 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. A significance lower than 0.05 at simple logistic analysis was required for independent variables to be entered into a multivariate logistic model.

Protein identification and validation: Two proteins of 11700 and 8589 m/z, chosen among the most prominent predictors included in the classification Tree, were isolated, identified and validated by ELISA (β2-MG) and immunoprecipitation (ubiquitin), respectively (see online appendix for details of methods, available at http://care.diabetesjournals.org).

RESULTS

Differential diagnosis between DN and nd-CKD: We first tested BPS® ability to differentiate DN from non-diabetic CKD (figure 1 A). Initially, urine samples from 54 DN and 57 non-diabetic patients with nd-CKD were distributed into the training set (31 DN and 41 nd-CKD) and the testing set (23 DN and 16 nd-CKD). To minimize the influence of pre-analytical biases on the classification approach, urine samples of DN patients collected in 3 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 independent blinded set (testing set) of urine samples, to validate its discriminatory power. DN and nd-CKD were correctly classified with 78.2 % and 87.5% sensitivity and specificity, respectively (figure 1 B). Eighteen mass peaks (8586, 13593, 13687, 8515, 8665, 11724, 8423, 13902, 13780, 19335, 6320, 13422, 4115, 4049, 4024, 4308, 4370, 3086 m/z) were identified as the best predictors for the diagnosis of DN. Moreover, Receiver Operator Characteristic (ROC) analysis showed 80% accuracy in the diagnosis of DN (figure 1 C). Then, we were interested in examining the discriminatory power of the classification Tree towards diabetic patients with CKD other than DN. To this purpose, DN urine signature was scored on a second independent testing set including 11 newly
recruited DN patients and 10 diabetic patients with nd-CKD. DN classification Tree correctly distinguished DN from nd-CKD with 80% specificity, as further confirmed by ROC analysis (Figure 1, B and C). The accuracy of proteomic signature to discriminate DN from nd-CKD was thus largely independent from the presence, or the absence, of diabetes mellitus 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 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 DN 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).

**Set up of a classification Tree to discriminate NAD, MICRO and biopsy-proven DN:** We examined the ability of CART analysis to discriminate among NAD, MICRO and DN patients. Thirty-eight patients from all classes (16 DN, 10 MICRO and 12 NAD) were used to build up the training sample group, while the remaining 8 samples of each group were used as testing set. We chose to limit the analysis to 24 randomly selected DN out of 65 patients since a higher prevalence of DN 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 10533, 7919, 8185, 9072, 9135, 3396, 8982, 22735, 8847, 22245, 17084, 16710 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, 7/8 patients (87.5%) of MICRO and DN groups and 6/8 (75%) of NAD group where correctly classified (figure 2 A). ROC curve for DN showed a diagnostic power of 84% for the current analysis (figure 2 B).

Since 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 dependent polytomous variable, by logistic analysis. Nonetheless, none of the independent variables reached a significance lower than 0.05 at simple logistic analysis.

**Protein identification:** We examined and identified 2 mass peaks (~11700 and 8589 m/z) among the most prominent predictors included in the classification Tree for DN.

**β2 Microglobulin (β2MG):** The ~11700 m/z peak was identified as β2MG by MALDI-TOF/MS/MS mass spectrometry (figure A 1 on line appendix). To explore the relationship between β2MG urinary excretion and DN, we compared β2MG levels in the urine of diabetic patients with and without DN (figure 3 A upper panel) by SELDI profiling, and measured β2-MG urinary excretion in DN and nd-CKD patients by ELISA (figure 3 A lower panel). β2-MG urine excretion was considerably higher in diabetic patients with DN compared with NAD or MICRO, as well as with nd-CKD.

**Ubiquitin:** The 8589 m/z peak was provisionally identified as ubiquitin on the basis of its M.W. and of the available literature on putative biomarkers of DN. To confirm the identification, a recombinant ubiquitin standard was loaded on a blank spot of CM10 ProteinChip array and its mass and
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shape were compared with that of the 8589 m/z peak in the protein profiles of the patients (figure A 2 appendix on line). Further, we immunoprecipitated ubiquitin from the urine of 6 DN and 8 nd-CKD patients, and each immunoprecipitate was then analysed by CM10 ProteinChip array to confirm the increased excretion of ubiquitin in urine samples from DN (Figure 3 B).

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

Presently microalbuminuria is the only non-invasive marker of early DN. 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 DN occurred in up to 57% of cases [3]. Moreover, patients with type 2 diabetes can progress to a significant degree of renal impairment even if remaining normoalbuminuric, and this may reflect renal parenchymal diseases other than classic diabetic glomerulosclerosis [15]. Diabetic patients with non-diabetic 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 DN from non-diabetic 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 few years, only a few studies have adopted proteomic strategies aimed at identifying one or more urine biomarkers which would allow either the early detection of DN or its discrimination from other non-diabetic CKD, or the identification of normoalbuminuric type 2 diabetic patients prone to develop DN. [10-12;18,19] All of them, however, identified DN 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 over 1/3 of cases 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 is to identify biopsy-proven DN by urine proteomic fingerprint. We therefore started by comparing the urine proteome of 54 biopsy-proven DN with that of 57 non-diabetic 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 urine proteome contains a set of key information useful to accurately distinguish biopsy-proven DN from nd-CKD. Moreover, multivariate analysis allowed 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 DN in a further group of 21 diabetic patients, 11 with DN and 10 with nd-CKD. CART analysis identified DN patients with 80% accuracy, thus demonstrating the
ability of the urine proteomic fingerprint to discriminate DN from nd-CKD, both in the presence and in the absence of diabetes. However, we are aware that the small size of the explored dataset limits the possibility to draw any final conclusion.

We then applied SELDI protein profiling to the study of urine proteome of the whole population of diabetic patients and analysed urine profiles by both unsupervised and supervised learning methods. When SELDI spectra were analysed by unsupervised methods, a list of mass peaks showed a significantly different expression 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 analysed 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 % MICRO and biopsy-proven DN and 75 % NAD patients 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, RAS blockers 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 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 NAD patients were checked again after a median of 1.8 years, but none of them had progressed towards microalbuminuria neither did they show a deterioration of eGFR. For this reason we were unable to correlate the initial misclassification of the two NAD patients 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 NAD signature depicted here would serve as early predictive biomarkers of diabetic glomerulosclerosis in normoalbuminuric diabetes [11]. Interestingly, all biopsy-proven DN showed a conserved proteomic pattern, independently from the individual rate of urine albumin excretion, which would support the assumption that 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 DN might provide insight into the mechanisms underlying the disease, besides serving as candidate biomarkers to diagnose DN. Therefore, 2 proteins ~ 8589 and 11700 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 DN, compared with diabetics without DN and with nd-CKD, was confirmed by immunoprecipitation (ubiquitin) or ELISA (β2MG) and was found to be independent from the severity of daily proteinuria.

Our findings are consistent, at least in part, with those reported by Dhiazi et al [12]. These authors found a significant excretion of β2MG in proteinuric diabetics and a selective excretion of ubiquitin ribosomal fusion protein in micro- and macro-albuminuric diabetics, 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, a deranged excretion of ubiquitin is a novel and
potentially interesting biomarker of DN. At variance, increased levels of urine β2MG, a recognized marker of renal tubular damage, have long been described in diabetic patients with micro- and macro-vascular complications, and mainly in those with associated hypertension (20), as well as in diabetics with micro- or macro-albuminuria (12), but studies comparing β2MG release in DN patients versus nd-CKD are presently lacking.

In conclusion, we reported 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 to generate a robust multi-parametric panel of mass peaks that was able to reliably discriminate biopsy-proven DN from nd-CKD of both diabetic and non-diabetic 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 non-invasive differential diagnosis of renal lesions in diabetics, while 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 DN may suggest a role as candidate biomarkers and a possible involvement in the pathophysiology of the disease.

**Author contributions:** M.P. designed the study, performed SELDI analysis and data interpretation, wrote, reviewed and edited the manuscript. S.DP. designed the study, contributed to data interpretation, wrote, reviewed and edited the manuscript; R.M recruited DN patients at the University of Modena and reviewed the manuscript. O.L. recruited diabetic patients without nephropathy at the University of Foggia and analysed clinical data. A.DM. collected clinical data of DN patients recruited at the University of Foggia. A.DP. examined biopsy specimens collected at the University of Foggia. MT.R. identified β2MG by Mass Spectrometry and reviewed the manuscript. L. F. examined biopsy specimens collected at the University of Modena and Reggio Emilia. S.P. examined biopsy specimens collected at “Sant’Orsola Malpighi” Hospital, Bologna. S. DC recruited diabetic patients without nephropathy at “Casa Sollievo della Sofferenza” Hospital, San Giovanni Rotondo, and reviewed/edited the manuscript. M.C. contributed to the study design and reviewed the manuscript. L.G. designed the study, contributed to data interpretation, wrote and reviewed the manuscript.

**ACKNOWLEDGMENTS**
We gratefully acknowledge Dr. Simona Magaldi, Dr. Valentina d’Onofrio and Dr. Roberta Damiano (Research Center Bioagromed, University of Foggia, Italy) for their excellent technical support in sample collection, handling and analysis; Dr. Vittorio Albertazzi (Nephrology Unit, University of Modena and Reggio Emilia, Italy), Dr. Giada Cardinale and Dr. Ivan Raffaele Cincione (Endocrinology Unit, University of Foggia, Italy) for their contribution to the collection of the clinical records of the patients enrolled; Prof. Elena Ranieri (Clinical Pathology Unit, University of Foggia, Italy) and Prof. Antonio Santoro (Nephrology Unit, Sant’Orsola-Malpighi Hospital, Bologna, Italy) for their critical review of the present manuscript; and Chiara Di Giorgio (Bioagromed, University of Foggia) for the linguistic support as medical translator. The study was supported by the following grants: Ministry of Health (ex art 12 and 56: PI Gesualdo Loreto, Mauro Cignarelli and Salvatore De Cosmo).

**Disclosure:** The authors declare they have no relevant conflict of interest.
REFERENCES

FIGURE LEGENDS

**Figure 1 Classification and Regression Tree analysis of DN and nd-CKD**
A. Histological picture of one DN and one nd-CKD patient and their respective SELDI urine protein profile. B. Prediction success of CART analysis on the training set (upper table) and on the testing set with non-diabetic (intermediate table) and diabetic (lower table) nd-CKD patients. C. Receiver-operator-characteristic (ROC) analysis of the ability of the proteomic signature to identify DN.

DN= biopsy-proven diabetic nephropathy; nd-CKD1 = non diabetic patients with non-diabetic chronic kidney disease; nd-CKD2 = diabetic patients with non-diabetic chronic kidney disease

**Figure 2 Classification and Regression Tree analysis of NAD, MICRO and DN.**
A. Prediction success of the Classification and Regression Tree (CART) analysis for the training set (upper table), after 10-fold cross-validation, and the independent testing set (lower table). B. Receiver-operator-characteristic (ROC) analysis of the ability of the proteomic signature to identify DN.

HS= healthy subjects, NAD= normoalbuminuric diabetic patients, MICRO= microalbuminuric diabetics, DN= biopsy-proven diabetic nephropathy.

**Figure 3 Validation of β2-Microglobulin (β2MG) and Ubiquitin differential excretion.**
A. Upper panel: Representative SELDI spectra (gel view) showing β2MG excretion in DN compared with HS, NAD, Micro (left side) and with nd-CKD (right side); lower panel: β2-MG urine excretion as measured by ELISA (M ± SEM) in DN compared with nd-CKD B. Upper panel: ubiquitin urine excretion as measured by SELDI analysis on the whole urine profile (M ± SEM) in DN compared with nd-CKD; lower panel: SELDI profiling of urine ubiquitin immunoprecipitated by a specific monoclonal antibody (ubiquitin IP) and run on CM10 ProteinChip array. Representative SELDI spectra (gel view) from 6 DN and 8 nd-CKD patients are shown.

* p <0.05; Abbreviations as in figure 1 and 2
Figure 1

**A**

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**B**

**Prediction Success (training set)**

<table>
<thead>
<tr>
<th>Actual Class</th>
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**C**

**ROC (testing set)**

ROC Integral: 0.809

**ROC (independent testing set)**

ROC Integral: 0.827
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Figure 2

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Figure 3

A

B

A

B

Figure 2

Figure 3