ORIGINAL ARTICLE

Changes in urine proteome accompanying diabetic nephropathy progression

Andrzej Lewandowicz[1], Magdalena Bakun[2], Rafał Kohutnicki[1],
Agnieszka Fabijańska[2], Michał Kistowski[2], Jacek Imiela[1], Michał Dadlez[2,3]

1   Miedzyleski Specialist Hospital, Warsaw, Poland
2   Mass Spectrometry Laboratory, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland
3   Department of Biology, Warsaw University, Warsaw, Poland

ABSTRACT

INTRODUCTION   Owing to the prevalence of type 2 diabetes, diabetic kidney disease (DKD) becomes the major cause of end-stage renal disease. The current markers of diabetic nephropathy are based on albuminuria and clinical signs of retinopathy. Sensitive and specific noninvasive diagnostic tools, unbiased by the presence of comorbidities, are needed, especially to detect the early stages of diabetic complications.

OBJECTIVES   The aim of the study was to analyze changes in urinary protein excretion based on the stage of DKD using quantitative proteomics.

PATIENTS AND METHODS   A total of 27 healthy controls were age- and sex-matched to 72 diabetes patients classified into 3 groups: no signs of retinopathy or nephropathy (n = 33), retinopathy but no microalbuminuria (n = 15), and diabetic nephropathy (DN) based on overt albuminuria or microalbuminuria with retinopathy (n = 24). To assess the intergroup differences, samples were partially pooled, tagged using 8-plex iTRAQ reagents, and the resulting peptide mixture was resolved by isoelectrofocusing. The obtained fractions were analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS). Data were analyzed using the MASCOT software and dedicated in-house proteomic data analysis programs.

RESULTS   The changes in the urine proteome following DKD progression involved some known protein markers of DN and several other proteins. Decreased levels of some proteins are presumably related to impaired secretory function of other organs affected by diabetes. In particular, a diminished excretion of pancreatic amylase and deoxyribonuclease I suggested exocrine pancreatic insufficiency (EPI), coexisting with type 2 diabetes.

CONCLUSIONS   A decrease in the urinary excretion of some pancreatic enzymes suggests EPI associated with diabetes. This hypothesis is yet to be verified; nevertheless, renal and extrarenal confounders must be considered when interpreting the results of quantitative urinary proteomics.

Correspondence to:
Prof. Michał Dadlez, PhD, Instytut
Biochemii i Biofizyki Polskiej
Akademii Nauk, ul. Pawińskiego
5a, 02-106 Warszawa, Poland,
phone: +48-22-592-34-76, fax:
+48-22-658-47-66, e-mail:
michald@ibb.waw.pl;
Andrzej Lewandowicz, MD, PhD,
Klinika Chorób Wewnętrznych
i Kardiologii, Szpital Kliniczny
Warszawskiego Uniwersytetu
Medycznego im. Dzieciątka
Jezus, ul. Lindley'a 4, 02-005
Warszawa, Poland, phone/fax:
+48-22-502-11-44, e-mail:
alewandow@gmail.com

INTRODUCTION   Primary and secondary kidney diseases leading to end-stage renal disease (ESRD) are a serious health care problem around the world.[1,2] Owing to recent changes in lifestyle, diabetic kidney disease (DKD; diabetic nephropathy, DN) is now the major cause of deterioration of chronic kidney disease (CKD) leading to renal replacement therapy. By the end of 2012 in the United States, diabetic patients constituted 39.3% of the patients with ESRD, while in 14.1% of the cases, it was caused by glomerulonephritis, and in 4.6%[3]—by autosomal dominant polycystic kidney disease (ADPKD), also considered as a common cause of ESRD.[4] Patients with CKD, especially diabetic in etiology, suffer from a drastically increased risk of death due to cardiovascular causes.[5,6] Simple albuminuria combined with a reduced filtration rate is a recognized cardiovascular risk factor.[7] Early prevention and diagnosis of diabetic complications is the key to effective treatment.

On the other hand, in a selected group of patients with type 2 diabetes, DN was reported to develop within 10 years after a diagnosis of diabetes in only 30.2% of the patients (24.9% with microalbuminuria and 5.3% with

macroalbuminuria).[8] Progression has been linked to the genetic background,[9] and a special focus on these cases is of great clinical importance. Although the Kidney Disease Outcomes Quality Initiative (KDOQI) and Kidney Disease: Improving Global Outcomes guidelines recommend assessing microalbuminuria for DKD screening, they also underscore the insufficient sensitivity of this test. The intraindividual variability in urinary albumin excretion depends on numerous factors, including coexisting diseases, exertion, body mass, and temperature, making this type of screening potentially unreliable. Although diabetic retinopathy (DR) in the presence of microalbuminuria according to the KDOQI guidelines on DKD is considered an equivalent marker of DN (overt albuminuria),[10] the correlation between DR and DKD may be biased. A discordance has been reported, with a fraction of patients not exhibiting coexisting retinopathy and nephropathy, as verified by kidney biopsy.[11-12] However, these findings are consistent with the observed differences in the pathogenesis of retinopathy and nephropathy. The urine proteome provides an opportunity to search for a potential diagnostic tool for kidney disease and DN, though its application is currently hampered by numerous limitations, which still remain to be solved.[13-15]

In the present work, we performed a quantitative urinary proteome analysis of patients with type 2 diabetes using the iTRAQ technology. This approach, coupled with peptide prefractionation by isoelectrofocusing, has not been used previously for urinary proteomics in DN. Owing to variability in clinical characteristics, we distinguished between 4 categories. Changes in the urine proteome were correlated with various stages of diabetes complications, particularly the stage including DR without albuminuria and renal dysfunction.

PATIENTS AND METHODS **Patient selection and urine samples** The project was approved by the local ethics committee (approval number, KB/643/08). Patients enrolled in the study were selected from inpatients hospitalized at Miedzyleski Specialist Hospital in Warsaw. Informed consent was obtained before collecting urine samples. The study was performed in accordance with the Declaration of Helsinki. Patients selection protocol, inclusion and exclusion criteria, and urine sample preparation were described in detail in *Supplementary material online*.

Sample classification We distinguished 4 study subgroups based on the clinical characteristics of diabetic patients. Group C (control) was the control sample set collected from healthy individuals without diabetes or any of the exclusion criteria. Group D (type 2 diabetes with no nephropathy) comprised samples from patients with diabetes but no biochemical markers of DKD or ophthalmologic signs of DR. Patients with a diagnosis of diabetes established during hospitalization, were included in this group. Group D+R (type 2 diabetes with retinopathy) included samples from patients with diabetes and ophthalmologic signs of DR but no markers of nephropathy with regards to microalbuminuria, overt albuminuria, or decreased glomerular filtration rate (GFR). This group was considered as having "early diabetic complications" with respect to the lack of biochemical markers of impaired renal function. Group DN (overt DN) included samples from patients with diabetes and overt DN with albuminuria, indicating the presence of advanced diabetic damage to the nephrons. It included patients with albuminuria, characterized by a wide range of the albuminuria level, namely with macroalbuminuria defined as a urinary albumin-to-creatinine ratio (UACR) over 34 mg/mmol or microalbuminuria (3.4–34 mg/mmol), but in the latter case, with parallel signs of DR, in accordance with the KDOQI criteria.[10] Owing to the small size of the study group, patients with DR were pooled into 1 group without further assignment to specific subcategories (proliferative, nonproliferative, or diabetic macular edema).[16] Patients with DR were classified into the third or fourth subgroup, but the principal classifier was based on the verification of albuminuria.

Pooled sample studies A total of 27 patients were assigned to group C (control), 33 to group D (diabetes with no complications), 15 to group D+R (diabetes + retinopathy) and 24 to group DN (overt DN). The characteristics of the groups, including age, sex, biochemical parameters, diabetes duration, concomitant diseases, and administered drugs, are provided in TABLE 1.

Pooling samples, iTRAQ study design, qualitative MS/MS, and quantitative iTRAQ analysis The sample preparation protocol, pooling samples, iTRAQ study design, mass spectrometry settings, mass spectrometry qualitative data processing, and quantitative iTRAQ analysis were described in detail in *Supplementary material online*.

Quantitative analysis of proteins using label-free method To verify the experiment with pooled samples labeled by iTRAQ, we conducted a label-free quantitation of a selected set of proteins for individual (nonpooled) samples analyzed in a single liquid chromatography–mass spectrometry (LC-MS) run. Each LC-MS run was preceded by a blank run to ensure the lack of carry-over of material from the previous analyses. The label-free quantitative analysis and statistical analysis of quantitative MS results were described in detail in *Supplementary material online*.

Statistical analysis of amylase activity and urinary albumin-to-creatinine ratio The Kruskal–Wallis test was used to compare amylase activities and UACR since no normal distribution was confirmed in the Shapiro–Wilk test.

TABLE 1 Characteristics of study groups

Characteristics	Group C (healthy controls without diabetes)	Group D (diabetes and no diabetic kidney disease: no albuminuria and no diabetic retinopathy)	Group D+R (diabetes and diabetic retinopathy with no albuminuria)	Group DN (diabetes with overt diabetic nephropathy [albuminuria] or microalbuminuria with retinopathy)
No. of patients[a] (men/women)	27 (15/12)	33 (18/15)	15 (9/6)	24 (15/9)
age, y	48 ±14 (27–71)	60 ±12 (39–86)	72 ±11 (48–85)	68 ±8 (51–81)
urinary albumin-to-creatinine ratio, mg/mmol/dm³	0.77 ±0.9 (0.14–5)	1.21 ±1.08 (0–5)	1.40 ±0.9 (0.05–3.33)	57 ±94[b] (0.5–396)
eGFR, ml/min/1.73m²	93.5 ±24.9	96.3 ±30.4	87.3 ±48.8	49.9 ±24.3
HbA$_{1c}$, %	–	8.24 ±2.73 (assayed in 61% of subjects)	8.06 ±2.01 (assayed in 73%)	7.27 ±1.97 (assayed in 71%)
diabetic retinopathy	0%	0% and 15% hypertensive angiopathy	100%	42% and 16% hypertensive angiopathy, 42% not available
diabetes duration	–	1 month – 10 years	5–25 years	<1 year – 21 years
diabetic retinopathy	0%	0% and 15% hypertensive angiopathy	100%	42% and 16% hypertensive angiopathy, 42% not available
diabetes duration	–	1 month – 10 years	5–25 years	<1 year – 21 years
common coexisting diseases				
hypertension	33%	88%	80%	96%
stable congestive heart failure	0%	15%	27%	42%
benign prostate hypertrophy	4%	3%	20%	13%
patients taking drugs known to influence albuminuria/proteinuria				
ACEI/ARB	15%	77%	80%	96%
β-blockers	15%	59%	53%	79%
diuretics	4%	41%	67%	71%
statins	19%	65%	73%	54%
hypoglycemic drugs				
insulin	0%	35%	67%	58%
biguanids	0%	74%	47%	46%
sulphonylurea derivatives	0%	18%	20%	13%
other commonly used drugs				
acetylsalicylic acid	7%	47%	40%	46%
calcium channel blockers	11%	41%	40%	58%
α-blockers	7%	3%	20%	4%

Data are presented as number of patients, percentage of patients, or mean ± standard deviation (range).

a   During the MS experiment, each group was divided into 3 equal subgroups to triplicate MS assays on pooled samples.
b   Data are skewed due to the broad range of albuminuria in the samples. Standard deviation has no use as it is not a normal distribution in this case.

Abbreviations: ACEI, angiotensin-converting enzyme inhibitors; ARB, angiotensin receptor blockers; eGFR, estimated glomerular filtration rate (calculated using the Modification of Diet in Renal Disease formula); HbA$_{1c}$, hemoglobin A$_{1c}$; MS, mass spectrometry

RESULTS   Our data showed marked differences between the groups. The principal component analysis showed a separation of all pseudosamples in the plane of the 2 components. The close position of technical replicates (samples marked A and B) confirmed a very good technical quality of our results (FIGURE 1). Interestingly, the progression from control (C) to type 2 diabetes without nephropathy (D) and type 2 diabetes with retinopathy (D+R) was marked by a clear shift along the second component, indicating the presence of a specific group of features/proteins that correlate with disease progression to the D+R stage. In contrast, the overt DN group was clearly differentiated from the other 3 along the first component, and along the second component DN was much closer to D than to D+R. This observation indicates that a different group of proteins follows the progression of disease from C through D to D+R than the progression from D to DN (TABLE 2).

FIGURE 1 Principal component analysis; 3 pooled biological replicates of control (C), diabetes (D), diabetes + retinopathy (D+R), and diabetic nephropathy (DN) samples were analyzed based on 520 proteins
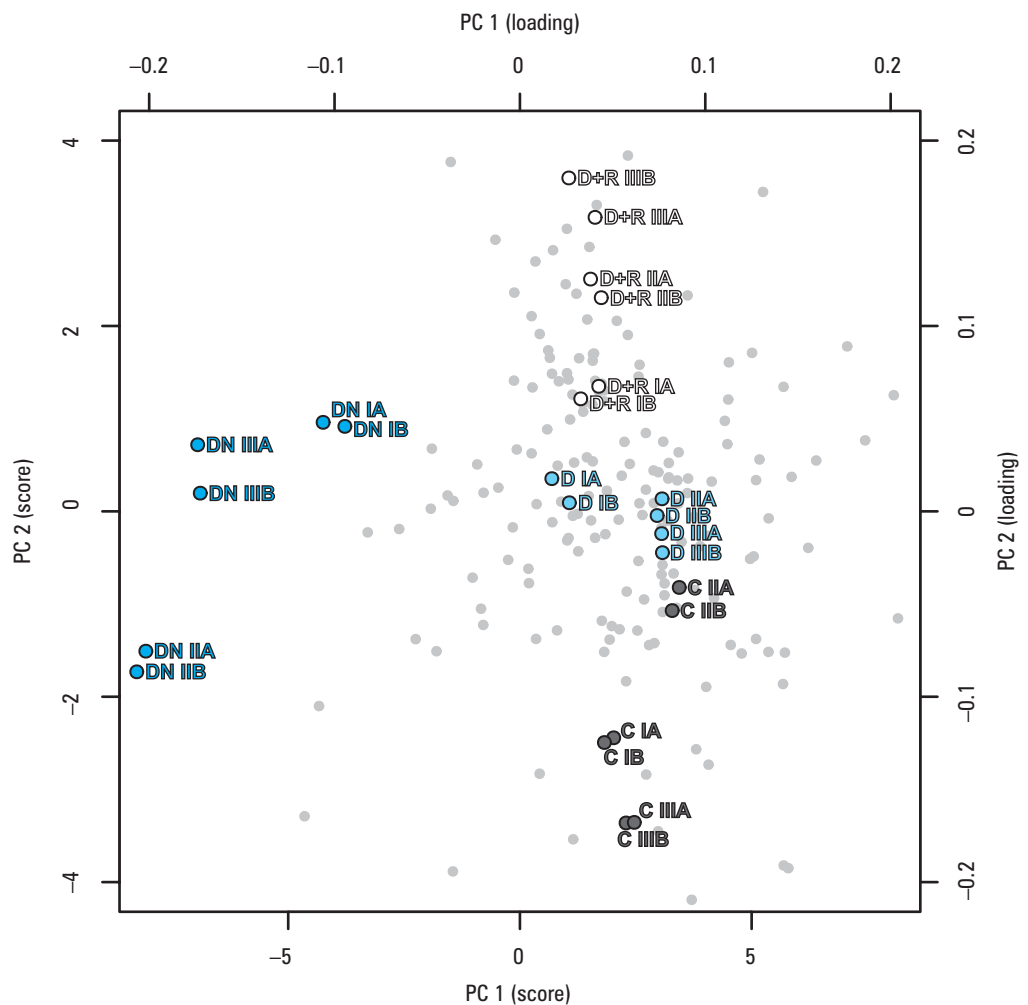
Some of these proteins have already been identified as altered in diabetic complications, whereas some have not been mentioned previously to be associated with the disease.

To test the pooling experiment results using an alternative analytical approach, we carried out a label-free analysis of individual samples assigned to 4 groups: C, 12 samples; D, 15 samples; D+R, 14 samples; and DN, 10 samples. To obtain quantitative information, 0.5-µg protein of each of the 51 samples was subjected to the LC-MS analysis (C, n = 12; D, n = 15; D+R, n = 14; DN, n = 10). Of the differentiating proteins identified in iTRAQ, only 4 were quantitated in the label-free analysis owing to smaller protein coverage expected in the case of a single LC-MS run. **FIGURE 2** shows the results of the label-free analysis for serum albumin, pancreatic α-amylase, proepidermal growth factor, and deoxyribonuclease I. Box-plots confirm the results of the analysis of pooled samples, showing the same trends of change and verifying the pooling experiment.

Validation of previously reported markers We found, among others, numerous proteins previously described as the markers of DN.[17] The results are presented in **TABLE 2**, and *q*-values indicate statistical significance. From a group of glycoproteins with a known pathophysiological role in diabetes and previously found to be upregulated

in diabetes compared with diabetes without albuminuria (ie, α-1B-glycoprotein, β-1B-glycoprotein [hemopexin], zinc-α-2-glycoprotein, and α-2-HS-glycoprotein), zinc-α-2-glycoprotein was differentiating in our study. In group D+R, the protein was slightly upregulated with a ratio of 1.21 compared with control. A ratio of 1.02 was obtained for DN, but the *q*-value was not significant. Also α-1-acid-glycoprotein was found upregulated in D+R (**TABLE 2**). Another extracellular glycoprotein from the serpin family, α1-antitrypsin, was elevated in DN, consistently with previous results for overt nephropathy.[18] Interestingly, for group D+R, we found reduced α1-antitrypsin levels. In agreement with a previous study,[18] we observed a decreased excretion of apolipoprotein A-I in groups D and D+R. On the other hand, the excretion of prostaglandin-H2 D-isomerase, an early marker of DKD, was upregulated in D+R (1.24) as anticipated, and not significantly decreased in DN.[19] The same trend was observed for osteopontin upregulated in groups D (1.32) and D+R (1.39). Albumin, the most abundant protein, was increased in DN (1.56), but almost unchanged in groups D (1.1) and D+R (1.05). We observed a significant pattern of increased urinary immunoglobulin chain secretion (Ig λ-3 chain C region, Ig κ chain C region, Ig κ chain V-I region BAN, Ig λ-7 chain C region, immunoglobulin λ-like polypeptide 5) in group D+R, 2 of them in group D

TABLE 2 Proteins previously reported as related to diabetic nephropathy with altered levels in pairwise comparisons

Protein	Protein ID	Number of peptides	Not complicated diabetes vs control (D vs C)		Diabetes with retinopathy vs control (D+R vs C)		Overt nephropathy vs control (DN vs C)	
			q-value	ratio	q-value	ratio	q-value	ratio
serum albumin	P02768	191	8×10^{-5}	1.1	0.67	1.05	1.6×10^{-4}	1.56
collagen α-2(IV) chain	P08572	NA						
collagen α-1(I) chain	P02452	18	1	1.09	0.73	0.94	5.9×10^{-4}	0.41
α-2-HS-glycoprotein	P02765	14	0.96	0.93	0.99	0.96	0.84	0.79
β-1B-glycoprotein (hemopexin)	P02790	20	0.40	0.94	0.92	1.09	0.68	0.93
α-1B-glycoprotein	P04217	29	0.98	1.06	0.98	0.97	0.45	0.89
α-1-acid glycoprotein	P02763	29	0.55	1.15	6×10^{-5}	1.46	0.87	1.25
α-1-antitrypsin	P01009	69	0.92	0.96	6×10^{-3}	0.82	0.01	1.57
zinc-α-2-glycoprotein	P25311	39	1	1.08	0.02	1.21	0.26	1.02
prostaglandin-H2 D-isomerase	P41222	55	0.03	1.09	1.6×10^{-3}	1.24	0.47	0.82
basement membrane-specific heparan sulfate proteoglycan core protein	P98160	49	0.02	0.85	0.03	0.88	4.6×10^{-2}	0.59
complement factor B	P00751	4	0.92	0.93	0.41	1.85	0.91	0.96
cathepsin D	P07339	28	0.21	1.13	6×10^{-5}	1.61	0.99	1.12
nephrin	O60500	NA						
osteopontin	P10451	35	8×10^{-5}	1.32	6×10^{-5}	1.39	0.79	0.73
vitamin D-binding protein	P02774	10	0.42	0.87	0.74	1.18	0.43	0.83
cystatin-C	P01034	5	0.29	1.2	0.98	1.06	0.38	1.29
neutrophil gelatinase-associated lipocalin	P80188	9	0.24	0.74	0.61	0.67	0.66	1.23
α-N-acetylglucosaminidase	P54802	30	0.78	0.99	0.97	0.76	0.91	1.04
fatty acid-binding protein	P07148	4	0.96	1.08	0.76	1.23	0.50	1.18
β-2-microglobulin	P61769	3	0.68	0.83	0.76	0.76	0.28	0.55
Ig λ-3 chain C regions	P0CG06	30	0.03	1.37	0.03	1.53	0.98	1.18
Ig κ chain C region	P01834	35	0.03	1.28	0.01	1.4	0.96	0.91
Ig λ-7 chain C region	A0M8Q6	20	0.06	1.37	0.03	1.67	0.97	1.17
Ig κ chain V-I region BAN	P04430	3	0.23	1.68	0.04	2.66	0.83	0.8
immunoglobulin λ-like polypeptide 5	B9A064	31	0.08	1.29	9×10^{-3}	1.58	0.90	1.18
low-affinity immunoglobulin γ Fc region receptor III-A	P08637	6	0.96	1.13	0.03	0.68	0.26	0.53
actin cytoplasmic 1	P60709	19	0.04	0.84	0.01	0.82	0.49	0.65
apolipoprotein A-I	P02647	12	0.03	0.77	1.7×10^{-3}	0.55	0.99	1.04

q-values <0.05 are shaded. The other values are given for comparison only, as their regulation status (ratio) did not reach the threshold of statistical validity.

Abbreviations: NA – not assayed

(Ig λ-3 chain C regions and Ig κ chain C region), but none in DN. Some urinary immunoglobulin fragments were previously reported as increased in diabetes and DN.[20] Interestingly, the level of low affinity immunoglobulin gamma Fc region receptor III-A was decreased in D+R.

Several proteins indicative of tubular damage, including cystatin C, neutrophil gelatinase-associated lipocalin, α-N-acetylglucosaminidase, fatty acid-binding protein, and $β_2$-microglubulin, were detected, but changes in their expression could not be confirmed because of insignificant corresponding q-values. Similarly, vitamin D-binding protein was detected but its level was not significantly altered. The exception was cathepsin D, a protein excreted from proximal tubules, the levels of which were increased almost 2-fold in group D+R. The cytoplasmic actin level was reduced in groups D and D+R. We did not detect collagen type IV (α-2 or α-1 chain), the main extracellular matrix protein with high urinary excretion recognized as an early marker of DN and associated with the deterioration of renal function in type 2 diabetic patients, even without overt proteinuria.[21,22] In the case of a complex matrix, global LC-MS analyses can detect only the most
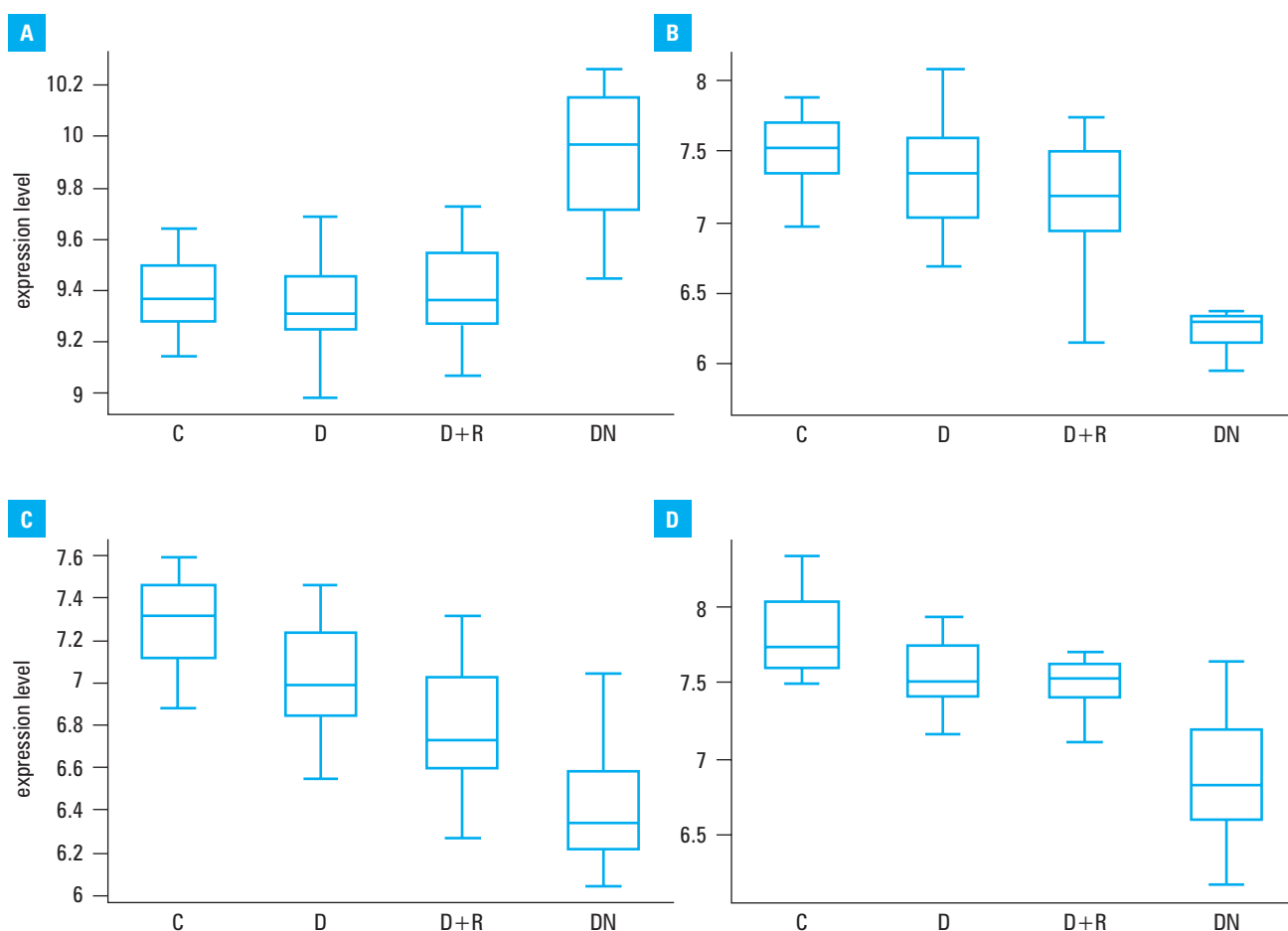
FIGURE 2 Box-plots of 4 selected urine proteins differentially expressed in iTRAQ and label-free analysis for serum albumin (**A**); pancreatic α-amylase (**B**); proepidermal growth factor (**C**); deoxyribonuclease I (**D**) in control (C), diabetes (D), diabetes + retinopathy (D+R), and diabetic nephropathy (DN) groups

abundant proteins, and a targeted analysis using a more sensitive multiple reaction monitoring mode should be applied for the detection of a predefined set of proteins. Our study focused on the identification of a new protein panel. However, we detected a decreased level of the collagen α-1(I) chain belonging to fibril-forming collagen as differentiating (ratio, 0.41) in the case of the DN group. It is consistent with data reported elsewhere that the urinary excretion of many collagen fragments detected by mass spectrometry is generally decreased in CKD, in contrast to proteins derived from serum.[18] This list includes fibril forming I, II, III, V, and XVII collagen types, as well as the type comprising subendothelium and basement membranes (VIII). The collagen-derived peptides were also observed to be relatively decreased in studies on CKD273 classifier for prognosis of CKD progression.[22] On the other hand, α-2 (IV) chain, an early marker of DKD, was described to be increased in immunoassays,[23] but was not reported in the published list.[18] Furthermore, the basement membrane-specific heparan sulfate proteoglycan core protein (perlecan) was detected and decreased in all analyzed groups.[24]

Proteins with elevated excretion in group D+R but not DN The panel of 14 proteins specifically up-regulated in group D+R deserves attention because it is potentially indicative of early diabetic complications (TABLE 3). Interestingly, only 3 other

proteins (prostaglandin-H2 D-isomerase, Ig λ-3 chain C regions, Ig κ chain C region) were similarly upregulated in group D, but none of them were found to be differentiating in group DN.

Proteins with decreased excretion in all analyzed groups Seven proteins were decreased in all 3 analyzed groups, including pancreatic α-amylase and deoxyribonuclease I related to the exocrine pancreatic system, α-amylase 2B, salivary α-amylase 1, proepidermal growth factor, mannan-binding lectin serine protease 2, and perlecan (TABLE 4). To verify the observed trend in the amylase ratio, the total urinary amylase activity was determined for 99 individual samples comprising all studied groups in parallel with urinary amylase-to-creatinine ratio (FIGURE 3). In the multiple Kruskal–Wallis comparison, we observed a significant difference between groups C and D ($P = 0.045$) and C and DN ($P = 0.007$). For the UACR, a significant difference was observed only in the case of group C vs group DN ($P = 0.0076$), but a decreasing trend was visible in group C vs group D ($P = 0.146$).

DISCUSSION In the present study, the list of changes in the urine proteome following the progression of DN includes some known protein markers of the disease, some specific markers for certain stages of the disease, and several proteins related to exocrine pancreatic function.

TABLE 3 Proteins with elevated excretion in diabetes with retinopathy vs control groups

Protein	Protein ID	Number of peptides	Not complicated diabetes vs control (D vs C)		Diabetes with retinopathy vs control (D+R vs C)		Overt nephropathy vs control (DN vs C)	
			q-value	ratio	q-value	ratio	q-value	ratio
prostaglandin-H2 D-isomerase	P41222	55	0.03	1.09	1.6×10^{-3}	1.24	0.47	0.82
acid ceramidase	Q13510	24	0.07	1.28	6×10^{-5}	1.78	1	0.96
α-1-acid glycoprotein 1	P02763	29	0.55	1.15	6×10^{-5}	1.46	0.87	1.25
α-1-acid glycoprotein 2	P19652	18	0.30	1.09	0.03	1.41	0.98	1.17
zinc-α-2-glycoprotein	P25311	39	1	1.08	0.03	1.21	1	1.02
leucine-rich α-2-glycoprotein	P02750	25	0.29	1.14	9.4×10^{-3}	1.34	0.98	0.93
lysosomal protective protein (cathepsin A)	P10619	12	0.18	1.45	5.4×10^{-3}	2.43	0.96	1.03
protein AMBP (α-1-microglubulin/ bikunin)	P02760	65	0.23	1.08	7.1×10^{-3}	1.17	0.74	0.77
β-galactosidase	P16278	22	0.81	1.09	0.02	1.51	0.73	0.89
cathepsin B	P07858	11	0.18	1.38	0.02	1.54	0.77	0.77
cathepsin D	P07339	28	0.21	1.13	6×10^{-5}	1.61	0.99	1.12
annexin A2	P07355	7	0.58	1.14	0.03	1.99	0.67	0.68
Ig λ-7 chain C region	A0M8Q6	20	0.06	1.37	0.03	1.67	0.98	1.17
Ig κ chain V-I region BAN	P04430	3	0.23	1.68	0.04	2.66	0.83	0.8
Ig λ-3 chain C regions	P0CG06	30	0.03	1.37	0.03	1.53	0.98	1.18
Ig κ chain C region	P01834	35	0.03	1.28	0.01	1.4	0.96	0.91
immunoglobulin λ-like polypeptide 5	B9A064	31	0.08	1.29	9.1×10^{-3}	1.58	0.90	1.18

q-values <0.05 are shaded.

This finding may indicate exocrine pancreatic insufficiency (EPI) overlapping the subsequent stages of DKD. According to our hypothesis and literature data, EPI may be causally related to diabetes. Although the proteins secreted by the exocrine pancreas and excreted into urine should be increased due to protein leakage in DN, we observed a reverse phenomenon. The observed decrease in amylase excretion suggests the presence of other processes accompanying renal clearance of proteins in DKD because diabetes affects other organs and their protein secretion.

Proteins with excretion specifically elevated in group D+R As mentioned above, protein markers in diabetes patients with retinopathy but without biochemical markers of renal impairment may have a crucial diagnostic meaning for early prevention. The proteins listed in TABLE 3, especially those upregulated in D+R, deserve interest as potential markers differentiating the stages of diabetic complications other than overt renal impairment. The excretion level of prostaglandin-H2 D-isomerase in group D+R (ratio 1.24) meets our expectations consistent with the results describing this protein as an early marker of DN.[19] However, a further decrease of this protein in group DN could not be confirmed due to an insufficient q-value for the observed ratio.

Several factors may obscure the expected tendency in protein excretion levels. As shown in TABLE 1, patients were burdened with numerous coexisting diseases and a complicated combination of drugs, especially with the more advanced stages of diabetic complications. These confounders affect the urinary proteome. The drugs known to reduce proteinuria can be selective against only some proteins because they are excreted with different filtration and reabsorption rates.[25]

Proteins consistently decreased in all progression stages
We also found proteins with consistent trends of decreased excretion following the analyzed stages: proepidermal growth factor, mannan-binding lectin serine protease 2, and basement membrane-specific heparan sulfate proteoglycan core protein (perlecan). Mannan-binding lectin serine protease 2 is associated with the lectin pathway of complement activation and also plays a role in IgA nephropathy.[26,27] Perlecan is an integral component of basement membranes and provides a fixed negative charge involved in charge-selective ultrafiltration. In DN, the expression of glycosaminoglycan side chains of perlecan in the glomerular basement membrane is reduced in proportion to the degree of proteinuria.[24] To the best of our knowledge, the urinary level of this protein has not been studied so far.

The proepidermal growth factor physiologically cleaved into epidermal growth factor (EGF) is a single-pass type I membrane protein expressed in the kidney, salivary gland, cerebrum, and prostate. EGF stimulates the growth of various epidermal and epithelial tissues and some fibroblasts. In addition, EGF acts as a magnesiotropic hormone stimulating magnesium reabsorption in the renal

TABLE 4 Downregulated urinary proteins associated with exocrine pancreatic function, salivary glands, and some other proteins whose level was decreased consistently in all analyzed groups; all results obtained with achieved statistical threshold

Protein	Protein ID	Number of peptides	Not complicated diabetes vs control (D vs C)		Diabetes with retinopathy vs control (D+R vs C)		Overt nephropathy vs control (DN vs C)	
			q-value	ratio	q-value	ratio	q-value	ratio
proteins associated with pancreatic exocrine function								
pancreatic α-amylase	P04746	46	$8×10^{-5}$	0.81	$6×10^{-5}$	0.7	$6.1×10^{-4}$	0.49
deoxyribonuclease-1	P24855	25	0.01	0.8	$2.6×10^{-3}$	0.71	$8.2×10^{-3}$	0.44
salivary isozyme of amylase								
α-amylase 1	P04745	40	$3.1×10^{-3}$	0.86	$3×10^{-4}$	0.74	$1.4×10^{-3}$	0.5
other proteins with decreased excretion								
α-amylase 2B[a]	P19961	41	$6.4×10^{-4}$	0.84	$6×10^{-5}$	0.69	$3×10^{-3}$	0.5
proepidermal growth factor	P01133	32	$8×10^{-5}$	0.69	$6×10^{-5}$	0.6	$1.6×10^{-4}$	0.46
mannan-binding lectin serine protease 2	O00187	11	0.02	0.75	0.03	0.64	$4.8×10^{-2}$	0.37
basement membrane-specific heparan sulfate proteoglycan core protein (perlecan)	P98160	49	0.02	0.85	0.03	0.88	0.04	0.59

[a] novel type amylase, expressed in tumorous tissue, probably liver-specific

distal convoluted tubule. Defects in EGF are the cause of hypomagnesemia type 4, known as renal hypomagnesemia with normocalciuria.[28] The significance of decreased excretion of this precursor protein is not clear.

Hypothesis of exocrine pancreatic insufficiency coexisting with diabetes   We observed decreasing trends in the levels of 3 amylase isozymes; pancreatic α-amylase, α-amylase 2B (novel type),[29] and salivary α-amylase 1. Compared with controls, these proteins exhibited decreased excretion in all 3 groups, unlike urinary albumin excretion, which was increased in DN. We also observed a diminished excretion of deoxyribonuclease I, secreted by the exocrine pancreatic glands. We did not detect lipases, phospholipases, or elastase-1. The lack of elastase-1 was expected knowing its excretion in an unchanged form in the feces.[30]

The possible diabetes-related pathophysiological explanation of the decreased excretion of pancreatic enzymes observed in our study was discussed in detail elsewhere.[31] That study found a correlation between exocrine pancreatic function and decreased secretion of amylases and lipases into the blood after pancreozymin-secretin stimulation in diabetic patients, as well as decreased fecal elastase-1. Pancreatic histology in autopsy studies has revealed substantial changes in the exocrine glands of diabetic patients, including chronic inflammation, approximately twice more frequent in diabetics than nondiabetics.[32] Autopsy also often shows pancreatic atrophy.[33] A smaller pancreas has also been shown in imaging studies of living patients with diabetes.[34] The authors discussed whether dysfunction in the exocrine pancreas associated with diabetes may be related to diabetic neuropathy, affecting the autonomic nervous system, which regulates exocrine function. They emphasized that "only very

few studies directly interested in possible correlations between diabetic neuropathy and exocrine pancreatic dysfunction are available" and further research is needed.[31] The hypothesis that the trophic action of insulin directed at acinar cells producing enzymes in the pancreas under conditions of insufficient insulin secretion affect exocrine function was discarded on the basis of the lack of changes in the pancreatic glands of most type 1 diabetes patients. Also, the fact that pancreatic changes observed in type 2 diabetes cannot be explained by the local lack of insulin, especially in early stages, does not support that hypothesis.[31] The causal relationship between diabetes and EPI is not well explored clinically, though recent work has pointed to an increased risk of overall and acute pancreatitis associated with diabetes.[35] Instead, a reverse cause–effect relationship between pancreatic β-cells dysfunction and chronic pancreatitis, progressing to diabetes, is more often considered.[36,37]

An alternative explanation for the obtained results could be the decreased glomerular filtration of amylases as well as increased tubular reabsorption. These two mechanisms play a role in renal clearance of pancreatic and salivary amylases but with more active reabsorption of salivary isozyme.[38] In general, the urinary secretion of amylases is considered in terms of the amylase creatinine clearance ratio (ACCR), which includes amylase and creatinine concentrations in urine and serum (ACCR = urine amylase/serum amylase × serum creatinine/urine creatinine × 100).[39] Previous studies point to an association between increased ACCR and renal insufficiency. However, in terms of urinary but not serum amylase concentration, the simple ratio of urinary amylase to urinary creatinine was not higher in patients with chronic renal insufficiency relative to healthy controls.[39] The literature data are

FIGURE 3 Total urinary amylase (**A**) and amylase activity-to--creatinine ratio (**B**) for control (C), diabetes (D), diabetes + retinopathy (D+R), and diabetic nephropathy (DN) groups; the box heights cover the 25–75 percentile range with whiskers indicating minimum–maximum values
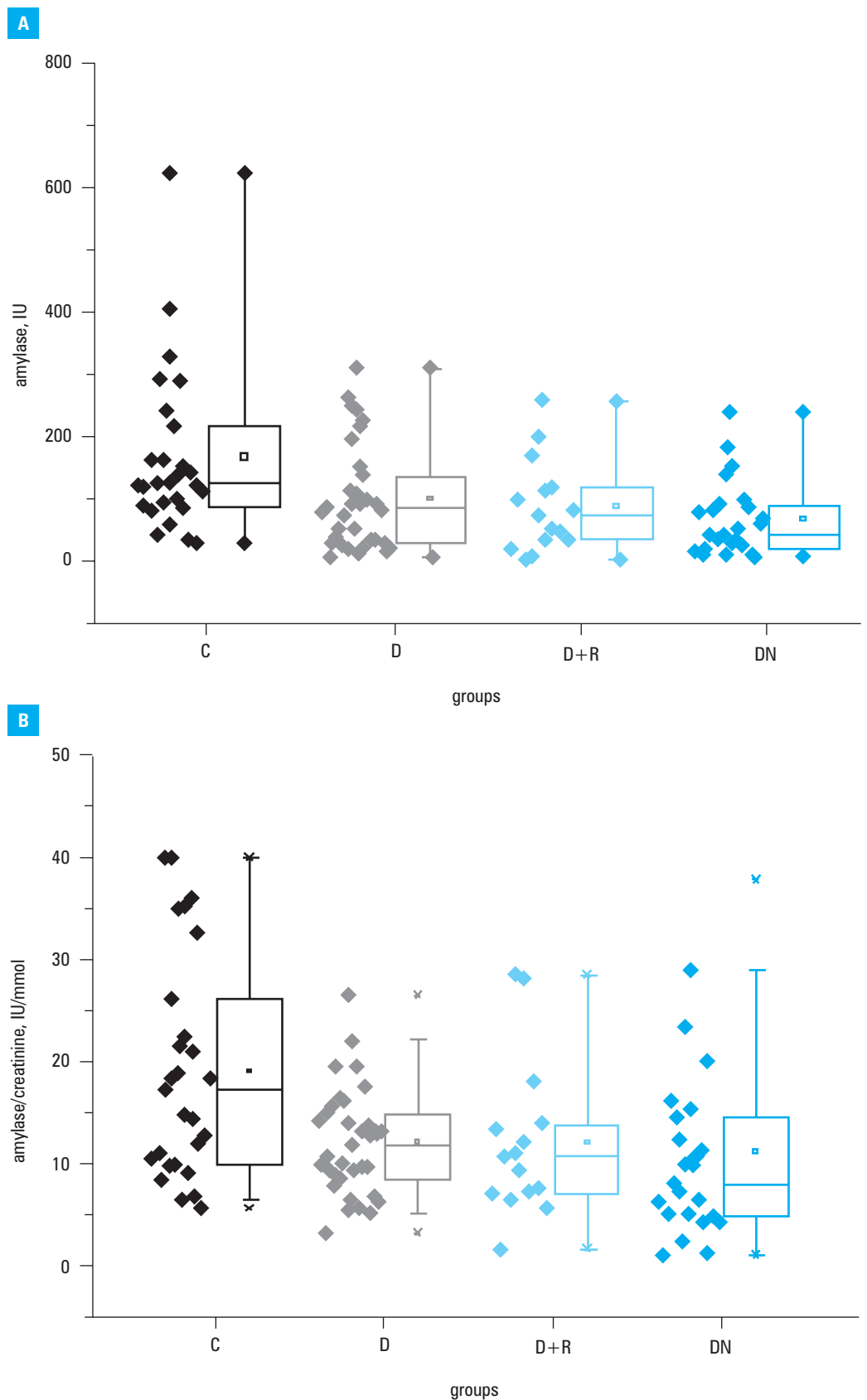
not conclusive regarding the ACCR in CKD. An increased ACCR was reported for both pancreatic and salivary isozymes in patients with noncompensated renal failure.[40] However, an elevated ACCR in CKD may be caused by increased serum creatinine rather than increased urinary amylase, which may be evident if creatinine clearance falls below 50 ml/min.[41]

Although the results obtained from MS quantitation are difficult to translate into classical terms of clearance, they show absolute underrepresentation of amylase compared to total excreted protein, arguing for the role of EPI. We also found a trend for decreasing amylase activity as diabetes progresses to DN. We found the alternative renal cause of decreased urinary amylase to be less

probable. The increased urinary protein leakage is expected in glomerular damage unless compensating reabsorption in proximal tubules increases to counteract protein loss, which is rather unlikely. The tubular contribution to amylase clearance was described previously for a case of acute pancreatitis. Its impairment due to renal insufficiency resulted in reduced amylase reabsorption and accounted for the elevated amylase excretion in regard to pancreatic and salivary isozymes.[42] The decreased level of deoxyribonuclease I observed in our study supports coexisting pancreatic dysfunction.

The reason for the decreased level of salivary amylase isozyme is less clear. A common mechanism that impairs the exocrine function of the pancreas in diabetes may consequently affect other organs including salivary glands. On the other hand, if the reason is on the kidney filtration–reabsorption side, the excretion trends would be different for pancreatic and salivary isozymes. Some studies have reported preferable reabsorption of salivary isozymes in proximal and distal tubules.[43]

Urinary excretion of differentiating proteins in other diseases Our laboratory has recently described the uroproteome characteristics of ADPKD[44] and IgA nephropathy (Bakun et al.; in preparation) using the standardized protocol. Differentiating proteins in these conditions can be compared to the results of the present study. Interestingly, similar relative deficits in pancreatic α-amylase (ratio, 0.7) and salivary α-amylase 1 (0.47) were found in ADPKD samples compared with controls. Such decreases in amylases were not observed in IgA nephropathy. Also, similar to DN, decreased levels of proepidermal growth factor and perlecan were found in both ADPKD and IgA nephropathy. However, the direction of change in other proteins was different in diabetes and other conditions. This reversed tendency was observed for apolipoprotein A-I (3.89 in ADPKD vs 0.55 in D+R) and α-1-antitrypsin (1.64 and 2.47 for ADPKD and IgA nephropathy, respectively, vs 0.82 in D+R). The β_2-microglobulin was also strongly upregulated in ADPKD (3.69), but not in DN. Serum albumin excretion was more increased in IgA nephropathy (3.56) and ADPKD (1.69) compared with the 3 diabetic groups in the present study (1.1, 1.05, and 1.56 for D, D+R, and DN, respectively). Thus, the urine proteome revealed changes that are common for different conditions, as well as some changes specific for a given disease. In the context of a discussion of amylase clearance in CKD, it is questionable to associate the decreased urinary amylase level with the decreased GFR observed in diabetes or in ADPKD. Reabsorption failure in renal tubules, concomitant with impaired filtration, can be speculated to increase amylase excretion.

The decreased excretion of pancreatic relevant proteins, observed in ADPKD, obviously does not exclude hypothesis of EPI as a cause of their diminished excretion in type 2 diabetes. The total observed effect of decreased amylase levels may reflect the synergy of several different factors. Pancreatic insufficiency is rather unlikely in ADPKD. One of the kidney diseases associated with pancreatic dysfunction is von Hippel–Lindau disease, which can mimic ADPKD and confuse the diagnosis.[45] However, although pancreatic cysts occur in 70% of the patients with von Hippel–Lindau disease and rarely cause exocrine and endocrine insufficiency, pancreatic cysts are found only in 9% of ADPKD patients. In light of the decreased urinary amylase excretion in ADPKD, the tendency of decreased pancreatic protein excretion in DN reveals a complex effect resulting from EPI and, possibly, from impaired renal function.

Study limitations It is obvious that pooling samples flattens biological diversity, which was the reason for verifying crucial protein contents in individual samples. Although the single reaction / multiple reaction monitoring techniques would be the method of choice, the label-free quantitation was applied in the frame of project budget for some proteins.

Summary DKD disturbs urinary protein excretion on various levels, including the urinary tract and prerenal factors. Although the glomerular and tubular parts of the nephron play a central role in the mechanism preserving protein balance, the prerenal contribution to the contents of protein preload is crucial when diabetes affects other body organs and may influence the urinary proteome. Using MS/iTRAQ, we have demonstrated decreased excretion of pancreatic amylases and deoxyribonuclease I, which may originate from EPI related to diabetes. The pathomorphological proof of this condition was described elsewhere. However, overlapping CKD as an alternative cause of the observed phenomenon should be confirmed, and the parallel decrease in urinary salivary isozyme elucidated.

Supplementary material online Supplementary material online is available with the online version of the paper at www.pamw.pl.

REFERENCES

1 Centers for Disease Control and Prevention (CDC). National chronic kidney disease fact sheet: general information and national estimates on chronic kidney disease in the United States, 2010. Atlanta, GA: U.S. Department of Health and Human Services (HHS).

2 Park CW. Diabetic kidney disease: from epidemiology to clinical perspectives. Diabetes Metab J. 2014; 38: 252-260.

3 United States Renal Data System, 2014 annual data report: An overview of the epidemiology of kidney disease in the United States. National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD, 2014; Volume 2 - ESDR, Table B7.

4 Wołyniec W, Jankowska MM, Król E, et al. Current diagnostic evaluation of autosomal dominant polycystic kidney disease. Pol Arch Med Wewn. 2008; 118: 767-773. Review.

5 Whaley-Connell A, Bomback AS, McFarlane SI, et al.; on behalf of the Kidney Early Evaluation Program Investigators. Diabetic Cardiovascular Disease Predicts Chronic Kidney Disease Awareness in the Kidney Early Evaluation Program. Cardiorenal Med. 2011; 1: 45-52.

6 Gosmanov AR, Wall BM, Gosmanova EO. Diagnosis and treatment of diabetic kidney disease. Am J Med Sci. 2014; 347: 406-413.

7 Solini A, Penno G, Bonora E, et al. Renal Insufficiency And Cardiovascular Events (RIACE) Study Group. Diverging association of reduced GFR and albuminuria with coronary and noncoronary events in patients with type 2 diabetes: the renal insufficiency and cardiovascular events (RIACE) Italian multicenter study. Diabetes Care. 2012; 35: 143-149.

8 Adler AI, Stevens RJ, Manley SE, et al. Development and progression of nephropathy in type 2 diabetes: the United Kingdom Prospective Diabetes Study (UKPDS 64). Kidney Int. 2003; 63: 225-232.

9 Trevisan R, Viberti G. Genetic factors in the development of diabetic nephropathy. J Lab Clin Med. 1995; 126: 342-349.

10 National Kidney Foundation. http://www2.kidney.org/professionals/KDOQI/guideline_diabetes/guide1.htm. Accessed August 2014. Kanauchi M, Kawano T, Uyama H, et al. Discordance between retinopathy and nephropathy in type 2 diabetes. Nephron. 1998; 80: 171-174.

11 Jawa A, Kcomt J, Fonseca VA. Diabetic nephropathy and retinopathy. Med Clin N Am. 2004; 88: 1001-1036.

12 Jantos-Siwy J, Schiffer E, Brand K, et al. Quantitative urinary proteome analysis for biomarker evaluation in chronic kidney disease. J Proteome Res. 2009; 8: 268-281.

13 Bramham K, Mistry HD, Poston L, et al. The non-invasive biopsy-will urinary proteomics make the renal tissue biopsy redundant? QJM. 2009; 102: 523-538.

14 Kalantari S, Nafar M, Rutishauser D, et al. Predictive urinary biomarkers for steroid-resistant and steroid-sensitive focal segmental glomerulosclerosis using high resolution mass spectrometry and multivariate statistical analysis. BMC Nephrol. 2014; 15: 141.

15 Arden GB, Sivaprasad S. The pathogenesis of early retinal changes of diabetic retinopathy. Doc Ophthalmol. 2012; 124: 15-26.

16 Rao PV, Lu X, Standley M, et al. Proteomic identification of urinary biomarkers of diabetic nephropathy. Diabetes Care. 2007; 30: 629-637.

17 Good DM, Zürbig P, Argilés A, et al. Naturally occurring human urinary peptides for use in diagnosis of chronic kidney disease. Mol Cell Proteomics. 2010; 9: 2424-2437.

18 Hirawa N, Uehara Y, Ikeda T, et al. Urinary prostaglandin D synthase (beta-trace) excretion increases in the early stage of diabetes mellitus. Nephron. 2001; 87: 321-327.

19 Bellei E, Rossi E, Lucchi L, et al. Proteomic analysis of early urinary biomarkers of renal changes in type 2 diabetic patients. Proteomics Clin Appl. 2008; 2: 478-491.

20 Kotajima N, Kimura T, Kanda T, et al. Type IV collagen as an early marker for diabetic nephropathy in non-insulin-dependent diabetes mellitus. J Diabetes Complications. 2000; 14: 13-17.

21 Siwy J, Schanstra JP, Argiles A, et al. Multicentre prospective validation of a urinary peptidome-based classifier for the diagnosis of type 2 diabetic nephropathy. Nephrol Dial Transplant. 2014; 29: 1563-1570.

22 Araki S, Haneda M, Koya D, et al. Association between urinary type IV collagen level and deterioration of renal function in type 2 diabetic patients without overt proteinuria. Diabetes Care. 2010; 33: 1805-1810.

23 van der Pijl JW, Daha MR, van den Born J, et al. Extracellular matrix in human diabetic nephropathy: reduced expression of heparan sulphate in skin basement membrane. Diabetologia. 1998; 41: 791-798.

24 Tojo A, Kinugasa S. Mechanisms of glomerular albumin filtration and tubular reabsorption. Int J Nephrol. 2012; 2012: 481520.

25 Roos A, Rastaldi MP, Calvaresi N, et al. Glomerular activation of the lectin pathway of complement in IgA nephropathy is associated with more severe renal disease. J Am Soc Nephrol. 2006; 17: 1724-1734.

26 Mucha K, Bakun M, Jaźwiec R, et al. Complement components, proteolysis-related, and cell communication-related proteins detected in urine proteomics are associated with IgA nephropathy. Pol Arch Med Wewn. 2014; 124: 380-386.

27 Groenestege W, Thebault S, van der Wijst J, et al. Impaired basolateral sorting of pro-EGF causes isolated recessive renal hypomagnesemia. J Clin. Invest. 2007; 117: 2260-2267.

28 Koyama I, Komine S, Iino N, et al. alpha-Amylase expressed in human liver is encoded by the AMY-2B gene identified in tumorous tissues. Clin Chim Acta. 2001; 309: 73-83.

29 Leeds JS, Oppong K, Sanders DS. The role of fecal elastase-1 in detecting exocrine pancreatic disease. Nat Rev Gastroenterol Hepatol. 2011; 8: 405-415.

30 Hardt PD, Ewald N. Exocrine pancreatic insufficiency in diabetes mellitus: a complication of diabetic neuropathy or a different type of diabetes? Exp Diabetes Res. 2011; 2011: 761950.

31 Blumenthal HT, Probstein JG, Berns AW. Interrelationship of diabetes mellitus and pancreatitis. Arch Surg. 1963; 87: 844-850.

32 Olsen RS. The incidence and clinical relevance of chronic inflammation in the pancreas in autopsy material. Acta Pathol Microbiol Scand A. 1978; 86: 361-364.

33 Bilgin M, Balci NC, Momtahen AJ, et al. MRI and MRCP findings of the pancreas in patients with diabetes mellitus: compared analysis with pancreatic exocrine function determined by fecal elastase 1. J Clin Gastroenterol. 2009; 43: 165-170.

34 Shen HN, Chang YH, Chen HF, et al. Increased risk of severe acute pancreatitis in patients with diabetes. Diabet Med. 2012; 29: 1419-1424.

35 Sikkens EC, Cahen DL, van Eijck C, et al. Patients with exocrine insufficiency due to chronic pancreatitis are undertreated: a Dutch national survey. Pancreatology. 2012; 12: 71-73.

36 Ewald N, Hardt PD. Diagnosis and treatment of diabetes mellitus in chronic pancreatitis. World J Gastroenterol. 2013; 19: 7276-7281.

37 Duane WC, Frerichs R, Levitt MD. Simultaneous study of the metabolic turnover and renal excretion of salivary amylase - 125I and pancreatic amylase - 131I in the baboon. J Clin Invest. 1972; 51: 1504-1513.

38 Keogh JB, McGeeney KF, Drury MI, et al. Renal clearance of pancreatic and salivary amylase relative to creatinine in patients with chronic renal insufficiency. Gut. 1978; 19: 1125-1130.

39 Maeda M, Otsuki M, Okano K, et al. Clinical evaluation of amylase-creatinine clearance ratio and amylase isoenzyme clearance in chronic renal failure. Gastroenterol Jpn. 1981; 16: 242-248.

40 Collen MJ, Ansher AF, Chapman AB, et al. Serum amylase in patients with renal insufficiency and renal failure. Am J Gastroenterol. 1990; 85: 1377-1380.

41 Johnson SG, Ellis CJ, Levitt MD. Mechanism of increased renal clearance of amylase/creatinine in acute pancreatitis. N Engl J Med. 1976; 295: 1214-1217.

42 Hegarty JE, O'Donnell MD, McGeeney KF, et al. Pancreatic and salivary amylase/creatinine clearance ratios in normal subjects and in patients with chronic pancreatitis. Gut. 1978; 19: 350-354.

43 Bakun M, Niemczyk M, Domanski D, et al. Urine proteome of autosomal dominant polycystic kidney disease patients. Clin Proteomics. 2012; 9: 13.

44 Chatha RK, Johnson AM, Rothberg PG, et al. Von Hippel-Lindau disease masquerading as autosomal dominant polycystic kidney disease. Am J Kidney Dis. 2001; 37: 852-858.

Zmiany w proteomie moczu towarzyszące progresji nefropatii cukrzycowej

Andrzej Lewandowicz[1], Magdalena Bakun[2], Rafał Kohutnicki[1],
Agnieszka Fabijańska[2], Michał Kistowski[2], Jacek Imiela[1], Michał Dadlez[2,3]

1 Międzyleski Szpital Specjalistyczny, Warszawa
2 Laboratorium Spektrometrii Mas, Instytut Biochemii i Biofizyki, Polska Akademia Nauk, Warszawa
3 Wydział Biologii, Uniwersytet Warszawski, Warszawa

SŁOWA KLUCZOWE

amylaza, cukrzycowa choroba nerek, nefropatia cukrzycowa, proteomika moczu, zewnątrzwydzielnicza niewydolność trzustki

STRESZCZENIE

WPROWADZENIE   Ze względu na rozpowszechnienie cukrzycy typu 2, cukrzycowa choroba nerek (*diabetic kidney disease* – DKD) staje się główną przyczyną schyłkowej niewydolności nerek. Wykorzystywane obecnie markery nefropatii cukrzycowej opierają się na albuminurii i klinicznych cechach retinopatii. Potrzebne są – zwłaszcza do wykrywania wczesnych stadiów powikłań – czułe i swoiste nieinwazyjne narzędzia diagnostyczne, niewrażliwe na choroby współistniejące.

CELE   Celem pracy była analiza zmian wydalania białek z moczem w zależności od stadium DKD za pomocą technik proteomiki ilościowej.

PACJENCI I METODY   27 zdrowych ochotników w grupie kontrolnej dopasowano pod względem wieku i płci do 72 pacjentów z cukrzycą, przydzielonych do trzech grup: bez cech retinopatii i nefropatii (n = 33); z retinopatią, ale bez albuminurii (n = 15); z nefropatią cukrzycową (*diabetic neuropathy* – DN), potwierdzoną na podstawie jawnej albuminurii lub mikroalbuminurii z retinopatią (n = 24). W celu określenia różnic międzygrupowych próbki zostały częściowo spulowane, oznakowane za pomocą odczynnika iTRAQ w wersji 8-kanałowej, a mieszanina wynikowych peptydów rozdzielona za pomocą izoelektroogniskowania. Otrzymane frakcje analizowano za pomocą metody chromatografii cieczowej sprzężonej z tandemową spektrometrią mas (LC-MS/MS). Zebrane dane analizowano za pomocą programu MASCOT i własnych programów do analizy danych proteomicznych.

WYNIKI   Zmiany w proteomie moczu towarzyszących postępowi DKD dotyczyły wielu znanych markerów DN oraz innych białek. Obniżony poziom wydalania kilku z nich wiąże się prawdopodobnie z upośledzoną funkcją wydzielniczą innych narządów dotkniętych cukrzycą. Zwłaszcza zmniejszone wydalanie amylazy trzustkowej i deoksyrybonukleazy I sugeruje zewnątrzwydzielniczą niewydolność trzustki (*exocrine pancreatic insufficiency* – EPI), nakładającą się na cukrzycę typu 2.

WNIOSKI   Spadek wydalania z moczem kilku enzymów związanych z trzustką sugeruje EPI na tle cukrzycy. Hipoteza ta wymaga dalszej weryfikacji, niemniej podczas interpretacji wyników ilościowej proteomiki moczu należy brać pod uwagę czynniki nerkowe i pozanerkowe.

Full description of patients and methods

Patient selection and urine samples

The project was approved by the local ethics committee (approval number KB/643/08).

Patients enrolled in the study were selected from inpatients hospitalized at Miedzyleski

Specialist Hospital in Warsaw. Informed consent was obtained before collecting urine

samples. The study was performed in accordance with the Declaration of Helsinki. The

control samples were collected from inpatients admitted for routine diagnosis or hospitalized

for reasons not affecting the urinary tract. A few healthy controls were also selected from

outpatients. Blood samples for routine examination of the health status were collected

according to a standard protocol required for hospitalization. Candidates were selected on the

basis of their medical records, physical examination, and laboratory tests, including red and

white cell counts, C-reactive protein, creatinine and urea levels, blood glucose concentration,

albumin, and total protein concentration. A general urine examination, including albuminuria

and urine creatinine for the urinary albumin-to-creatinine ratio (UACR), was also carried out.

Transaminase assays and hepatitis B and C tests were performed on admission to the hospital,

and patients with abnormalities were excluded from the study. All of the basic assays were

performed using the hospital's automated analyzer (Beckman Couler/Synchron System) and

standard protocols. The creatinine concentration was determined using the classic Jaffe

method [1]; total amylase activity, using AMY7 reagent [2]; and albuminuria, using the

turbidimetric method in the Synchron LX system.

Inclusion and exclusion criteria

Patients with diabetes are naturally burdened with several other diseases, with diabetes as a general cause of these complications on the background of metabolic imbalance and vascular damage. Thus, conditions concomitant to diabetes and diabetic kidney disease (DKD), including hypertension, dyslipidemia, congestive heart failure (stable New York Heart Association class up to class II), stable coronary artery disease, and atrial flutter/fibrillation, were not excluded. Candidates with conditions that influence the urine proteome, including urinary tract infections, suspected glomerulopathies other than those of diabetic origin, abnormalities revealed by liver markers, immunological and hematological clonal diseases, cancer, pneumonia, and patients in a bad general condition were excluded.

Sample classification

We distinguished 4 groups based on the clinical characteristics of diabetic patients. Group C (control) was the control sample set collected from healthy individuals without diabetes or any of the exclusion criteria. Group D (type 2 diabetes with no nephropathy) comprised samples from patients with diabetes but no biochemical markers of DKD or ophthalmologic signs of diabetic retinopathy (DR). Patients with a diagnosis of diabetes established during hospitalization, were included in this group. Group D+R (type 2 diabetes with retinopathy) included samples from patients with diabetes and ophthalmologic signs of DR but no markers of nephropathy with regards to microalbuminuria, overt albuminuria, or decreased glomerular filtration rate (GFR). This group was considered as having "early diabetic complications" with respect to the lack of biochemical markers of impaired renal function. The diabetic nephropathy (DN) group (overt DN) included samples from patients with diabetes and overt DN with mandatory albuminuria, indicating the presence of advanced diabetic damage to the

nephrons. Here, we assigned patients with albuminuria, characterized by a wide range of the albuminuria level, namely with macroalbuminuria defined as a UACR over 34 mg/mmol or microalbuminuria (3.4–34 mg/mmol), but in the latter case the latter case, with parallel signs of DR, in accordance with the Kidney Disease Outcomes Quality Initiative (KDOQI) criteria [3]. Simultaneously, elevated creatinine and decreased GFR were markers of chronic kidney disease, rather than factors affecting classification, according to the definition of DKD independent of GFR, which can be even elevated in the hyperfiltration stage. The assessment of albuminuria in controls after 3 to 6 months was not technically possible, though it is recommended by KDOQI for the diagnosis of DKD. In the fourth group, roughly half of the patients (42%) had signs of DR and 16%—hypertensive angiopathy. In 42% of the subjects, an ophthalmologic examination was not available, but they had overt macroalbuminuria. Owing to the small size of the study group, patients with DR were pooled into 1 group without further assignment to specific subcategories (proliferative, nonproliferative, or diabetic macular edema) [4]. Patients with DR were classified into the third or fourth subgroup, but the principal classifier was based on the verification of albuminuria.

Pooled sample studies

Twenty-seven patients were assigned to group C (control); 33, to group D (diabetes with no complications); 15, to group D+R (diabetes + retinopathy); and 24, to group DN (overt DN). The exact characteristics of the groups, including age, sex, biochemical parameters, diabetes duration, concomitant diseases, and administrated drugs, are provided in Table 1 of the main text.

Sample preparation

All included patients provided clean-catch urine samples (~80 ml) at random time (11 AM – 2 PM). The time interval between sample collection and preceding urination was at least 2 hours, but no more than 4 hours. Urine samples were stored at room temperature for less than 1 hour and processed using a modified standard protocol [5,6]. Briefly, 4 ml of 1 M HEPES (pH 7.2) buffer was added to 46 ml of urine in a Falcon tube to a final volume of 50 ml, vortexed for 2 minutes, and centrifuged at 3000 rcf for 10 minutes to remove cellular debris. The supernatant was filtered through 0.45 μm Roth syringe filters, then thoroughly mixed, aliquoted in 1-ml portions, and frozen at –80°C for further analysis. Frozen samples were transported in dry ice and thawed only once for further preparation.

Sample filtration

Cutoff membrane filters (10 kDa, Amicon Ultra-0.5, Millipore) were washed twice with MilliQ water prior to use. Urine was centrifuged through the membrane at $14000 \times g$ (15 min). Next, 500 μl of water was added to the retentate and centrifugation was repeated. To recover the concentrated and desalted sample, the filter was placed upside down in a clean microcentrifuge tube and centrifuged for 2 minutes at $1000 \times g$. The protein concentration was measured using the Bradford method and aliquots stored at –80°C.

Pooling samples and iTRAQ study design

Samples from each study group were divided into 3 pools: n = 9 each in the control subgroups (C I–III), n = 11 each in the diabetes subgroups (D I–III), n = 5 in diabetes with rethinopathy group subgroups (D+R I–III), and n = 8 in diabetic nephropathy subgroups (DN I–III). From each sample within the subgroup, aliquots corresponding to 100 μg of protein were pooled into pseudosamples. Two aliquots of each pseudosample were obtained, representing 2 technical replicates (marked A and B), resulting in a set of 24 pooled pseudosamples to be

compared after iTRAQ labeling (the set of the 3 pools within each of the 4 study groups were evaluated twice). Because 8-plex iTRAQ was used, 2 technical replicates of each group were compared in one liquid chromatography–tandem mass spectrometry (LC-MS/MS) experiment. To analyze 24 samples, we conducted a set of 3 independent LC-MS/MS experiments.

iTRAQ labeling

Reagents for iTRAQ labeling were provided in the Applied Biosystems iTRAQ kit. Before labeling, protein aliquots were evaporated to dryness in a speedvac, dissolved in 20 µl dissolution buffer with 0.1% sodium dodecyl sulphate, reduced with tris(2-carboxyethyl)phosphine, cysteine-blocked with methyl methanethiosulfonate, and digested overnight with trypsin (Promega). Each group of samples was differentially labeled with 1 of the 8 iTRAQ tags (113 and 114 for C samples, 115; 116 for D, 117; 118 for D+R, 119; 121 for DN) for 2 hours according to the manufacturer's protocol. The reaction was quenched by 100 µl H_2O.

For each of the 3 LC-MS/MS experiments, 2 C, 2 D, 2 D+R, and 2 DN iTRAQ-labeled samples were combined and 340 µl buffer added (8M urea, 0.2% IPG buffer pH 3–11 NL [GE Healthcare, Fairfield CT, USA] 0.002% bromophenol blue in 50 mM Tris-HCl, pH 8.0). The solution was applied to an 18-cm IPG strip with 3–11 NL pH gradients (GE Healthcare) for isoelectrophocusing: 340 µl of sample/strip, corresponding to 400 µg of protein. The immobilized pH gradient (IPG) strip was rehydrated overnight in an IPG box). The next day, the strips underwent IEF using an Ettan IPGphor 3 electrophoresis system. Two electrophoresis steps were used. The first step consisted of a 5-hour prerun at 500 V. During this step, the conductivity decreased and salts and other highly conductive compounds moved towards the electrode (anode). In the second step, a long gradient focusing program was used: 1 h at 500 V, 9 h at 1000 V, and 30 h at 8000 V with a final current of 5 µA.

After IEF, the strip was removed from the tray, and the overlay oil blotted with a paper tissue. The strip was wrapped in parafilm and stored at –80°C. For sectioning, the tissue was placed on a tray, cooled with dry ice, cut into 7-mm sections, and transferred into individual 1.5-ml siliconized Eppendorf tubes. In total, 18-cm-long gel strips were sliced into 26 sections. Peptides were extracted from gel sections by 2 cycles: addition of 60 µl 0.1% trifluoroacetic acid and 2% acetonitrile, and vortexing the tubes for 40 min at room temperature. Aliquots with extracted peptides were stored at –80°C for LC-MS/MS analysis.

Mass spectrometry: LC-MS/MS settings

The peptide mixture (20µl) was applied to a nanoACQUITY UPLC Trapping Column (Waters) using water containing 0.1% formic acid as the mobile phase and then transferred to a nanoACQUITY UPLC BEH C18 Column (Waters, 75 µm/250-mm) using an acetonitrile gradient (3–33% over 150 min) in the presence of 0.1% formic acid at a flow rate of 250 nl/min. The column outlet was directly coupled to the ion source of the LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific, USA) working in the regime of data-dependent MS to MS/MS switch. Higher-energy collisional dissociation (HCD) fragmentation was used. Other Orbitrap parameters were as follows: one MS scan followed by max. 5 MS/MS scans, capillary voltage 1.5 kV, data acquired in positive polarity mode.

Mass spectrometry: qualitative MS/MS data processing

The acquired MS/MS data were preprocessed with Mascot Distiller (version 2.3.2.0, Matrix Science). A database search using the MASCOT search engine was carried out in a 3-step procedure described elsewhere [7] to calculate MS and MS/MS measurement errors and to recalibrate the data for the repeated MASCOT search. The initial search parameters were set as follows: enzyme, semitrypsin; fixed modification, cysteine modification by MMTS and

iTRAQ labeling of the N-terminus of peptides and lysine side chains; variable modifications, oxidation (M); max missed cleavages, 1. The Swiss-Prot database was searched with the taxonomy restricted to *Homo sapiens* (20249 sequences). For the repeated search, the recalibrated data from all gel sections were merged into 1 input file and, using MASCOT, searched against the Swiss-Prot database supplemented with a decoy database to obtain a statistical assessment of the identification of each peptide by a joined target/decoy database search strategy [8]. This procedure provided q-value estimates for each peptide spectrum match (PSM) in the dataset. All PSMs with q-values exceeding 0.01 were removed from further analysis. A protein was regarded as confidently identified if at least 2 of its peptides were found. Proteins identified by a subset of peptides from another protein were excluded from analysis. Proteins that exactly matched the same set of peptides were clustered into one group/cluster. MS/MS spectra of peptides meeting the above acceptance criteria were subjected to quantitative analysis to obtain a list of proteins differentially populated between a set of groups (Differential Protein List).

Mass measurement error correction

The maximum mass deviation (MMD) for precursor and fragment ions was established in a procedure involving 2 database searches separated by a mass recalibration step. Data from each LC-MS/MS run was searched in the first pass with permissive MMD settings (precursor ions, ±40 ppm; fragment ions, ±0.8 Da). The resulting Mascot DAT files were next imported to an in-house Java tool (DATViewer, http://proteom.ibb.waw.pl/datviewer/index.html), which implements a procedure for systematic mass errors elimination and high accuracy MMD estimation. Only PSMs with a score value exceeding the smaller of the Mascot identity and homology thresholds were used for the mass accuracy assessment. For each LC-MS/MS run, the mass correction was performed by fitting a smooth LOESS curve to the scatterplot of

the observed mass errors vs precursor ion masses. The obtained normalization function was then used for the entire set of precursor ions. A separate calibration function was also calculated for fragmentation spectra on the basis of the masses of singly charged y-series ions. The new MMD values were estimated as 3-fold the standard deviation of the mass errors after recalibration. Finally, the mass-corrected spectra were exported as Mascot Generic File format files and resubmitted to the database search engine to obtain final peptide and protein identifications.

Identification estimation *q*-value

For the statistical assessment of peptide assignments, the fragmentation spectra were matched against a joined target/decoy database [8]. The target part of the database containing true protein sequences was concatenated with a decoy part composed of reversed versions of these sequences. All peptide PSMs, from the forward and reverse database, were sorted according to their score modified by subtracting the smaller of the Mascot identity and homology thresholds. The number of false positive identifications associated with a specific position P in the sorted list was estimated by doubling the number PSMs from the decoy part of the database at positions not greater than P. The position-related false discovery rate (FDR) was calculated by dividing the estimated number of false positives by the total number of PSMs at preceding positions. To address the fact that FDR itself is not a function of the underlying score (ie, FDR can decrease with increasing position in the sorted list), the position-related FDRs were next converted to *q*-values, as described by Kall et al.[9]. The presented analysis was performed using a proprietary software tool implemented in the Java programming language (MScan, http://proteom.ibb.waw.pl/mscan/index.html).

iTRAQ quantitative analysis

For protein quantitation, only unique peptides (peptides belonging to only 1 protein/cluster) were included. In the first step, iTRAQ reporter ion peaks were detected in the preprocessed MS/MS spectra. Next, the intensities were corrected for isotope impurity using the information provided by the reagent manufacturer. For each spectrum, the geometric means of 2 reporter ion intensities belonging to 1 study group were calculated separately. The ratio of those mean values was reported as the peptide ratio. If more than 1 spectrum was obtained for a peptide in a single LC-MS/MS experiment, the median peptide ratio for all spectra was used. Prior to calculating the protein ratio, peptide ratios were median-normalized to remove systematic bias. Protein ratios were calculated as the median ratio of their peptide ratios. The significance of a single protein ratio was assessed by Diffprot [10]. In this program, the statistical validity of the expression status of the protein, represented by its calculated protein ratio, is based solely on a statistical analysis of the MS/MS datasets from a given experiment without assumptions about the character of the distribution of peptide ratios in a dataset (its normality). The probability of obtaining a given protein ratio by random selection from the dataset was tested by calculating protein ratios for a large number of permuted decoy datasets in which the peptide-protein assignment was scrambled. Calculated p-values were adjusted for multiple testing using a procedure controlling for the FDR. The significance threshold for iTRAQ quantitative analysis was set at q-value of less than 0.05.

Label-free quantitative analysis

LC-MS settings

To verify the experiment with pooled samples labeled by iTRAQ, we conducted a label-free quantitation of a select set of proteins for individual (nonpooled) samples analyzed in a single LC-MS run. Each LC-MS run was preceded by a blank run to ensure a lack of carry-over of material from the previous analyses.

First, qualitative analyses (peptide and protein identification) were performed on pooled urine samples in a data-dependent MS-to-MS/MS acquisition mode. Up to 5 MS/MS processes were allowed for each MS scan. To increase the number of peptide identifications, 3 LC-MS/MS runs were performed per pooled sample, each covering 1 of 3 ranges of m/z values: 300–600, 500–900, and 800–2000. This approach was found to substantially improve the coverage of protein identification.

Quantitative analyses of individual samples were carried out in separate survey scans of LC-MS runs with an m/z measurement range of 300 to 2000 using the same acetonitrile gradient as in the qualitative LC-MS/MS runs. The data-dependent MS-to-MS/MS switch was disabled, and the spectrometer resolution was set to 15000.

Qualitative MS data processing and database search

Qualitative MS data processing and a database search were conducted as described for iTRAQ-labeled samples, but with the following differences in search parameters: fixed modification, carbamidomethylation (C); variable modifications, oxidation (M). Only PSMs with q-values of 0.01 or lower were regarded as confidently identified. Proteins identified by a subset of peptides from another protein were excluded from analysis, and proteins matching the same set of peptides were clustered into single groups.

Quantitative MS data processing

The list of peptide identifications from the LC-MS/MS runs was overlaid onto 2-dimensional maps generated from the LC-MS profile data for individual samples. A more detailed description of the feature extraction is provided elsewhere [11]. Briefly, the list of identified peptides was used to tag the corresponding peptide-related ion spectra on the basis of m/z value, the deviation from the predicted elution time, and the match between theoretical and

observed isotopic envelopes. The relative abundance of each peptide ion was determined as the height of a 2-dimensional fit with the most prominent peak of the tagged isotopic envelope.

After the feature extraction, a filter was applied to the dataset to remove peptide ions with missing quantitative values in more than 20% of the samples. To avoid the removal of strictly differential peptides (ie, detected or missing in only one of the studied groups of samples), the χ^2 test was used to assess significant inequalities in the distribution of the missing values in the sample groups. Next, a modified k-nearest neighbors procedure was used to input the remaining missing values. The applied modifications included the usage of variable size neighborhoods and correlation-based metric instead of the Euclidean distance.

To minimize the effects of nonbiological sources of variation, log-transformed peptide abundance was normalized by fitting a robust locally weighted regression smoother (LOESS) between the individual samples and a median pseudosample. The parameters of the fit were established using a set of features exhibiting low variance in the unnormalized data and then applied to the whole data set. Finally, the normalized peptide-level data was rounded up to relative protein abundance. The procedure involved rescaling the abundance of peptides that originate from the same protein to a common level, followed by computing their median value.

Statistical analysis of quantitative MS results from label-free analysis

To select differentially expressed proteins, a nonparametric analysis of variance-based resampling test was used. The resulting P values were corrected for multiple hypothesis testing using a 2-step Benjamini–Hochberg procedure that controls for FDR [12]. Relative protein abundances with adjusted P values (≤ 0.01) and fold change values of 2 and higher were considered significantly changed in at least one of the studied groups. All statistical

analyses were performed using a proprietary software running in the MATLAB environment (MathWorks; MStat, http://proteom.ibb.waw.pl/).

Statistical analysis of amylase activity and UACR

The Kruskal–Wallis test was used to compare amylase activities and UACR since no normal distribution was proven in the Shapiro–Wilk test.

References

1 Jaffe M. Ueber den Niederschlag welchen Pikrinsäure in normalen Harn erzeugt und über eine neue reaction des Kreatinins. Z Physiol Chem. 1886; 10: 391-400.

2 International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) et al. IFCC primary reference procedures for the measurement of catalytic activity concentrations of enzymes at 37 degrees C. Clin Chem Lab Med. 2006; 44: 1146-1155.

3 National Kidney Foundation. http://www2.kidney.org/professionals/KDOQI/guideline_diabetes/guide1.htm. Accessed August 2014.

4 Arden GB, Sivaprasad S. The pathogenesis of early retinal changes of diabetic retinopathy. Doc Ophthalmol. 2012; 124: 15-26.

5 Rao PV, Lu X, Standley M, et al. Proteomic identification of urinary biomarkers of diabetic nephropathy. Diabetes Care. 2007; 30: 629-637.

6 Tyan YC, Yang MH, Chen SC, et al. Urinary protein profiling by liquid chromatography/tandem mass spectrometry: ADAM28 is overexpressed in bladder transitional cell carcinoma. Rapid Commun Mass Spectrom. 2011; 25: 2851-2862.

7 Mikula M, Gaj P, Dzwonek K, et al. Comprehensive analysis of the palindromic motif TCTCGCGAGA: a regulatory element of the HNRNPK promoter. DNA Res. 2010; 17: 245-260.

8 Elias JE, Gygi SP. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. Nat Methods. 2007; 4: 207-214.

9 Kall L, Storey JD, MacCoss MJ, Noble WS. Assigning significance to peptides identified by tandem mass spectrometry using decoy databases. J Proteome Res. 2008; 7: 29-34.

10 Malinowska A, Kistowski M, Bakun M, et al. Diffprot – software for non-parametric statistical analysis of differential proteomics data. J Proteomics. 2012; 75: 4062-4073.

11 Bakun M, Karczmarski J, Poznanski, et al. An integrated LC-ESI-MS platform for quantitation of serum peptide ladders. Application for colon carcinoma study. Proteomics Clin Appl. 2009; 3: 932-946.

12 Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Royal Statistical Soc B. 1995; 57: 289-300.