Urinary Proteomics for Early Diagnosis in Diabetic Nephropathy

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Diabetic nephropathy (DN) is a progressive kidney disease, a well-known complication of long-standing diabetes. DN is the most frequent reason for dialysis in many Western countries. Early detection may enable development of specific drugs and early initiation of therapy, thereby postponing/preventing the need for renal replacement therapy. We evaluated urinary proteome analysis as a tool for prediction of DN. Capillary electrophoresis–coupled mass spectrometry was used to profile the low–molecular weight proteome in urine. We examined urine samples from a longitudinal cohort of type 1 and 2 diabetic patients (n = 35) using a previously generated chronic kidney disease (CKD) biomarker classifier to assess peptides of collected urines for signs of DN. The application of this classifier to samples of normoalbuminuric subjects up to 5 years prior to development of macroalbuminuria enabled early detection of subsequent progression to macroalbuminuria (area under the curve [AUC] 0.93) compared with urinary albumin routinely used to determine the diagnosis (AUC 0.67). Statistical analysis of each urinary CKD biomarker depicted its regulation with respect to diagnosis of DN over time. Collagen fragments were prominent biomarkers 3–5 years before onset of macroalbuminuria. Before albumin excretion starts to increase, there is a decrease in collagen fragments. Urinary proteomics enables noninvasive assessment of DN risk at an early stage via determination of specific collagen fragments. ***Diabetes*** **61:3304–3313, 2012**

Chronic kidney disease (CKD) is usually characterized by a slow and asymptomatic but progressive impairment of renal function. It generally takes several years from the onset of clinically detectable disease until end-stage renal disease occurs. Patients suffering from end-stage renal disease require renal replacement therapy (dialysis or renal transplantation) for the rest of their lives. Owing to organ shortage, renal transplantation is not an option for all patients. The most common cause of CKD in North America, Europe, and Asia today is diabetic nephropathy (DN), followed by hypertension and glomerulonephritis (1).

DN represents a major and growing public health problem, which may be improved upon by more accurate detection at an early stage (2,3). Early detection may enable development of specific drugs and early initiation of appropriate preventive therapy that can delay or prevent progression to later stages of disease.

In clinical practice, DN is diagnosed by presence of proteinuria and/or changes in serum creatinine indicating decline in the glomerular filtration rate (4). Although these tests are appropriate in patients with advanced DN, high interindividual variability and, as a consequence, moderate specificity and sensitivity at early stages of disease are major limitations for early diagnosis with these standard tests (5). In general, development of DN is characterized by a progressive increase of urinary albumin excretion rate (UAER) from normo- to micro- to macroalbuminuria (6). Microalbuminuria is considered a risk marker indicating possible onset of DN and is today the best predictor of DN available in the clinic (7,8). However, microalbuminuria may be a marker rather than a predictor of DN. It is not specific for DN and is highly variable within an individual, further compromising its specificity (9,10). Originally, it was reported that 80% of patients with type 1 diabetes and microalbuminuria would develop DN, but our previous results show that only ~34% of type 1 diabetic patients with microalbuminuria progress further to macroalbuminuria and 15–20% even regress to normoalbuminuria (11). In addition, the onset of impaired renal function in the absence of overt albuminuria has been reported in almost one-half of a cohort of type 1 diabetic patients (12), indicating also a lack of sensitivity. Significant reduction of glomerular filtration rate is certainly a clear indicator of DN but only at a late stage of disease, when success of treatment is severely compromised by the presence of advanced structural damage (13).

Therefore, new markers are needed for early diagnosis to allow early treatment of DN. A number of urinary proteins were analyzed in the last years for investigation of their role as predictors of DN (14–16). In comparison with other proteomic methods, capillary electrophoresis coupled to mass spectrometry (CE-MS) offers several advantages (for more details, see recent revs. in 17–19). A main advantage is the good reproducibility, which enabled the generation of >10,000 comparable individual datasets, substantially easing identification and validation of biomarkers. Hence, CE-MS has recently been used to analyze urine samples from healthy volunteers and patients with various kidney diseases in several studies at different centers (20–23).

In a previous study (24), 65 DN biomarkers were identified with a proteomic approach using CE-MS. In an independent validation cohort (n = 70), DN was classified with 97% sensitivity and specificity. This DN biomarker classifier derived from 65 biomarkers was further validated in a multicenter study cohort (n = 145) with 93.8% sensitivity and 91.4% specificity (25). However, only 34 of the 65 DN peptide biomarkers have been sequenced thus

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far. Most of these were fragments of collagen downregulated in the urine of patients with DN. We therefore speculated that changes in the collagen metabolism are closely linked with renal damage.

Good et al. (26) recently reported on a classifier based on a panel of 273 urinary biomarkers that was shown to be indicative with high specificity and sensitivity for CKD, irrespective of the underlying pathology, and thus different from the 65-marker DN classifier. In this multicenter effort, a total of 889 urine samples from 339 patients with various biopsy-proven CKDs and 550 control subjects were analyzed using CE-MS to establish a CKD-specific biomarker classifier. This support vector machine (SVM)-based CKD273 classifier showed satisfactory performance for the discrimination between presence and absence of CKD as indicated by specificity and sensitivity values of 97.8 and 85.5%. All 273 peptide biomarkers were identified by tandem mass spectrometry (27), and 74% of the biomarker peptides are collagen fragments. Furthermore, the biomarkers of the CKD273 classifier changed significantly after treatment (28), where the most prominent were changes of the collagen fragments. These changes were not observed in the placebo-treated individuals. It is tempting to speculate that this decrease in urinary collagen–derived peptides is related to an increase in extracellular matrix deposition, which is a major complication in diabetes. Therefore, urinary proteome analysis might enable noninvasive assessment of this process at an early stage via determination of specific collagen fragments.

Since the biomarkers of the CKD273 classifier were identified in an extensive cohort and all biomarkers have been sequenced, we applied this classifier to a longitudinal cohort for the diagnosis of DN to further validate it and to specifically examine whether the proteomic analysis can detect DN at early stages (before onset of microalbuminuria). In addition, we investigated which of the 273 biomarkers are most suitable for early diagnosis of DN.

RESEARCH DESIGN AND METHODS

The local ethics committees approved the study, and all participants gave informed consent. The study was performed in accordance with the Declaration of Helsinki. From an inception cohort of type 1 diabetic patients followed from onset of diabetes at Steno Diabetes Center (11), samples from urine collection were available from 5 case subjects with normoalbuminuria later developing persistent macroalbuminuria and 9 matched control subjects remaining normoalbuminuric during follow-up and from a cohort of 21 normoalbuminuric diabetic patients (19 type 2 diabetic patients and 2 type 1 diabetic patients), of whom 11 progressed to macroalbuminuria from Austin Health Center. Samples were available almost yearly from each patient over a period of ~10–15 years (UAER: normoalbuminuria <20 μg/min, microalbuminuria 20–200 μg/min, and macroalbuminuria >200 μg/min). Patients are categorized based on urine samples. Patients' characteristics are shown in Supplementary Table 2.

Sample preparation. Urine samples were stored in the clinical centers. After arrival, they were prepared essentially as previously described (29). Briefly, for CE-MS analysis 0.7 mL aliquot was thawed immediately before use and diluted with 0.7 mL 2 mol/L urea and 10 mmol/L NH_4OH containing 0.02% SDS. For removal of high–molecular weight polypeptides, samples were filtered using Centrisart ultracentrifugation filter devices (20 kDa molecular weight cutoff; Sartorius, Goettingen, Germany) at 3,000*g* until 1.1 mL filtrate was obtained. Subsequently, filtrate was desalted using a PD-10 column (GE Healthcare, Stockholm, Sweden) equilibrated in 0.01% NH_4OH in high-performance liquid chromatography–grade water. Finally, samples were lyophilized and stored at −20°C. This procedure results in an average recovery of sample in the preparation procedure of ~85% (22). Shortly before CE-MS analysis, lyophilisates were resuspended in high-performance liquid chromatography–grade water to a final protein concentration of 0.8 μg/μL checked by BCA assay (Interchim, Montlucon, France).

CE-MS analysis. CE-MS analysis was performed as previously described (30,31). The limit of detection was ~1 fmol, and mass resolution was >8,000, enabling resolution of monoisotopic mass signals for $z \leq 6$. After charge deconvolution, mass deviation was <25 ppm for monoisotopic resolution and <100 ppm for unresolved peaks ($z > 6$). The analytical precision of the platform was assessed by *1*) reproducibility achieved for repeated measurement of the same replicate and *2*) reproducibility achieved for repeated preparation and measurement of the same urine sample; details on analytical precision have recently been reported (26). To ensure high data consistency, a minimum of 800 peptides/proteins had to be detected with a minimal mass spectrometry resolution of 8,000 in a minimal migration time interval of 10 min.

Data processing. Mass spectral ion peaks representing identical molecules at different charge states were deconvoluted into single masses using MosaiquesVisu software (32). Both capillary electrophoresis migration time and ion signal intensity (amplitude) showed variability, mostly owing to different concentrations of ions in the sample, and were consequently normalized. Reference signals of 1,770 urinary polypeptides were used for capillary electrophoresis time calibration by local regression. For normalization of analytical and urine dilution variances, mass spectrometry signal intensities were normalized relative to 29 internal standard peptides generally present in at least 90% of all urine samples with small relative SD. For calibration, linear regression was performed (33). The obtained peak lists characterized each polypeptide by its molecular mass (Da), normalized capillary electrophoresis migration time (minutes), and normalized signal intensity. All detected peptides were deposited, matched, and annotated in a Microsoft SQL database, allowing further statistical analysis.

CKD273 classifier analysis. CE-MS measurement of the urine samples and data processing resulted in a maximum of 5,010 distinct peptides, which described the human urinary low–molecular weight proteome (27,34). The CKD273 classifier is an SVM-based classification model (35–37), which allows the classification of samples in the high-dimensional parameter space using MosaCluster software (version 1.7.0) (38). When the CKD273 classifier was applied to CE-MS data of unknown samples, MosaCluster calculated classification scores based on the amplitudes of the 273 CKD biomarkers. Classification is performed by determining the Euclidian distance (defined as the SVM classification score) of the 273-dimensional vector to a 272-dimensional maximal margin hyperplane, which was defined previously (26). The cutoff of the classification score was determined with the result of the biomarker discovery cohort in the study by Good et al. (26). Patients with urine samples who had classification factors >0.343 were classified as CKD273 classifier–positive case subjects, and patients with urine samples scoring <0.343 were classified as CKD273 classifier control subjects.

Statistical analysis. Sensitivity, specificity, and area under the curve (AUC) of the previously defined CKD273 classifier and 95% CIs were calculated using receiver operating characteristic (ROC) plots (MedCalc version 12.1.0.0; MedCalc Software, Mariakerke, Belgium [www.medcalc.be]) (39). Furthermore, Mann-Whitney *U* test (for independent samples) was performed to develop Box-and-Whisker plots, and logistic regression was used to calculate the (adjusted) odd ratios (ORs) of the CKD273 classifier with this software. For analysis of differences of individual peptides between case and control subjects, statistical significance was assumed at $P < 0.05$.

RESULTS

Early identification of patients at risk for DN. To systematically assess whether the CKD273 classifier allows identification of patients who develop DN in the future while clinical indicators like UAER are still normal, we retrospectively analyzed 316 urine samples collected in a cohort of initially normoalbuminuric diabetic patients. Samples were available from patients with type 1 (n = 16) or type 2 (n = 19) diabetes from two different centers (Steno Diabetes Center, Gentofte, Denmark, and Austin Health, Victoria, Australia). We analyzed mean ± SD 9 ± 3 urine samples from each patient over a period of 9.1 ± 3.3 years. (Baseline patient data and *P* values are summarized in Table 1 and Table 2.) We defined patients who had an increase in UAER from <20 μg/min (normalbuminuric) at baseline to >200 μg/min (macroalbuminuric) over the observed period as "progressors" and patients who retained a UAER of <20 μg/min as "nonprogressors." At baseline, most potential risk factors, i.e., UAER, estimated glomerular filtration rate (eGFR), age, diabetes duration, and blood pressure, showed no significant differences between progressors and nonprogressors. In type 1 diabetic patients (Table 1), only HbA_{1c} and systolic blood pressure

TABLE 1
Patient characteristics at baseline of longitudinal study of type 1 diabetic patients

	n	Sex (male/female)	Age (years)	Diabetes duration (years)	HbA_{1c} (%)	Glucose (mmol/L)	Cholesterol (mmol/L)	Triglyceride (mmol/L)	UAER (μg/min)	eGFR (mL/min/1.73 m^2)	SBP (mmHg)	DBP (mmHg)
Nonprogressors	10	8/2	41 ± 7	9 ± 3	7.4 ± 0.8	10.6 ± 4.9	n.d.	n.d.	5 ± 2	105 ± 13	123 ± 9	78 ± 6
Progressors	6	3/3	49 ± 12	11 ± 6	8.9 ± 1.3	7.0 ± 3.3	n.d.	n.d.	12 ± 9	102 ± 19	146 ± 14	81 ± 11
P (*t* test)		0.299*	0.132	0.356	0.029	0.167			0.123	0.765	0.018	0.575

Data are means ± SD unless otherwise indicated. DBP, diastolic blood pressure; n.d., no data available; SBP, systolic blood pressure. *Fisher exact test.

were different, and in type 2 diabetic patients (Table 2) glucose concentrations were different between groups. However, observation of the clinical parameters at end point of the longitudinal study (data not shown) showed that no risk factor except UAER and eGFR showed significant differences between progressors and nonprogressors. Proteome analysis was performed without the laboratory having any knowledge of the clinical characteristics or outcome (single blinded).

In this longitudinal study, all urine samples from diabetic patients were measured with CE-MS. The calibrated CE-MS data of each urine sample was matched to the urinary database. Applying the CKD273 classifier to the CE-MS data of each urine sample resulted in a classification score defined by SVM-based analysis. The resulting classification factors are listed in Supplementary Table 1. The CKD273 classifier score was associated with CKD events in univariate analysis (OR 25.6 [95% CI 2.5–265.4], P=0.007). When adjustment was made for other known CKD risk factors and potential confounders, the CKD273 score remained significantly associated with CKD, as shown in Table 3.

Owing to the fact that proteomic analysis of body fluids may be troubled by sample degradation/proteolysis over time, it is instructive to demonstrate that the classification score of the CKD273 classifier is irrespective of the storage time. Long-term stability was analyzed with a standard sample (40) over 35 months. With the regression analysis of these 1,229 data, we extrapolated a possible shift of the CKD273 classifier owing to the long-term storage of the samples in the clinical centers (Supplementary Fig. 1). The longest storage time (analysis date minus sampling date) was calculated to be 259 months. The potential shift of −0.286 was within the intermediate precision of the CKD273 classifier (10 ± 0.300%), which has been analyzed before (26).

Progression was defined as change in UAER from normo- to microalbuminuria (Supplemental Fig. 2). Of type 2 diabetic patients who progressed to microalbuminuria during follow-up (n = 10), seven had classification factors above the cutoff before they developed microalbuminuria. Of the type 1 diabetic patients who progressed to microalbuminuria during follow-up (n = 6), four showed classification factors above the cutoff before developing microalbuminuria. In contrast, only two of nine type 2 diabetic patients (and none of the type 1 diabetic patients) who did not progress to microalbuminuria had classification factors above the cutoff.

To determine how many years before the development of macroalbuminuria the CKD273 classifier showed a positive result and to compare this with microalbuminuria, we performed paired t tests for all progressors (n = 16). With use of the CKD273 classifier, the diagnosis of macroalbuminuria were 4.9 ± 2.2 years earlier compared with 3.4 ± 2.1 years for microalbuminuria (P = 0.016) (Fig. 1). The difference of 1.5 years means a relative improvement of 44% for the urinary proteomic analysis compared with the commonly used microalbuminuria.

To investigate the predictive value of the CKD273 classifier in comparison with UAER with respect to diabetic nephropathy, we analyzed UAER and CKD273 classifier of each patient at −5, −4, −3, −2, and −1 year before onset of macroalbuminuria (Fig. 2**)**. In Fig. 2*A*, progressors are on average around the cutoff (0.343) and nonprogressors are lower. In Fig. 2*B*, the mean of the classification factors is above the cutoff except at −5 years before onset of

TABLE 2
Patient characteristics at baseline of longitudinal study of type 2 diabetic patients

	n	Sex (male/female)	Age (years)	Diabetes duration (years)	HbA_{1c} (%)	Glucose (mmol/L)	Cholesterol (mmol/L)	Triglyceride (mmol/L)	UAER (μg/min)	eGFR (mL/min/1.73 m^2)	SBP (mmHg)	DBP (mmHg)
Nonprogressors	9	1/8	56 ± 11	n.d.	8.9 ± 1.6	15.6 ± 3.1	6.1 ± 1.5	2.2 ± 2.9	8 ± 5	77 ± 16	131 ± 13	83 ± 10
Progressors	10	6/4	63 ± 10	n.d.	8.6 ± 1.1	11.5 ± 3.2	5.5 ± 1.2	2.0 ± 1.0	10 ± 3	72 ± 27	142 ± 13	79 ± 10
P (*t* test)		0.057*	0.192		0.728	0.038	0.462	0.906	0.357	0.635	0.149	0.471

Data are means ± SD unless otherwise indicated. DBP, diastolic blood pressure; n.d., no data available; SBP, systolic blood pressure. *Fisher exact test.

macroalbuminuria, where the SD of progressors and nonprogressors overlap. The UAER showed a good separation of progressors and nonprogressors at −1 and −2 years before onset of macroalbuminuria (Fig. 2*C* and *D*). Furthermore, as depicted in Fig. 2*D*, the mean UAER of the progressors is below the cutoff (20 μg/min) from −4 and −5 years, and the SD overlaps from −3, −4, and −5 years before onset of macroalbuminuria.

For the differentiation of progressors from nonprogressors, an ROC analysis of all urine samples where 5-year follow-up resulted in either macroalbuminuria (progressor) or normoalbuminuria (nonprogressor) was performed using UAER or proteomic scoring as a continuous variable (Fig. 3*A*). Based on 214 urine samples, this ROC analysis resulted in an AUC of 0.93 (95% CI 0.89–0.96) for the proteomic scores compared with 0.86 (0.81–0.90) for the UAER in the same samples ($P = 0.022$). To obtain more information on the predictive value of the CKD273 classifier, we examined only urine samples that were normoalbuminuric at the sampling date ($n = 150$). The ROC analysis resulted in an AUC of 0.92 (95% CI 0.86–0.96) for the CKD273 classifier versus 0.67 (0.59–0.75) for UAER ($P = 0.0001$) (Fig. 3*B*).

To examine the sensitivity of the CKD273 classifier in relation to type of diabetes, we performed a stratified ROC analysis. For urine samples ($n = 128$) from type 2 diabetic patients, AUC values of 0.94 (95% CI 0.88–0.97) for the CKD273 classifier and 0.80 (0.72–0.86) for UAER were observed ($P < 0.001$). For urine samples ($n = 86$) from type 1 diabetic patients, ROC analysis resulted in AUC values of 0.91 (0.82–0.96) for the CKD273 classifier and 0.93 (0.86–0.98) for the UAER ($P = 0.607$).

Time course of urinary biomarker levels. In the former section, we demonstrated that the urinary biomarkers enabled detecting development of DN with high sensitivity and specificity ~4 years before clinical manifestation. The aim of the subsequent assessment was to examine which of the 273 biomarkers showed altered urinary excretion at this early time point. Therefore, we compared urinary biomarker levels at "early" (−5 to −3 years) and "late" (−2 to 0 years) time points before clinical manifestation of DN. Subsequently, we examined the CKD273 classifier, UAER, and selected representative specific peptides (with the highest AUC values in their group) of the CKD273 classifier in patients at late and early stage before clinical manifestation of macroalbuminuria in type 1 (Supplemental Fig. 3) and type 2 (Supplemental Fig. 4) diabetic patients. This was done to assess the performance of each analysis/biomarker with respect to diagnosis over time. As previously demonstrated in type 1 diabetic patients, also at late and early stages, the CKD273 classifier showed AUC values ($P = 0.0889$ and 0.9570) similar to those of UAER. In type 2 diabetic patients, the subdivision in late and early stages demonstrated significant higher AUC values ($P = 0.0348$ and 0.0156) of the CKD273 classifier and UAER.

In urine of patients of both diabetes types, fragments of collagens, polymeric-immunoglobulin receptor, clusterin, CD99 antigen, and uromodulin had lower amplitude levels in progressors than in nonprogressors. In the majority of the case subjects, the AUC values at later stage were higher than in the early stage prior to clinical diagnosis of DN. In contrast, fragments of abundant blood proteins, such as α-1-antitrypsin or serum albumin, had higher amplitude levels in progressors than in nonprogressors.

The results of the statistical analysis of each CKD biomarker peptide per patient group (progressors or nonprogressors)

TABLE 3
ORs of CKD predicted by CKD273 classifier at baseline

Factors adjusted for	OR for CKD273 classifier	95% CI	*P*
Univariate	25.6	2.5–265.4	0.007
Age (years)	17.9	1.5–217.0	0.024
Sex	27.0	2.7–274.1	0.005
Diabetes type	32.5	2.5–420.5	0.008
UAER (μg/min)	21.3	1.8–246.6	0.014
eGFR (mL/min/1.73 m^2)	25.9	2.4–282.2	0.005
SBP (mmHg)	22.0	1.6–296.6	0.000
DBP (mmHg)	44.7	3.5–569.8	0.001
HbA_{1c} (%)	48.1	2.6–878.0	0.001
Glucose (mmol/L)	30.8	2.8–343.2	0.003

DBP, diastolic blood pressure; SBP, systolic blood pressure.

at early and late stages stratified by diabetes type are shown in Supplementary Table 2. In the urine of type 1 diabetic patients, 66 of the 273 biomarkers were significant at early stages, whereas 69 biomarkers were significant at late stages. In type 2 diabetic patients, more biomarkers were significant: while 85 of 273 biomarkers showed significant alterations ($P < 0.05$) at the early time point, urinary levels of 130 of 273 biomarkers were significantly altered at the late time point. As depicted in Fig. 4*A* and *B*, AUC values of collagen fragments were higher than those of other protein fragments in type 1 (late: $P = 0.0166$; early: $P = 0.0355$) and type 2 (late: $P = 0.0001$; early: 0.0073) diabetic patients. Interestingly, urinary fragments of classical serum biomarker candidates showed almost no differences at early time points. While in urine of type 1 diabetic patients 1 of 18 α-1-antitrypsin fragments were significantly altered, no serum albumin fragments were significantly elevated (Supplementary Table 2). In urine of type 2 diabetic patients, 2 of 9 serum albumin fragments and no α-1-antitrypsin fragments were significantly changed.

Furthermore, we observed the best AUC values of the biomarkers at early and late stages in type 1 and 2 diabetic patients. Therefore, we searched for the highest AUC value of a protein fragment at late stage and compared it with the AUC value at early stage (Fig. 5*A* and *C*). This procedure was also performed with the highest AUC values at early stages (Fig. 5*B* and *D*). This assumption indicated that collagen peptides, especially collagen α-1(I) and collagen α-1(III) fragments, have the highest AUCs at both stages compared with peptides of other protein groups. In some cases (e.g., uromodulin, polymeric immunoglobulin receptor), the same peptides had the highest AUC values at late and early stages. Furthermore, almost all peptides with high AUCs at late stage also had AUC values >0.7 at early stage and vice versa.

DISCUSSION

In this study, we demonstrate that the previously generated CKD273 classifier, when applied to normoalbuminuric patients, identifies patients who will develop diabetic nephropathy during follow-up, performing better than or equal to UAER. In the investigated cohort, the CKD273 classifier was able to predict development of DN before patients developed microalbuminuria. The classification factors of the CKD273 classifier in patients who developed DN over the time showed consistently higher values than in patients who did not develop DN. Furthermore, the CKD273 classifier identified progressors in 65% of the case subjects earlier than the classical parameter UAER; on average, the CKD273 classifier was 1.5 years earlier than microalbuminuria. Interestingly, the performance of UAER

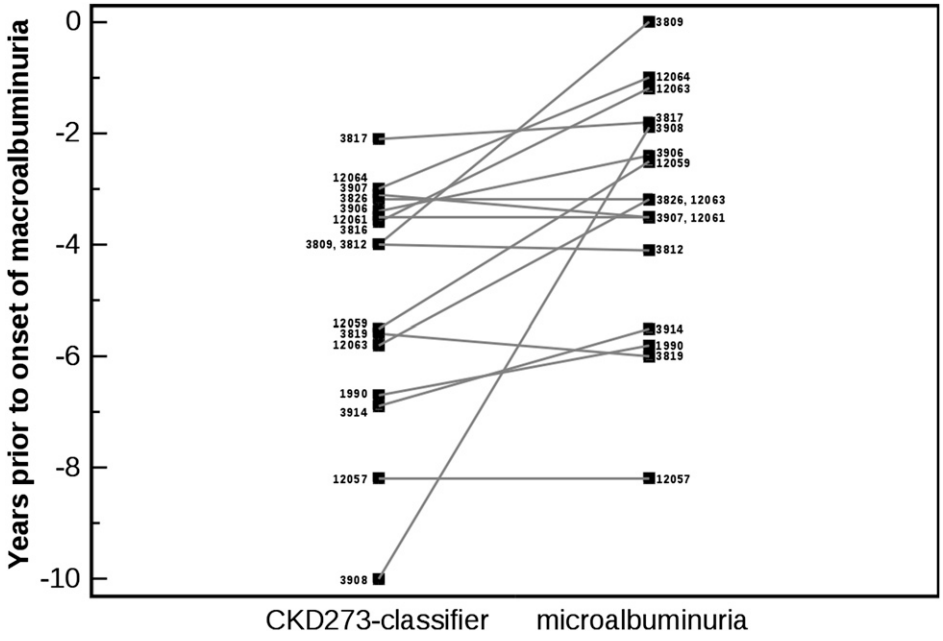

FIG. 1. Dot-and-line plot of the comparison between early diagnoses of the CKD273 classifier and microalbuminuria in the longitudinal cohort. The *y*-axis depicts the years prior to onset of macroalbuminuria. The years prior to the onset of macroalbuminuria is the difference in examination date (Supplementary Table 1) of the first urine sample with UAER >200 μg/min, the examination date of the first sample with a classification factor >0.343 in the case of the CKD273 classifier, and the examination date of the first sample with UAER >20 μg/min in the case of microalbuminuria, respectively. The numbers next to the dots correspond with the number of patients in the longitudinal cohort.

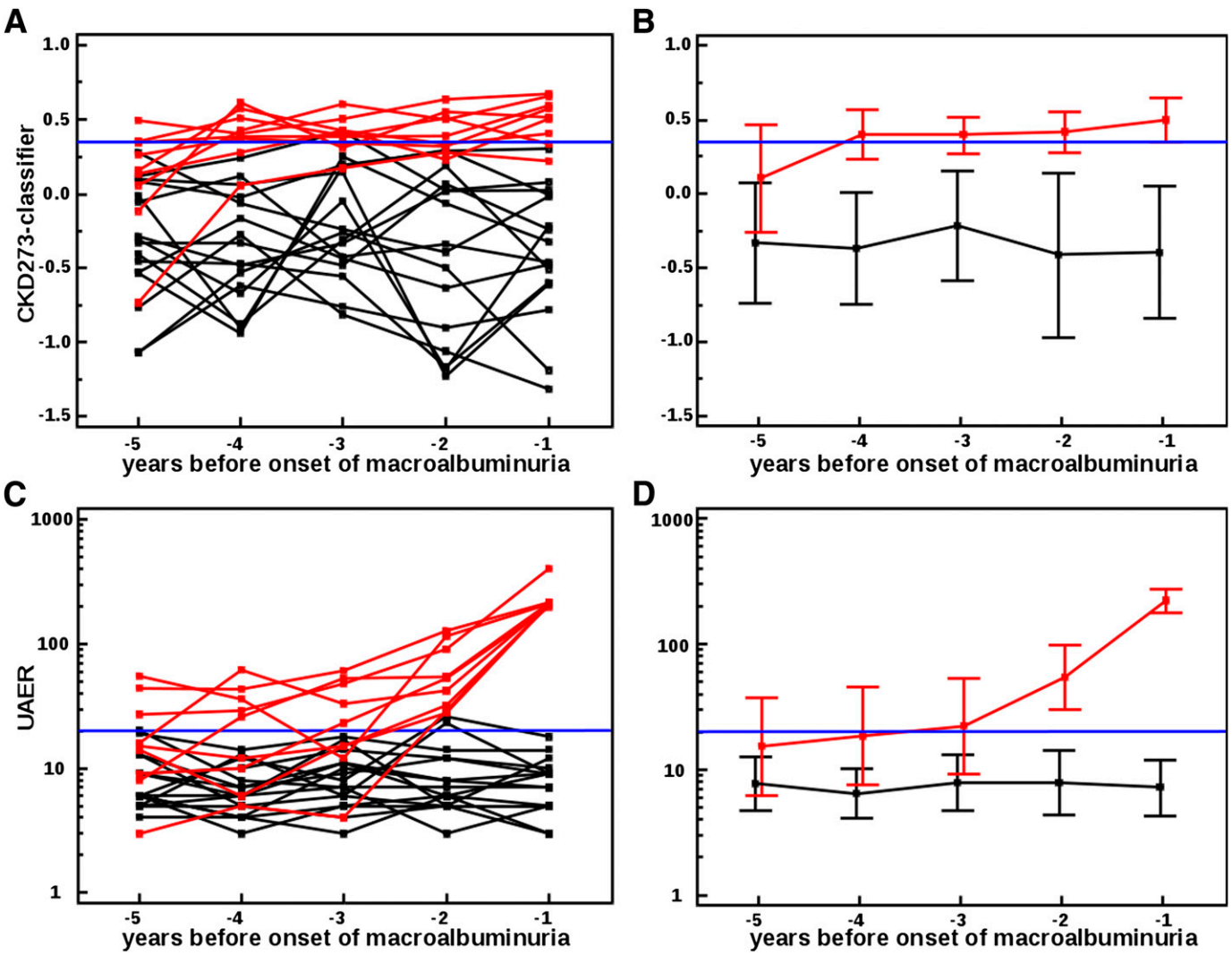

FIG. 2. Dot-and-line plots of the CKD273 classifier and UAER of progressors and nonprogressors of DN in the longitudinal data analysis. The *x*-axis shows the years prior to onset of macroalbuminuria. The blue line depicts the cutoff (0.343) of the CKD273 classifier and the cut off (20 μg/min) from normo- to microalbuminuria. The plots in the *left panel* show the progress of the CKD273 classifier and the UAER over the years prior to onset of macroalbuminuria. The *right panel* indicates the mean ± SD of the CKD273 classifier and UAER.

was lower in type 2 diabetic patients, whereas the AUC values of the CKD273 classifier were similar with stratification for diabetes type. This effect may be due to the fact that albuminuria is a good predictor for development of CKD in younger patients, but its accuracy is compromised in the elderly.

Our data indicate that normoalbuminuric patients with the CKD273 classifier–positive pattern are at increased risk for development of DN and can be identified as such before patients develop microalbuminuria (at least in the type 2 diabetic patients). This suggests that the pattern could be used in the clinic to identify patients in need of optimal renoprotective treatment at an even earlier stage than is possible today. In clinical trials, the CKD273 classifier can potentially be used to identify high-risk normoalbuminuric subjects for intervention trials aiming for prevention of DN, thereby targeting intervention to those who need it and reducing the number of subjects needed for enrollment (41). Previous attempts at early prevention of development of microalbuminuria with blockade of the renin-angiotensin-aldosterone system have only shown limited (42) or no (43) beneficial effect, perhaps because intervention has to be targeted at high-risk normoalbuminuric patients, such as CKD273 classifier–positive patients.

Statistical analysis of the 273 biomarkers depicted the regulation of each peptide in the urine with respect to diagnosis of DN over time. In the urine of diabetic patients, collagen fragments played the most important role even 3–5 years before onset of macroalbuminuria. Collagen fragments, especially fragments of the collagen α-1(I) chain, are major constituents of the low–molecular weight urinary proteome (27). These peptides are likely the result of normal physiological turnover of the extracellular matrix. Hence, it has been assumed that diminished activity of matrix metalloproteinases may be responsible for the accumulation of proteins in the extracellular matrix and collagens that typify the fibrotic kidney (44). This effect may be interpreted as an indication of increased tissue levels of inhibitors of matrix metalloproteinases. Accumulation of extracellular matrix as predominantly observed in diabetic nephropathy was recently shown to be associated with decreased excretion of several specific collagen fragments (45). The data of this longitudinal study

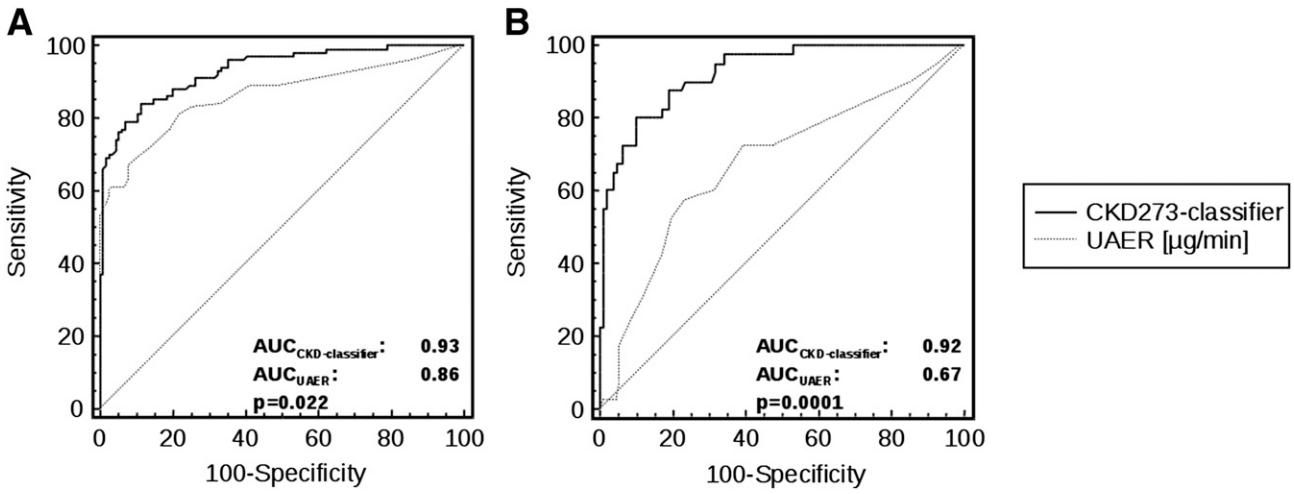

FIG. 3. Comparison of ROC curves of the classification results from longitudinal collected urine samples. *A*: ROC analysis of all urine samples of diabetic patients at sampling date up to 5 years prior to onset of macroalbuminuria (DN). *B*: ROC analysis of all samples of diabetic patients who are normalbuminuric at sampling date up to 5 years prior to onset of DN. The black line shows the ROC curve from the CKD273 classifier and the dashed line from the UAER.

support the hypothesis that collagens play an important role in the development of diabetic nephropathy and may be suitable targets for therapeutic effects of novel drugs. It seems obvious that the use of a mass spectrometry–independent validation approach with available antibody reagents will allow the assessment of the concentration of full-length collagen versus collagen fragments. This would support the conclusion that the change in urinary collagen fragment concentration is generated by a disease process with decreased protease activity in urine. Unfortunately, the generation of antibodies for collagen fragments is difficult. Owing to the fact that almost all collagen fragments are modified with several hydroxylated proline residues, the use of an immuno-based assay would typically only allow the detection of either the modified or the unmodified form—not the comparison of the abundance of each form when using only a single antibody (46). However, the next step will be to provide any evidence of alteration in protease activity, e.g., in experimental models.

Furthermore, other protein fragments show changes in the urine at an early stage of diabetic nephropathy: uromodulin expression is essentially restricted to the tubules. Excretion of uromodulin has been reported to be downregulated in diabetic nephropathy (24,47,48), and reduced

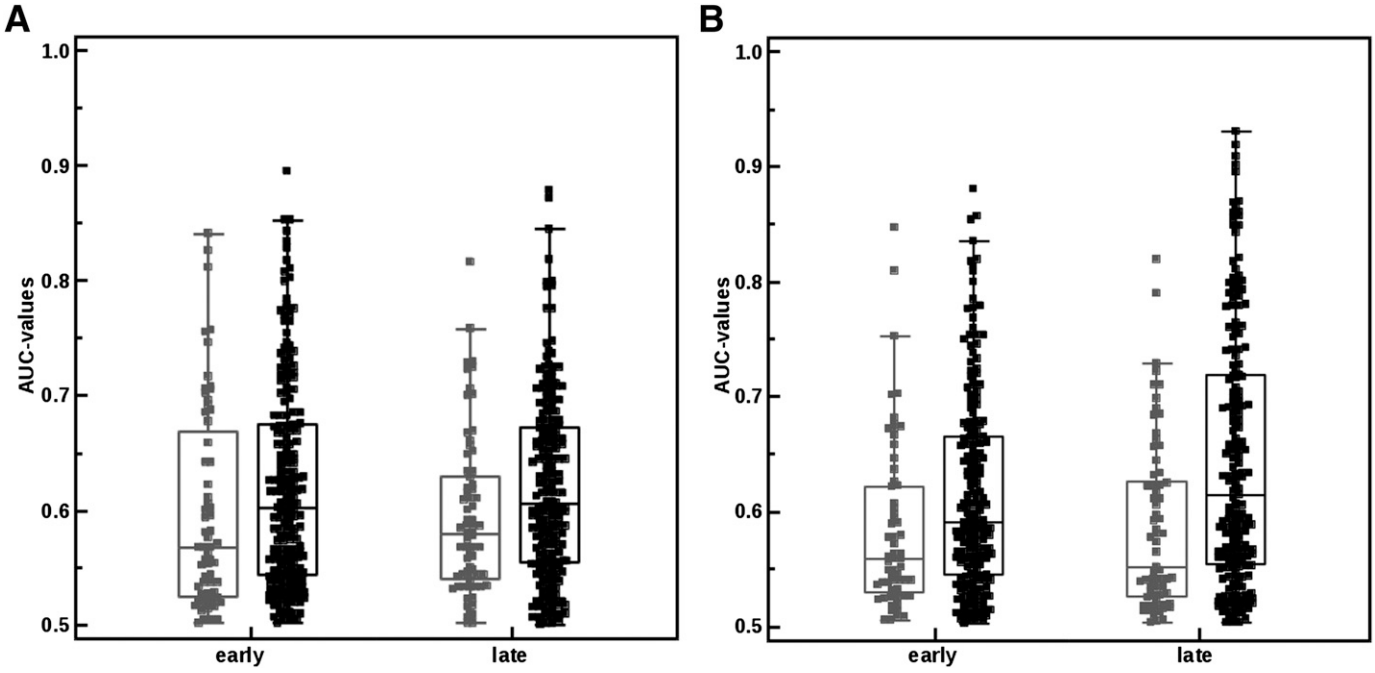

FIG. 4. Box-and-Whisker plots of AUC values from biomarkers of the CKD273 classifier in type 1 (*A*) and type 2 (*B*) diabetic patients separated in collagen-derived and non–collagen-derived peptides. The black plot and dots depict the AUC values of the collagen fragments, and the gray plot and dots depict the AUC values of the noncollagen fragments.

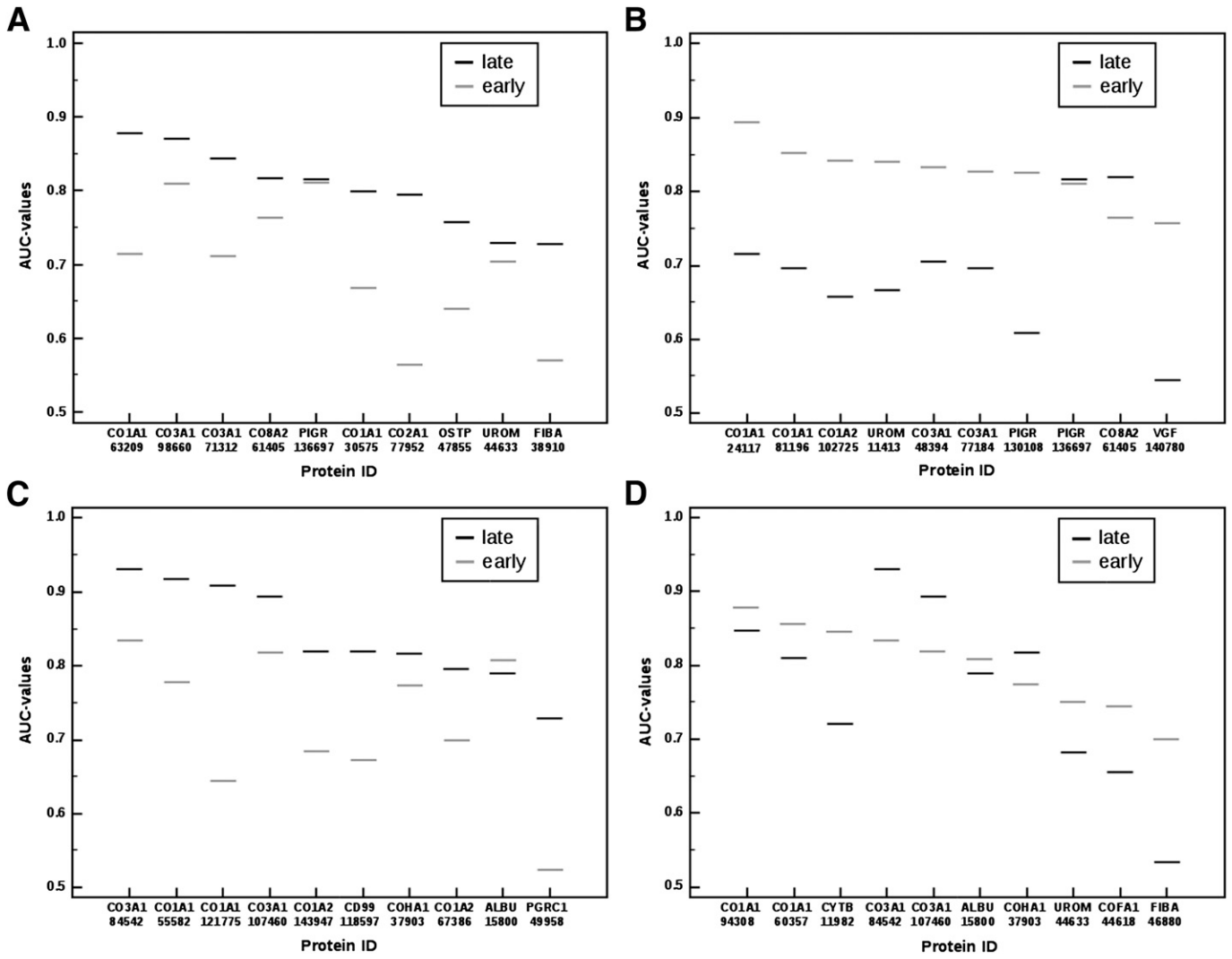

FIG. 5. Distribution of highest AUCs of CKD273 classifier biomarkers for type 1 diabetic patients at late stage (−5 to −3 years prior to onset on macroalbuminuria) (*A*) and early stage (−2 to 0 years prior to onset of macroalbuminuria) (*B*) and for type 2 diabetic patients at late stage (*C*) and early stage (*D*). For this figure, best AUCs (>0.7) were selected at late and at early stage in type 1 and 2 diabetic patients, respectively (Supplementary Table 2). In case subjects in whom more than two peptides of a protein were identified, only two peptides are depicted. The AUC values of late stage are depicted with black lines and of early stage with gray lines. The markers are characterized by protein ID and the SwissProt name.

excretion of specific uromodulin fragments has also been observed in other forms of CKD (22). Clusterin (apolipoprotein J) has been known to be associated in diabetes (49), and a number of papers have been published on the possible use of urinary clusterin concentration analysis in the assessment of proteinuria and renal function disorders (50,51). α-1-antitrypsin seems to be a good marker for the differentiation between DN case and control subjects (26), but for the early diagnosis of DN the amplitude levels are not significantly different in the progressors compared with nonprogressors.

A shortcoming of the study reported here is the relatively low number of patients included. Unfortunately, it is very difficult to obtain longitudinal collected samples over a large period. However, the results were consistent within each group and statistically significant. The ultimate proof of the benefit of urinary proteomics in management of DN would be a clinical trial that aims at identification of patients at risk for developing DN at an early stage (before microalbuminuria) based on urinary proteomics, followed by a targeted intervention (41). Such a proteomics-driven intervention trial has started in Europe with the use of the CKD273 classifier (http://eu-priority.org).

In summary, collagens showed the major role in the initiation of DN, even at an early time point. The progress in urinary proteomics and the use of multiple biomarker classifiers, like the CKD273 classifier, open the possibility of establishing new tools adapted to different clinical needs. Some of them have been built up to differentiate etiology and others to screen for DN and DN progression. Although not routinely available, urinary proteomics is becoming more and more accessible with increasing automation, easing application, and reducing costs. In our study, urinary proteomics enabled identification of new biomarkers for early detection of DN with promising clinical value.

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P.Z. wrote the manuscript and researched data. G.J. contributed urine samples and reviewed and edited the manuscript. P.H. contributed urine samples and reviewed and edited the manuscript. R.J.M. reviewed and edited the manuscript. H.M. contributed to discussion and reviewed and edited the manuscript. S.E.N. reviewed and edited the manuscript. S.P. reviewed and edited the manuscript. F.P. reviewed and edited the manuscript. P.R. contributed urine samples and reviewed and edited the manuscript. P.Z. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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