

# A urinary peptide biomarker set predicts worsening of albuminuria in type 2 diabetes mellitus

S. S. Roscioni · D. de Zeeuw · M. E. Hellemons ·  
H. Mischak · P. Zürlbig · S. J. L. Bakker ·  
R. T. Gansevoort · H. Reinhard · F. Persson · M. Lajer ·  
P. Rossing · H. J. Lambers Heerspink

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## Abstract

**Aims/hypothesis** Microalbuminuria is considered the first clinical sign of kidney dysfunction and is associated with a poor renal and cardiovascular prognosis in type 2 diabetes. Detection of patients who are prone to develop micro- or macroalbuminuria may represent an effective strategy to start or optimise therapeutic intervention. Here we assessed the value of a urinary proteomic-based risk score (classifier) in predicting the development and progression of microalbuminuria.

**Methods** We conducted a prospective case–control study. Cases ( $n=44$ ) and controls ( $n=44$ ) were selected from the PREVEND (Prevention of Renal and Vascular End-stage Disease) study and from the Steno Diabetes Center (Gentofte, Denmark). Cases were defined by transition from normo- to microalbuminuria or from micro- to macroalbuminuria over a follow-up of 3 years. Controls with no transitions in albuminuria were pair-matched for age, sex and albuminuria status. A model for the progression of

albuminuria was built using a proteomic classifier based on 273 urinary peptides.

**Results** The proteomic classifier was independently associated with transition to micro- or macroalbuminuria (OR 1.35 [95% CI 1.02, 1.79],  $p=0.035$ ). The classifier predicted the development and progression of albuminuria on top of albuminuria and estimated GFR (eGFR, area under the receiver operating characteristic [ROC] curve increase of 0.03,  $p=0.002$ ; integrated discrimination index [IDI]: 0.105,  $p=0.002$ ). Fragments of collagen and  $\alpha$ -2-HS-glycoprotein showed significantly different expression between cases and controls.

**Conclusions/interpretation** Although limited by the relatively small sample size, these results suggest that analysis of a urinary biomarker set enables early renal risk assessment in patients with diabetes. Further work is required to confirm the role of urinary proteomics in the prevention of renal failure in diabetes.

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S. S. Roscioni (✉) · D. de Zeeuw · M. E. Hellemons ·  
H. J. L. Heerspink  
Department of Clinical Pharmacology,  
University Medical Center Groningen, University of Groningen,  
Antonius Deusinglaan, 1,  
9713 AV Groningen, the Netherlands  
e-mail: s.s.roscioni@umcg.nl

H. Mischak  
BHF Glasgow Cardiovascular Research Center,  
University of Glasgow,  
Glasgow, UK

H. Mischak · P. Zürlbig  
Mosaiques Diagnostics GmbH,  
Hannover, Germany

S. J. L. Bakker · R. T. Gansevoort  
Department of Nephrology, University Medical Centre Groningen,  
Groningen, the Netherlands

H. Reinhard · F. Persson · M. Lajer · P. Rossing  
Steno Diabetes Center,  
Gentofte, Denmark

P. Rossing  
Faculty of Health Science, University of Aarhus,  
Aarhus, Denmark

P. Rossing  
Novo Nordisk Foundation Center for Basic Metabolic Research,  
Copenhagen University,  
Copenhagen, Denmark

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### Abbreviations

|         |  |
|---------|--|
| ACEi    | ACE inhibitor  |
| ARB     | Angiotensin receptor blocker                           |
| CE-MS   | Capillary electrophoresis coupled to mass spectrometry |
| CKD     | Chronic kidney disease                                 |
| eGFR    | Estimated GFR  |
| ESRD    | End-stage renal disease                                |
| IDI     | Integrated discrimination index                        |
| MDRD    | Modification of diet in renal disease                  |
| PREVEND | Prevention of renal and vascular end-stage disease     |
| RAAS    | Renin–angiotensin–aldosterone system                   |
| ROC     | Receiver operating characteristic                      |
| UAE     | Urinary albumin excretion                              |

### Introduction

According to the diabetes atlas of the International Diabetes Federation, 366 million people were affected with diabetes in 2011 and diabetes prevalence is expected to rise to 552 million by 2030 [1]. About 35% of diabetic patients have progressive renal function loss [2]. Microalbuminuria, defined as a urinary albumin excretion (UAE) between 30 and 300 mg/day, is associated with vascular dysfunction and is commonly considered to be the first sign of kidney dysfunction and diabetic nephropathy [3, 4]. Despite important progress in optimising therapy, many patients with type 2 diabetes still experience progressive worsening of albuminuria towards micro- or macroalbuminuria (the latter defined as  $\text{UAE} \geq 300$  mg/day), ultimately leading to end-stage renal disease (ESRD) [5]. One of the potential explanations for this high renal risk despite therapy is that intervention is initiated too late, only when significant vascular complications have already been established. Early detection of patients who are prone to develop elevated albuminuria may therefore be an efficacious and safe approach to ultimately reduce the occurrence of adverse renal outcomes.

Several renal and cardiovascular risk factors, including hypertension, hyperglycaemia and dyslipidaemia, as well as age, have been associated with increase in albuminuria [6, 7]. However, risk stratification based on these variables is insufficient, highlighting the need for novel and more disease-specific biomarkers [8]. Urinary proteomics has gained considerable attention in the last decade due to the capacity to identify proteins/peptides associated with pathophysiological

changes, particularly at an early stage of disease [9, 10]. The analysis of the urine proteome from healthy and diseased individuals has revealed several biomarkers associated with renal and cardiovascular outcomes [11].

A urinary peptide classifier, consisting of 273 defined urinary peptides, was recently discovered as a good classifier in patients with chronic kidney disease (CKD) and therefore named the CKD273 classifier [12]. However, the predictive ability of this classifier could not be assessed owing to the cross-sectional design of the study, which analysed urine samples at time points when CKD was already established. Furthermore, the population enrolled in that study encompassed a broad range of advanced CKD patients, many of them already showing macroalbuminuria, consequently precluding the validation of the CKD273 classifier as an early clinical biomarker of renal disease progression. Therefore, we assessed whether the use of the CKD273 classifier may also predict early changes in renal function, using albuminuria progression as an endpoint.

Here we report on the evaluation of the prognostic value of the CKD273 classifier in prospectively collected urine samples from patients with type 2 diabetes.

### Methods

*Patients and methods* A case–control study was designed that enrolled individuals participating in the Prevention of Renal and Vascular End-stage Disease (PREVEND) study or who visited the Steno Diabetes Center in Copenhagen. The PREVEND study is a prospective community-based cohort study conducted in the city of Groningen in the Netherlands, initiated in 1997. The goal of this study is to monitor the natural course of UAE and its relationship with long-term renal and vascular end-stage disease. Details of the PREVEND study protocol have been published elsewhere [13]. Individuals with type 2 diabetes, defined as the use of oral glucose-lowering treatment (self-reported or by information retrieved from the regional pharmacy database), a fasting plasma glucose  $>7.0$  mmol/l (126 mg/dl) or non-fasting plasma glucose  $>11.1$  mmol/l ( $>200$  mg/dl), were eligible for this case–control study. At Steno Diabetes Center, patients from a prospective study initiated in 2006 following 200 patients with type 2 diabetes with elevated UAE rate ( $>30$  mg/day), normal plasma creatinine and no known history of cardiovascular disease were included [14].

*Selection of cases and controls* Patients who transitioned from normo- to microalbuminuria or from micro- to macroalbuminuria between two consecutive study visits were defined as cases. Patients who had a stable albuminuria level during the entire follow-up period were selected as controls. Transition in albuminuria stage was defined as a progression

from normo- to micro- or from micro- to macroalbuminuria with at least 30% increase in UAE from baseline. Cases/controls were matched 1:1 based on diabetes, age, sex and albuminuria stage at baseline (normo- or microalbuminuria). Participants attending the Steno Diabetes Center were also matched based on duration of diabetes. In PREVEND, patients were matched on the study visit when diabetes was newly diagnosed. The use of antihypertensive agents intervening in the renin–angiotensin–aldosterone system (RAAS) (i.e. ACE inhibitors [ACEis] or angiotensin receptor blockers [ARBs]) was allowed, but the type of drug and its dose had to remain stable during the study period. Patients were not allowed to discontinue their RAAS medication or initiate new treatment. Participants at the Steno Diabetes Center who were on RAAS blockers were treated with irbesartan 300 mg daily. The study consisted of 44 case/control pairs. The normo- to microalbuminuria group consisted of 24 case/control pairs from the PREVEND study and the micro- to macroalbuminuria group consisted of 20 case/control pairs of which 12 came from the Steno Diabetes Center and 8 from the PREVEND study.

**Urinary proteomics** The analysed urine samples from these patients were from the visit that had taken place before the transition from normo- to microalbuminuria or from micro- to macroalbuminuria. Samples were derived from 24 h urine collections, stored in aliquots at  $-20^{\circ}\text{C}$  for 2–5 years, which were prepared as described previously [15]. A 0.7 ml aliquot was thawed immediately before use, diluted with 0.7 ml urea (2 mol/l) and  $\text{NH}_4\text{OH}$  (10 mmol/l) containing 0.02% sodium dodecyl sulphate (SDS) and filtered using Centriscart ultracentrifugation filter devices (20 kDa molecular mass cut-off; Sartorius, Goettingen, Germany) at 3,000g until 1.1 ml of filtrate was obtained. This was desalted using a PD-10 column (GE Healthcare, Stockholm, Sweden) equilibrated in 0.01%  $\text{NH}_4\text{OH}$  in HPLC-grade water. Finally, samples were lyophilised and resuspended in HPLC-grade water to a final protein concentration of 0.8  $\mu\text{g}/\mu\text{l}$ , checked by BCA assay (Interchim, Montlucon, France). Capillary electrophoresis coupled to mass spectrometry (CE-MS) analysis was performed as previously described [16]. The limit of detection was  $\sim 1$  fmol, with mass resolution above 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 ( $z > 6$ ). Details on analytical precision were reported recently [17]. A minimum of 950 peptides/proteins had to be detected with a minimal MS resolution of 8,000 in a minimal migration time interval of 10 min. Mass-spectral ion peaks representing identical molecules at different charge states were deconvoluted into single masses using MosaiquesVisu software ([www.proteomiques.com](http://www.proteomiques.com)). Reference signals of 1,770 urinary polypeptides were used for CE-time calibration by linear regression. For normalisation

of analytical and urine dilution variances, linear regression was performed based on the data of 29 ‘housekeeping’ peptides generally present in urine samples with small relative SD [18]. All detected peptides were deposited, matched and annotated in a Microsoft SQL database. The datasets were examined with respect to scoring in the urinary peptide classifier, CKD273. This support vector machine-driven classifier combines the data on 273 distinct urinary peptides, found to be significantly altered in CKD, to give a continuous numeric variable that represents the probability of disease being present [12]. CE-MS analysis was performed blinded on all samples. The data were evaluated using the CKD273 classifier and the results of the classification were reported back to the study centre. Subsequently, the data were unblinded.

**Routine laboratory measurements** Urinary albumin concentration was determined by nephelometry (Siemens, Munich, Germany). Twenty-four-hour albuminuria excretion is given as the average of two 24 h urinary excretions. BP was measured twice, in the supine position, every minute for 10 min with an automatic device (Dinamap XL Model 9300; Johnson-Johnson Medical, Tampa, FL, USA) (PREVEND) or in the sitting position as the mean of three oscillometric measurements by means of an electronic BP device (UA 779; A&D Instruments, Abingdon, UK) (Steno Diabetes Center). Serum creatinine was measured by dry chemistry (Eastman Kodak, Rochester, NY, USA). Estimated GFR (eGFR) was calculated using the modification of diet in renal disease (MDRD) formula [19].  $\text{HbA}_{1c}$  was measured by HPLC (Bio-Rad, Copenhagen, Denmark) with normal range 4.1–6.4% (21.3–46.4 mmol/mol) [20].

**Statistical analysis** Analyses were performed using Stata version 11.2 (StataCorp LP, Lakeway Drive, TX, USA). A case–control study with 50 cases and 50 controls will provide at least 80% power to detect an OR of 1.5 assuming a type 1 error of 5% and no residual confounding after matching cases and controls ([www.bioconductor.org](http://www.bioconductor.org)). Differences between the case/control pairs were tested with paired-samples  $t$  test for continuous variables and  $\chi^2$  test on paired proportions for categorical variables. Differences in the urinary peptide classifier between the different cases and controls were additionally tested with ANOVA followed by Bonferroni post hoc test.

Conditional logistic regression was used to investigate the association between the CKD273 classifier and the transition in albuminuria stage. In multivariate analyses, we adjusted for differences in baseline  $\log_{10}$  UAE and eGFR between cases and controls. We tested for interaction between patients who made a transition from normo- to microalbuminuria and those who made a transition from micro- to macroalbuminuria by adding an interaction term for baseline albuminuria status and CKD273 classifier in the model. The associations of the CKD273 classifier with changes in, and follow-up values of, UAE and eGFR were tested by linear

regression analysis. Non-parametric Wilcoxon test was used to determine the difference in individual peptide amplitude between cases and controls. To assess the degree of the association between the individual peptides and the changes in UAE and eGFR, as well as with follow-up values of UAE and eGFR, correlation analysis was performed and Spearman's  $\rho$  values were reported.

To assess whether the CKD273 classifier (the biomarker) improved risk prediction of the outcome (albuminuria-stage transition), we determined the area under the receiver operating characteristic (ROC) curve and the integrated discrimination index (IDI). Both measures evaluate the discriminative ability of a marker to distinguish between people who will or will not develop the outcome [21–23]. The predictive ability of the CKD273 classifier was measured as the additive discriminative value obtained by adding the biomarker to the model already including baseline  $\log_{10}$  UAE and eGFR. For all analyses, two-sided  $p$  values  $<0.05$  were considered statistically significant.

## Results

**Patient characteristics** The baseline characteristics of the cases and the controls in the type 2 diabetes cohort are depicted in Table 1. Cases and controls in the normo- to microalbuminuria subgroup and in the micro- to macroalbuminuria subgroup were generally well balanced for baseline characteristics. Although patients were matched for albuminuria stage, we observed significant differences in the levels of baseline UAE between cases and controls in both subgroups (Table 1, Fig. 1a, b). Furthermore, the eGFR was lower in cases than in respective controls, but this difference was statistically significant only in the micro- to macroalbuminuria subgroup (Table 1). Changes in UAE in cases and controls during follow-up are shown in Fig. 1a, b. Patients with transition from normo- to microalbuminuria and from micro- to macroalbuminuria had a median percentage increase in albuminuria of 142% and 334%, respectively. Normoalbuminuric controls and microalbuminuric controls had a median percentage increase in albuminuria of 1.2% and -11.6%, respectively (Table 1).

**Association between the CKD273 classifier and transition in albuminuria stage** All samples included in the study could be successfully analysed using CE-MS, passing the pre-specified quality control criteria. Mean values of the CKD273 classifier in cases and controls are depicted in Fig. 2. The values of the CKD273 classifier in cases were significantly higher than in respective controls ( $0.01 \pm 0.36$  vs  $-0.29 \pm 0.38$  [ $p=0.01$ ] for the normo- to microalbuminuria transition and  $0.41 \pm 0.23$  vs  $0.03 \pm 0.36$  [ $p<0.001$ ] for the micro- to macroalbuminuria transition). Moreover, values of the classifier were significantly different between

normoalbuminuric controls and microalbuminuric controls ( $p=0.016$ ) and also between the patients who developed microalbuminuria and the ones who developed macroalbuminuria ( $p=0.001$ ) (Fig. 2).

**Association of the CKD273 classifier with changes in UAE and eGFR** The values of the CKD273 classifier were significantly associated with the changes in UAE between follow-up and baseline (see electronic supplementary material [ESM] Fig. 1a,  $p=0.008$ ) and also with the values of follow-up  $\log_{10}$  UAE (ESM Fig. 1b,  $p<0.001$ ), even when adjusting for baseline  $\log_{10}$  UAE ( $p=0.029$ , data not shown). A significant negative correlation was observed between the CKD273 classifier and changes in eGFR between follow-up and baseline (ESM Fig. 1c,  $p=0.001$ ) and also with the values of eGFR at follow-up (ESM Fig. 1d,  $p<0.001$ ).

**Predictive performance of the CKD273 classifier for transition in albuminuria stage beyond conventional risk markers** As shown in Table 2, the CKD273 classifier was associated with transition in albuminuria stage. This association remained significant after adjustment for baseline  $\log_{10}$  UAE and eGFR (Table 2). Adjustment for BMI (which was slightly but not statistically significantly different between cases and controls) did not affect the association significantly (not shown). A decimal increment in the CKD273 classifier raised the odds of developing albuminuria-stage transition from 1.36 before adjustment ( $p=0.001$ ) to 1.32 after adjustment for baseline  $\log_{10}$  UAE and eGFR ( $p=0.014$ ). Further adjustment for ACEi/ARB use resulted in an OR of 1.35 ( $p=0.035$ ) (Table 2). There was no statistically significant difference between the odds for transition from normo- to microalbuminuria and from micro- to macroalbuminuria and increments in CKD273 classifier ( $p$  for interaction= $0.153$ ) indicating that baseline albuminuria stage (i.e. normo- or microalbuminuria) did not modify the predictive performance of the CKD273 classifier.

The ability of the CKD273 classifier to predict the transition in albuminuria stage was assessed by measuring the area under the ROC curves and IDI (Table 3) on top of baseline  $\log_{10}$  UAE and eGFR. Addition of the CKD273 classifier to the model increased the area under the ROC curve by 0.03 ( $p=0.002$ ) and resulted in an IDI of 0.105 ( $p=0.002$ ). Adjustment for BMI and antihypertensive drugs still resulted in a significant improvement in both C-statistic and IDI (data not shown).

**Analysis of individual peptide biomarkers** We subsequently aimed to examine which of the 273 biomarkers were the main predictors of transition in albuminuria stage. We compared the urinary levels of the individual peptides between cases and controls in the two subgroups of patients (ESM Table 1). We found 14 urinary peptides for which the levels were solely significantly different between normoalbuminuric controls and patients who progressed from normo- to

**Table 1** Baseline characteristics for cases and controls

| Patient characteristic   | Normo- to microalbuminuria |                         | Micro- to macroalbuminuria |                        |
|--|----------------------------|-------------------------|----------------------------|------------------------|
|  | Cases                      | Controls                | Cases                      | Controls               |
| Number, <i>n</i>   | 24                         | 24                      | 20                         | 20                     |
| Age, years   | 64.8±10                    | 62.3±9.5                | 65.6±7.1                   | 62.5±8.8               |
| Male sex, <i>n</i> (%)   | 17 (71)                    | 17 (71)                 | 17 (85)                    | 17 (85)                |
| Smoking, <i>n</i> (%)  | 5 (21)                     | 5 (21)                  | 6 (30)                     | 5 (25)                 |
| BMI, kg/m <sup>2</sup>   | 30±7                       | 28±4                    | 33±5                       | 29±5                   |
| Systolic BP, mmHg  | 136±14                     | 134±21                  | 136±15                     | 129±14                 |
| Diastolic BP, mmHg   | 75±9                       | 76±8                    | 73±10                      | 74±9                   |
| Follow-up time, years  | 2.7 (2.4–4.1)              | 2.8 (2.4–4.1)           | 3.6 (2.8–4.4)              | 2.9 (2.6–3.6)*         |
| Duration of diabetes (Steno), years                            | N/A                        | N/A                     | 18.5 (10.0–21.0)           | 17.0 (9.5–23)          |
| Laboratory variable  |                            |                         |                            |                        |
| UAE, mg/24 h   | 20 (15–22)                 | 7 (6–13)***             | 164 (117–201)              | 81 (45–122)***         |
| eGFR, mlmin <sup>-1</sup> (1.73 m <sup>2</sup> ) <sup>-1</sup> | 78±17                      | 84±17                   | 71±16                      | 82±22*                 |
| Total cholesterol, mmol/l                                      | 5.1±1.2                    | 5.1±1.4                 | 4.3±1.3                    | 4.6±1.3                |
| Fasting plasma glucose (PREVEND), mmol/l                       | 7.7±1.8                    | 7.2±1.2                 | 6.9±1.9                    | 7.1±1.3                |
| HbA <sub>1c</sub> (Steno), %                                   | N/A                        | N/A                     | 8.1±1.4                    | 8.2±1.0                |
| HbA <sub>1c</sub> , mmol/mol                                   |                            |                         | 65.6±10.7                  | 64.8±15.4              |
| UAE changes, %   | 1.2 (–20.4, 30.1)          | 142.1*** (101.1, 206.7) | –11.6 (–35.8, 69.7)        | 334.0** (146.2, 700.0) |
| Treatment, <i>n</i> (%)  |                            |                         |                            |                        |
| Antihypertensive drugs   | 16 (66.7)                  | 8 (33.3)*               | 18 (90)                    | 15 (75)                |
| ACEis/ARBs   | 10 (42.0)                  | 2 (8.3)**               | 15 (75)                    | 12 (60.0)              |
| Oral glucose-lowering drugs                                    | 18 (75)                    | 15 (62.5)               | 14 (70)                    | 15 (75)                |
| Lipid-lowering drugs   | 13 (54.2)                  | 4 (16.7)**              | 14 (70)                    | 15 (75)                |

Data are means±SD for normally distributed variables and median (interquartile range) for non-normally distributed variables

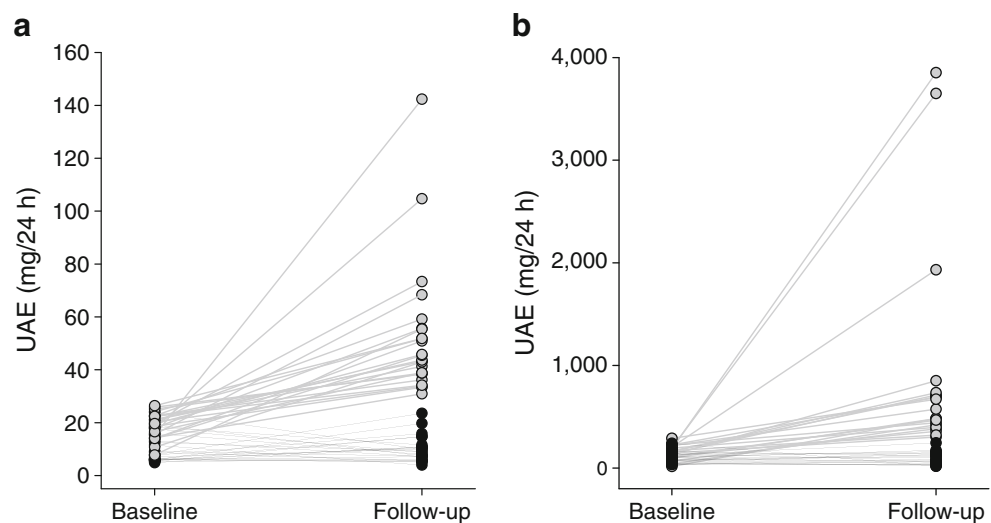
Median percentage changes in UAE are calculated as:  $([UAE_{follow-up} - UAE_{sample}] / UAE_{sample}) \times 100\%$ . Follow-up represents the time from the baseline measurement until the next study visit

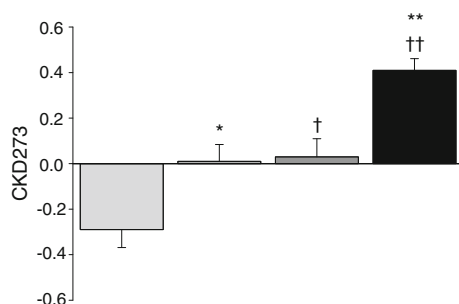
\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  cases vs controls

To convert HbA<sub>1c</sub> from % to mmol/mol use the formula:  $HbA_{1c}(mmol/mol) = [HbA_{1c}(\%) - 2.15] \times 10.929$

N/A, not applicable

**Fig. 1** Baseline and follow-up UAE in cases and controls. Individual courses of UAE for cases (grey lines) and controls (black lines) in type 2 diabetes are shown for the normo- to microalbuminuria subgroup (a) and the micro- to macroalbuminuria subgroup (b)





**Fig. 2** The CKD273 classifier in cases and controls. Data are means  $\pm$  SD,  $n=24$  for the normo- to microalbuminuria subgroup and  $n=20$  for the micro- to macroalbuminuria subgroup. \* $p<0.05$ , \*\* $p<0.01$  cases vs controls; † $p<0.05$  controls vs controls; †† $p<0.01$  cases vs cases. Light grey bar, normoalbuminuria control group; grey bar, normo- to microalbuminuria case group; dark grey bar, microalbuminuria control group; black bar, micro- to macroalbuminuria case group

microalbuminuria. Of these peptides, nine were less abundant and five were more abundant in cases compared with controls. All the less abundant peptides were type I collagen  $\alpha$ -1 fragments whereas  $\alpha$ -2-HS-glycoprotein was found in a higher concentration. A total of 40 peptides were exclusively different in the subgroup of patients that made a transition from micro- to macroalbuminuria. Most of these peptides were found in lower concentrations (the majority being type I and III collagen  $\alpha$ -1 fragments) in cases compared with controls. Only one peptide overlapped in the two subgroups,  $\alpha$ -2-HS-glycoprotein, which was more abundant in cases of both subgroups (ESM Table 1). Collectively, these results suggest that distinct pathophysiological mechanisms may occur in the early and later stage of renal disease.

We then assessed the degree of the linear association between the individual urinary peptides and the changes in UAE and eGFR, as well as the follow-up values of UAE and eGFR (ESM Tables 2 and 3). In ESM Table 2, Spearman's  $\rho$  values of all significant correlations between UAE and the biomarker peptides are shown. All collagen fragments and two uromodulin fragments showed a negative correlation with UAE whereas  $\alpha$ -2-HS-glycoprotein, serum albumin, and  $\alpha$ -1-antitrypsin positively correlated with UAE. Collagen fragments showed a positive correlation with eGFR whereas serum albumin and  $\alpha$ -1-antitrypsin had a negative correlation

**Table 2** ORs for the CKD273 classifier and transition in albuminuria per decimal unit increase in the level of the biomarker for patients with type 2 diabetes mellitus

| Classifier                    | OR   | $\beta$ | 95% CI $\beta$ | $p$ value |
|-------------------------------|------|---------|----------------|-----------|
| CKD273, crude                 | 1.36 | 0.306   | 0.126, 0.486   | 0.001     |
| CKD273, adjusted <sup>a</sup> | 1.32 | 0.274   | 0.054, 0.494   | 0.014     |
| CKD273, adjusted <sup>b</sup> | 1.35 | 0.302   | 0.020, 0.584   | 0.035     |

<sup>a</sup>Analysis was adjusted for baseline  $\log_{10}$  UAE and eGFR

<sup>b</sup>Analysis was adjusted additionally for ACEi/ARB use

with eGFR (ESM Table 3). No uromodulin or glycoprotein fragments were found to be correlated with eGFR.

## Discussion

In this study we showed that a urinary proteomic biomarker classifier previously cross-sectionally associated with CKD was able to predict transition in albuminuria stage in patients with type 2 diabetes on top of albuminuria and eGFR. Our findings suggest the use of this proteomic risk score as a tool to identify and treat patients with type 2 diabetes at risk of renal disease progression in order to halt its devastating consequences [24].

Urinary proteomic-based biomarker discovery represents a novel strategy to improve disease diagnosis, prognosis and treatment. Compared with blood samples, urinary samples are easy to collect (no requirement of a venepuncture) and the urinary proteome is very stable [25, 26]. Fuelled by the introduction of novel sophisticated technologies, the analysis of the urinary proteome pattern has recently led to the discovery of sets of biomarkers able to assess renal diseases with high accuracy [11], including diabetic nephropathy [9, 10]. We here investigated the value of a previously reported urinary peptide set (CKD273 classifier) [12] in predicting the onset of the first signs of renal damage (i.e. incidence of microalbuminuria) in individuals with type 2 diabetes.

Microalbuminuria is an early marker of kidney dysfunction and vascular damage and is an important predictor of renal and cardiovascular outcome in the diabetic population [5, 27]. Early detection of patients at risk of developing microalbuminuria may represent a key strategy for successful treatment outcome in patients with diabetes. Several attempts have been made to identify people at risk by using genetic analysis or concomitant risk factors usually associated with microalbuminuria such as hypertension, hyperglycaemia and dyslipidaemia [6, 28]. However, these conventional markers do not fully explain individual variation in renal and cardiovascular risk [29]. Our findings show that distinct changes in the urinary proteome of patients at an early stage of diabetes can distinguish patients at risk for progressive renal function loss. This has important clinical implications and suggests that urinary proteomics may represent a valuable tool to detect patients at high risk of renal and cardiovascular complications even when no other evident clinical signs of renal dysfunction are present.

Ideally, the prognostic value of biomarkers should be assessed using accepted hard endpoints. However, since hard renal endpoints take years to manifest—particularly when studying a population with preserved renal function and normoalbuminuria as we have done—the use of transition in albuminuria stage has been regarded as an adequate surrogate and has been used in other studies [30]. In our study, the CKD273

**Table 3** Area under the ROC curve and IDI for conditional logistic regression models predicting transition in albuminuria stage in patients with type 2 diabetes mellitus

| Model                      | AUC ROC | 95% CI     | <i>p</i> value | IDI   | 95% CI       | <i>p</i> value |
|----------------------------|---------|------------|----------------|-------|--------------|----------------|
| Control model <sup>a</sup> | 0.91    | 0.85, 0.98 | Ref            | Ref   |              | Ref            |
| + CKD273                   | 0.94    | 0.89, 0.99 | 0.002          | 0.105 | 0.038, 0.172 | 0.002          |

<sup>a</sup>Control model includes baseline log<sub>10</sub> UAE and eGFR

Ref, reference

classifier not only significantly predicted the progression and the development of albuminuria during a follow-up of about 3 years in type 2 diabetes but also correlated with the changes in eGFR during follow-up, supporting a direct association of the CKD273 set with changes in kidney function.

Based on their discovery study, Good et al [12] indicated that the optimal cut-off value of the CKD273 classifier to distinguish between controls and cases of established CKD is 0.343. This specific cut-off was not able to discriminate accurately between cases and controls in our study, and most of the CKD273 scores were below the cut-off value (data not shown). However, these results are not surprising as lower values of the CKD273 score are expected in a population of early-stage diabetic patients not yet having established CKD, such as the patients enrolled in our study. Hence, validation studies in large populations are required to determine the optimal cut-off for the CKD273 classifier in early stages of renal disease.

One could argue that the CKD273 score, which was developed in a heterogeneous CKD population, may include peptides that are not relevant in diabetic nephropathy. However, the CKD273 set contains protein and peptides that are clearly associated with the pathogenesis of diabetes, including collagens fragments (e.g. type I and III collagen  $\alpha$ -1), tubular proteins (e.g. uromodulin) and glycoproteins. We therefore performed additional analysis to investigate the differential urinary expression of these peptides and to assess their potential relationship with progressive renal dysfunction.

Several fragments of type I and III collagen were found in a lower concentration in patients with increased albuminuria levels at follow-up and this decrease was also positively correlated with the decline in eGFR. Importantly, the decrease of collagen fragments in the urine of diabetic patients has recently been associated with the accumulation of extracellular matrix and increased fibrosis [9].

Decreased levels of uromodulin have previously been shown in diabetes and other forms of CKD [31–33]. In particular, this impairment has been associated with damage of the thick ascending limb epithelium, which is responsible for uromodulin secretion [31]. In our study, the decrease in uromodulin correlated with the increase in albuminuria at follow-up, which likely reflects the progressive dysfunction of the nephrons.

Several glycoproteins were also found to be associated with worsening of albuminuria. Although the presence of

glycoproteins in the urine of diabetic patients may simply reflect the hyperglycated state of these proteins in the blood, glycoproteins such as  $\alpha$ -2-HS-glycoprotein have also been associated with inflammation and tubular damage, which occur in diabetes [34, 35]. The previously shown association between albuminuria and tubular damage markers [36] may explain why in our study  $\alpha$ -2-HS-glycoprotein was positively associated with increased follow-up albuminuria. The same association with albuminuria was found for  $\beta$ <sub>2</sub>-microglobulin, a protein that is normally filtered through the glomerulus and almost completely reabsorbed by proximal tubular cells [37]. The increased abundance of this protein in the urine of diabetic patients and the correlation with increased UAE may be regarded as a sign of progressive tubular dysfunction. Our small sample size precluded further detailed analyses of the individual urinary peptides. Nevertheless, the present data confirm that urinary proteomics provide useful insight into the underlying disease-specific pathophysiology [17]. Compared with the discriminative performance of the CKD273 classifier, one may find it surprising that only a limited number of single peptides actually showed significantly different concentration in cases and controls. However, biological, individual and measurement variability may severely affect the individual peptide analysis. For this reason the evaluation of a multi-marker panel consisting of clearly defined, discriminating molecules appears a better approach for identification of risk than using single peptides, as a multi-marker panel compensates for variability in individual analytes without compromising diagnostic power [12].

We recognise some limitations in our study. First, we must acknowledge the limited statistical power associated with the relatively small sample size of our study due to the stringent selection criteria used to define cases and controls. This weakens the implications of our findings, precludes a more detailed statistical analysis of potential confounders and may possibly hamper the identification of individual peptides with significantly different levels between cases and controls. However, the significant association of the classifier with the outcome, together with its additional discriminative capacity, strongly points to the potential of this proteomic tool. Larger prospective cohort studies are required to validate the present results. Second, despite the matching we found statistically significant differences between cases and controls in baseline

albuminuria, eGFR and specific therapy including ACEis/ARBs. Indeed, pharmacological inhibition of the RAAS has been shown to cause changes in the urine proteome pattern in diabetic patients [38] and may act as a confounder in our study. Importantly, despite the baseline difference in ACEi/ARB use, patients using ACEis/ARBs were not allowed to discontinue therapy and patients not on ACEi/ARB therapy were not allowed to initiate treatment with these medications during the study period. Furthermore, the association between the CKD273 classifier and the progression in albuminuria remained significant even after adjustment for ACEi/ARB use, ruling out a possible interference with our results. However, we cannot exclude that different types, doses and duration of antihypertensive treatment may have influenced the scoring of the classifier. Adjustment for baseline albuminuria and eGFR showed that the association of the CKD273 classifier with albuminuria-stage transition was independent of baseline levels of these variables. It should be emphasised that cases and controls were well balanced for all other clinical variables associated with renal function loss and worsening albuminuria, including BP and plasma levels of cholesterol, glucose and HbA<sub>1c</sub> [7, 9].

Our findings provide some evidence that routine analysis of urine samples could be used for improved renal risk assessment in diabetic patients with no clinical signs of renal impairment. Novel clinical trials guided by urinary proteomics have been proposed and initiated [39] and the results of these trials will provide further evidence of the applicability of urinary proteomics as a tailor-made tool for primary prevention and early intervention to reduce the human and economic burden of diabetic renal disease.

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**Duality of interest** H. Mischak is founder and co-owner of Mosaiques Diagnostics, which developed the CE-MS technology and the MosaiquesVisu software. P. Zürgbig is employed by Mosaiques Diagnostics.

**Contribution statement** SSR, MEH, HM, PZ and HJLH participated in the study design, acquisition of data and data entry, prepared the database and performed the statistical analysis. PR provided data from the Steno Center; RTG and SJLB provided data from the PREVEND study. DdZ, MEH, HM, PZ, SJLB, RTG, HR, FP, ML, PR and HJLH participated in the interpretation of the data and critically reviewed drafts of the manuscript. SSR wrote the first draft, edited several versions of the manuscript and finalised it. All authors read and approved the final version of the manuscript.

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