Original Paper

Urinary Matrix Metalloproteinase Activity in Diabetic Kidney Disease: A Potential Marker of Disease Progression

Nagi Altemtam   Meguid El Nahas   Tim Johnson

Sheffield Kidney Institute (Academic Nephrology Unit), School of Medicine and Biomedical Sciences, Sheffield, UK

Abstract

Background: Progressive kidney fibrosis, associated with chronic kidney disease (CKD), results from an imbalance in extracellular matrix (ECM) homeostasis. Reduced matrix metalloproteinases (MMP) activity causing lower clearance of ECM proteins has been implicated mainly through an overproduction of tissue inhibitors of metalloproteinases (TIMP), but also by reduced MMP synthesis. We tested the hypothesis that MMP activity can be measured in human urine and can be used as a potential biomarker of the progression of diabetic kidney disease (DKD). *Methods:* An observational prospective study was performed on 102 DKD patients using 21 diabetic patients without kidney disease and 21 healthy volunteers as controls. The Molecular Probes EnzChek Gelatinase/Collagenase Assay Kit were used to determine urinary MMP activity using DQ™ Gelatin (total MMPs), DQ™ Collagen I (interstitial collagenases) and DQ™ Collagen IV (gelatinises) substrates. A broad-spectrum synthetic inhibitor of all MMP, 1,10-phenanthroline, was used to confirm that the proteolytic activity is due to MMP activity. All MMP values were expressed per unit of urine creatinine. *Results:* Overall urinary MMP activity (DQ Gelatin substrate) was significantly elevated in DKD patients (14.76 ± 3.65 Δ fl/h/mmol creatinine) compared to diabetes mellitus controls (7.09 ± 2.12 Δ fl/h/mmol creatinine) and healthy volunteers (1.87 ± 0.74 Δ fl/h/mmol creatinine) (ANOVA p = 0.01). Within the DKD cohort, there was an approximate threefold higher urinary MMP activity in nonprogressive DKD patients compared to those with progressive disease (p = 0.002). The urinary MMP activity:creatinine ratio was significantly higher in normoalbuminuric and microalbuminuric DKD compared to macroalbuminuric DKD.

Dr. Tim Johnson

Academic Nephrology Unit and Sheffield Kidney Institute
Room GU24, Sir Henry Wellcome Laboratories for Biomedical Research
Medical School, University of Sheffield, Beech Hill Road, Sheffield S10 2RZ (UK)
Tel. +44 114 271 2842, E-Mail t.johnson@sheffield.ac.uk

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Positive correlations were observed between the rate of total MMP activity and interstitial collagenases (r = 0.75, p < 0.0001) and gelatinases (r = 0.59, p = 0.0001). The accuracy of MMP activity to predict the rate of annual eGFR decline (ROC analysis) was 77% compared to 64% for albuminuria. **Conclusions:** Total MMP activity can be easily measured in human urine. Surprisingly and in contrast to MMP activity in the kidney, urine MMP activity is elevated in DKD. However, there is a significantly lower MMP activity in patients with progressive DKD. ROC analysis demonstrates that single urine MMP activity estimation is superior to albuminuria in predicting DKD patients with progressive disease.

Introduction

Approaching epidemic levels, diabetic kidney disease (DKD) is now the leading cause of end-stage renal disease (ESRD) [1]. In the United States, the US Renal Data System annual report demonstrates that the incidence of new cases of ESRD due to diabetes mellitus (DM), which has been rising for the past 20 years, has levelled off since 2000, but it is still predicted that the prevalence of ESRD due to DM will increase 70% by 2030 [2].

One of the major clinical problems when evaluating patients with DKD is an early evaluation of the rate of decline of kidney function. Currently determining the rate of loss of GFR typically requires several months, while using proteinuria or albuminuria to predict patients with progressive DKD offers little greater than a 50% chance of a correct prediction [3]. Further such markers require considerable kidney damage to have occurred before changes are detectable [4]. Thus there is a clear need not only to develop better biomarkers of patients with a greater chance of ESRD, but also detect diabetic patients developing kidney disease earlier. Ideally a molecule involved in the early pathology of DKD that is related to the rate of the remodelling process would provide the best marker of progression. The hallmark of the pathogenesis of DKD is an excessive extracellular matrix (ECM) accumulation causing thickening of the glomerular and tubular basement membranes (GBM and TBM), mesangial expansion and sclerosis as well as tubulointerstitial fibrosis [5] making systems involved in this process particularly attractive markers of disease if they can be measured noninvasively.

The regulation of ECM levels is maintained by a homeostatic balance between ECM deposition and clearance [6]. Central to the breakdown and clearance of both glomerular and tubulointerstitial fibrosis is the matrix metalloproteinases (MMP) system. The MMP family is composed of 28 zinc-dependent enzymes that are involved in the breakdown of all ECM components [7]. The ability to degrade type IV collagen, the major component of the GBM, is favoured by gelatinases (MMP-2 and -9), whereas collagen I and III are predominantly hydrolysed by interstitial collagenases (MMP-1, -8 and 13) [8]. Stromelysins (e.g. MMP-3) specifically degrade fibronectin and laminin [9]. Due to their powerful degradative capacity, MMP activity is tightly regulated by a specific class of natural inhibitors known as tissue inhibitors of metalloproteinases (TIMP), as well as other proteinase inhibitors, such as α-2-macroglobulin and tissue factor pathway inhibitor-2 [7, 10]. The kidney expresses at least 10 MMPs (MMP-1, -2, -3, -9, -13, -14, -24, -25, -27, -28) as well as TIMP-1, -2 and -3 [11].

During progressive kidney scarring an imbalance in ECM homeostasis occurs leading to ECM accumulation. Reduced MMP activity contributes to this imbalance mainly due to overproduction of TIMP, but also partly through reduced MMP synthesis [12].

Numerous in vitro studies highlighted the critical role of hyperglycaemia in DKD, where glucose and advanced glycation end products (AGEs) modulate the regulation of MMP expression [13, 14]. In fact, dysregulation of MMP activity has been implicated in the patho-

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physiology of several diabetic complications, such as diabetic retinopathy and peripheral vascular disease [15, 16]. However, the exact roles of MMP and TIMP within the context of DM remain controversial. The expression of glomerular MMP-2 was found to be reduced, and TIMP-2 to be increased, in the kidney tissues of early diabetic patients [17]. On the other hand, clinical studies carried out on human urine showed that the MMP levels were significantly higher in patients with nephropathy compared to healthy volunteers [18, 19].

Therefore, we decided to test the hypothesis that changes in kidney MMP activity in DKD might be reflected in the urine and that a measure of urinary MMP activity can better predict the rate of progression of DKD than current clinical markers of kidney damage.

To test this hypothesis we firstly assayed total MMP activity as well as specific, gelatinase and interstitial collagenase activity in baseline urine from patients with DKD using whole molecule gelatin, collagen I and IV substrates. We secondly correlated baseline MMP activity with the subsequent rate of loss of kidney function and albuminuria, and thirdly evaluated the potential value of urinary MMP in predicting DKD progression.

Subjects and Methods

Study Population

A total of 102 adult type 2 DM (T2DM) patients with CKD at stages 3 and 4 (eGFR <60 to 15 ml/min/1.73 m^2) according to KDOQI classifications [20] were recruited from patients attending the renal outpatient clinic at Sheffield Kidney Institute (SKI) for routine check-up visits, and followed up for 3 years from the time of the first enrolments. All diabetic patients who received any form of renal replacement therapy and those with causes of CKD other than T2DM were excluded. Twenty-one diabetic patients without kidney disease (i.e. normal kidney function, negative urine dipsticks for proteinuria and normal albumin-creatinine ratios, ACRs) who attended the diabetes clinic at the Northern General Hospital (NGH) were included as a control group. Another 21 apparently healthy subjects who constituted a second group of controls were recruited from staff at the SKI laboratory in this observational study.

This work was done under ethics application reference STH15020 issued by the North Sheffield ethics committee and hosting organisations (Sheffield teaching hospital NHS Trust). Each participant was provided with a patient information sheet and a written informed consent. The study was conducted in line with the Declaration of Helsinki for health care research in humans, the International Conference on Harmonization and the Good Clinical Practice Guidelines of the European Union.

Sample and Data Collection

A detailed medical history was obtained from each participant. All demographic, clinical and biochemical parameters was collected from the unit's electronic database and entered into an anonymised bio-repository database established at the SKI to provide catalogued storage of biological material for this and future biomarker studies. About 200 ml of a mid-morning, mid-stream urine sample was collected from all study participants in sterile containers and immediately placed on ice. This was centrifuged for 10 min at 4°C at 2,000 g to pellet cells and the supernatant aliquoted and stored at –80°C as cell-free urine (CFU). Spot urine ACRs were calculated for all subjects and ACR cutoff value used according to recommended standard guidelines [21]. Accordingly, DKD patients were classified into 3 subgroups; normoalbuminuria (ACR <2.5 mg/mmol for males and <3.5 mg/mmol for females), microalbuminuria (ACR >2.5 mg/mmol for males, >3.5 for females and up to 30 mg/mmol) and macroalbuminuria (>30 mg/mmol). Finally, venous blood samples were tak-

en and analysed for full blood count, urea, creatinine, electrolytes, cholesterol, triglycerides, HbA1c, calcium, phosphorus and PTH using standard protocols at the clinical chemistry laboratories, NGH, Sheffield.

DKD Progression

Estimated GFR was calculated using the Modification of Diet in Renal Disease (MDRD) study equation [22]: eGFR (ml/min/1.73 m^2) = 186 × (standardized serum creatinine)$^{-1.154}$ × (age)$^{-0.203}$ × 0.742 (for women) × 1.212 (if the subject is black).

The progression of DKD was evaluated by the slope pattern of eGFR change (ml/min/1.73 m^2/year) for each patient throughout the entire 3-year follow-up period (at least six measurements) by regression coefficient analysis using all calculated eGFR values [23]. Patients with progressive DKD were defined as having a significant negative slope with loss of greater than 2 ml/min/year over the 3-year prospective follow-up, or being stable or nonprogressors if the slope was neutral, i.e. with a decline rate between +2 and –2 [23]. This was intentional as we considered average age-related decline to be around –1 ml/min/year [24] and considering variation in serum creatinine measurement we did not want to contaminate our progressor group with some age-related borderline progressors. We were concerned that reliance on two serum creatinine values (baseline and end of study) could be misleading. This has been achieved by including patients with 3 years of follow-up and at least 6 eGFR measurements; the requirements to establish a valid rate of decline in renal function.

Urinary MMP Activity Assay Measurement

The Molecular Probes EnzChek® Gelatinase/Collagenase Assay Kit (Invitrogen Corporation, EvoQuest™ Laboratory Services, USA) was used to determine MMP proteolytic activity. Three different fluorescein conjugate substrates were used. DQ™ Gelatin (E-12054) was used as a general MMP substrate, whereas DQ™ Collagen I (E-12060) was used as an interstitial MMP substrate for MMP-1, -8, and -13 and DQ™ Collagen IV (E-12052) was used as a gelatinase substrate for MMP-2 and -9. These substrates are heavily labelled with fluorescein (FITC) and a quenching agent. The enzymatic cleavage of the substrate molecule results in the separation of the quencher from the fluorochrome resulting in an increase in fluorescence that is proportional to the proteolytic activity. Analysis was performed using a 96-well plate approach and all assays were done in duplicate. Each well was set up with 200 μl reaction volume containing either 20 μl DQ Gelatin (12.5 μg/ml) or 20 μl DQ Collagen I (25 μg/ml) or 20 μl DQ Collagen IV (25 μg/ml), 100 μl of CFU from each participant or 100 μl of control enzyme (purified collagenase type IV from *Clostridium histolyticum*) and made up to 200 μl/well with 80 μl of reaction buffer (0.5 M Tris-HCl, 1.5 M NaCl, 50 mM CaCl$_2$ and 2 mM sodium azide with pH 7.6). The reaction was allowed to proceed for up to 72 h at room temperature and protected from light. Readings were taken every 15 min using a 96-well fluorescent plate reader (Fusion plate reader, Packard Bioscience Company, USA) with excitation/emission at 485/530 nm, respectively. All values were corrected against a collagenase standard curve (Δ fl/h). Background fluorescence was subtracted for each sample. The broad spectrum MMP inhibitor 1,10-phenanthroline (0.4 mM) was added to a parallel reaction for each sample to confirm the extent of the proteolytic activity specifically related to MMP activity as gelatin and collagen substrates can also be digested by other proteases. To account for day-to-day variation in urine volume, all MMP values were adjusted for creatinine concentration and expressed as Δ fl/h/mmol creatinine.

Three samples of known MMP proteolytic activity in DKD urine were assayed on each plate to assess inter- and intra-assay precision. The intra-assay coefficient of variation of total MMP was 3.8%, while the inter-assay coefficient was 9.6%.

Statistical Analysis

All analyses and calculations were performed using Statistical Package for Social Sciences for Windows version 16.0.1 (SPSS, Inc., Chicago, Ill., USA) and GraphPad Prism software (version 5.03, San Diego, Calif., USA). Data were reported as mean and standard deviation (SD) for normally distributed data or median (25th–75th centile) for those with skewed distribution. Standard t test was used to compare between subgroups of DKD patients. When the data was not normally distributed, it was compared by Mann-Whitney U test. Comparison of categorical variables was done by the chi-square (χ^2) test. One-way analyses of variance (ANOVA) with Bonferroni's post hoc analysis or Kruskall-Wallis test for multiple comparisons were used to evaluate differences among groups. Correlations between two parameters were generated using the Spearman's correlation coefficient (r). Linear and multivariate regressions were used as indicated to assess the impact of different study parameters on the annual rate of decline of eGFR. Finally, receiver operating characteristic (ROC) curve analysis was used to assess the sensitivity and reliability of MMP activity to predict DKD progression. All analyses were done with 95% confidence intervals (CI) and all p values were two tailed and the level of significance was determined at $p < 0.05$.

Results

Baseline Characteristics

Among 102 DKD patients, 20 patients were normoalbuminuric, 48 were microalbuminuric and 34 were macroalbuminuric based on their urinary ACRs (table 1). Patients with normo-, micro-, and macroalbuminuria were well-matched with respect to age, diabetes duration, arterial blood pressure parameters, BMI, WHR and most of the chemical parameters. Kidney function in terms of baseline eGFR and serum creatinine was significantly impaired in patients with macroalbuminuria compared to the other two groups. A trend towards higher HbA1c in the macroalbuminuric group was also evident, although the difference did not reach statistical significance (p = 0.06). The known duration of the T2DM was 13.6 ± 10.24 years. More than half of the patients had cardiovascular complications (54%), while diabetic retinopathy and neuropathy were documented in 48 and 35%, respectively. The majority of the patients (86.3%) were treated with statins, while ACE inhibitor/ARBs were used by 71.5% of the study population. Forty-two patients (41.2%) had progressive DKD (mean –4.4 ± 2.8 ml/min/1.73 m^2/year), leaving 60 patients (58.8%) with stable kidney function (mean –1.2 ± 0.89 ml/min/1.73 m^2/year, p = 0.0001) as assessed by the slope pattern of eGFR change (ml/min/1.73 m^2/year) by regression coefficient analysis using all calculated eGFR values over the 3-year follow-up (table 2).

The mean age for diabetic control patients was 64.76 ± 13.07 and 8/21 were males. The mean serum creatinine was 77 μM, urea was 4.89 mM and eGFR was 85.4 ml/min/1.73 m^2. The mean HbA1c was 7.46%. The mean age for the healthy volunteer group was 42.5 (29–56) years and 12 of them were males. All diabetic controls and healthy subjects were found to be normoalbuminuric as they had negative dipsticks which were confirmed by ACRs level.

Urinary MMP Activity

The Gelatinase/Collagenase proteolytic assay readily detected MMP activity in urine from patients with DKD. Over 90% of MMP activity was inhibited by addition of the general MMP inhibitor 1,10-phenanthroline, confirming degradation was due to MMP activity. Subsequently the activity retained after addition of 1,10-phenanthroline was subtracted from all measurements to account for no-MMP activity against the substrate.

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Table 1. Demographical and clinical characteristics of 3 subgroups of DKD patients

Variable	Normoalbuminuria	Microalbuminuria	Macroalbuminuria	p value
Number	20	48	34	
Age, years	72 ± 8	73 ± 9	63 ± 11	0.0001
Males, n (%)	8 (40)	31 (65)	27 (79)	0.01
Smokers, n (%)	13 (65)	21 (44)	15 (44)	0.2
Diabetes duration, years	12 ± 8	15 ± 10	18 ± 11	0.1
BMI	31.5 ± 5	32 ± 6	31 ± 5	0.5
Waist circumference, cm (range)	100.4 (92–112)	100 (79–142)	99 (81–117)	0.9
WHR (range)	0.93 (0.90–0.98)	0.94 (0.85–1.1)	0.94 (0.86–1.01)	0.9
eGFR, ml/min/1.73 m^2	36 ± 13	32 ± 11	28 ± 9	0.05
SBP, mm Hg	144 ± 14	149 ± 19	149 ± 18	0.6
DBP, mm Hg	69 ± 7	72 ± 10	72 ± 8	0.4
PP, mm Hg	75 ± 13	78 ± 19	77 ± 17	0.9
MAP, mm Hg	94 ± 8	97.5 ± 11	98 ± 10	0.40
Serum urea, mmol/l	13 ± 8	12.7 ± 5.3	16 ± 7.4	0.08
Serum creatinine, μmol/l	159 ± 59	177 ± 51	215 ± 69	0.002
HbA1c, %	8 ± 1.7	7.7 ± 1.3	8.5 ± 1.5	0.06
Serum cholesterol, mmol/l	4 ± 1.4	3.4 ± 1.3	4 ± 1.2	0.07
Serum triglycerides, mmol/l	1.8 ± 0.8	1.8 ± 1.2	2 ± 1.2	0.7
Serum calcium, mmol/l	2.4 ± 0.2	2.3 ± 0.1	2.4 ± 0.1	0.3
Serum phosphorus, mmol/l	1.2 ± 0.3	1.1 ± 0.2	1.3 ± 0.3	0.001
Serum Ca × PO$_4$, mmol/l	2.8 ± 0.8	2.6 ± 0.4	3.1 ± 0.5	0.002
PTH, pg/ml	170 ± 128	149 ± 91	165 ± 126	0.8
Hb, g/dl	12.8 ± 1.4	12.4 ± 1.7	12 ± 1.6	0.2

BMI = Body mass index; WHR = waist-hip ratio; SBP = systolic blood pressure; DBP = diastolic blood pressure; PP = pulse blood pressure; MAP = mean arterial blood pressure; HbA1c = glycosylated haemoglobin; PTH = parathyroid hormone; Hb = haemoglobin. Data presented as mean ± SD unless otherwise stated.

The total urinary MMP activity:creatinine ratio of healthy volunteers (n = 21) was 1.87 ± 0.74 Δ fl/h/mmol creatinine. Surprisingly this was 4-fold higher (7.09 ± 2.12 Δ fl/h/mmol creatinine, p = 0.003) in the diabetic control group and 8-fold higher in the urine of patients with DKD (14.76 ± 3.65 Δ fl/h/mmol creatinine, p < 0.0001, fig. 1a). The 2-fold increase in DKD patients compared to the diabetic control was not significant (p = 0.44, fig. 1a).

Total urinary MMP activity was significantly elevated in normoalbuminuric (14.57 ± 3.89 Δ fl/h/mmol creatinine) and microalbuminuric DKD (22.55 ± 7.41 Δ fl/h/mmol creatinine) compared to patients with macroalbuminuric DKD (3.87 ± 0.81 Δ fl/h/mmol creatinine, ANOVA p < 0.0001, fig. 1b). However, there was no difference in the urine MMP activity between patients treated or not treated with RAAS inhibitors. No differences were also detected in the statin- versus non-statin-treated subjects concerning urinary MMP activity.

Importantly, nonprogressors or stable DKD patients had an approximately 3-fold higher baseline urinary MMP activity (16.69 ± 4.55 fl/h/mmol creatinine) compared to those with a rapid decline in kidney function (5.41 ± 1.64 fl/h/mmol creatinine) over the period of study (p = 0.002, fig. 1c).

With respect to gender, the urinary MMP activity:creatinine ratio was significantly higher in females (26.60 ± 8.12) compared to males (8.16 ± 3.28 Δ fl/h/mmol creatinine, p = 0.01, fig. 2a), irrespective of albuminuria. In contrast, no differences were observed in the

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Table 2. Comparison between baseline characteristics of DKD progressors and nonprogressors (n = 102)

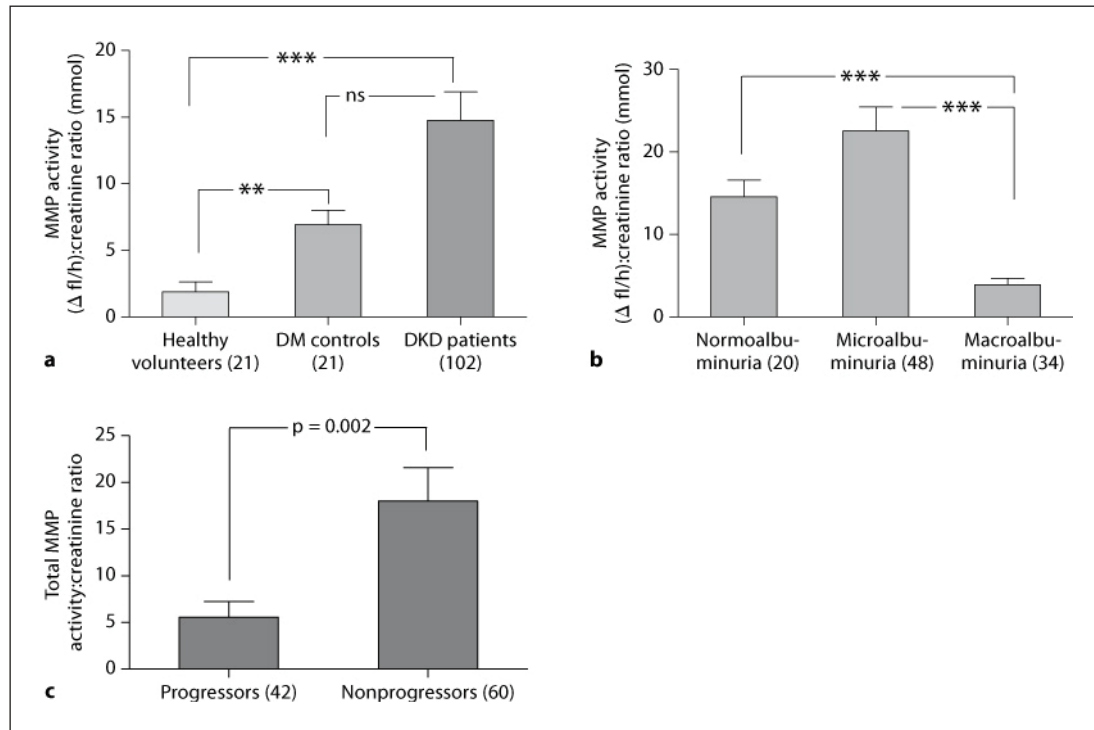| Variables	Progressors	Nonprogressors	p value
Number	42	60	–
Age, years	66 ± 11	71 ± 10	0.06
Males, n (%)	28 (67)	38 (63)	0.9
BMI	32 ± 5	31 ± 6	0.5
WHR	0.9 ± 0.04	0.93 ± 0.03	0.3
CVD, n (%)	28 (67)	27 (45)	0.007
eGFR, ml/min/1.73 m^2	27 ± 9	33 ± 11	0.01
Serum creatinine, μmol/l	211 ± 75	177 ± 55	0.02
Proteinuria, median (25th–75th), g/l	0.5 (0.2–1.2)	0.2 (0.1–0.5)	0.009
Albuminuria, median (25th–75th), mg/l	251 (21–1,070)	61 (21–169)	0.02
HbA1c, %	8.4 ± 2	8 ± 1.2	0.2
Serum cholesterol, mmol/l	3.8 ± 1.1	3.7 ± 1.3	0.5
Serum triglycerides, mmol/l	1.9 ± 1.2	1.8 ± 1.1	0.5
Baseline eGFR, ml/min/1.73 m^2	35 ± 10	32 ± 10	0.2
SBP, mm Hg	149 ± 19	146 ± 18	0.5
DBP, mm Hg	72 ± 8	69 ± 11	0.2
PP, mm Hg	77 ± 18	77 ± 16	0.9
MAP, mm Hg	98 ± 10	95 ± 11	0.3
Hb, g/dl	12 ± 1.5	12.6 ± 1.6	0.05

Abbreviations see table 1. Data presented as mean ± SD unless otherwise stated.

urine MMP activity between age categories <65 years (11.03 ± 2.071) and ≥65 years (14.13 ± 2.07 Δ fl/h/mmol creatinine) of the DKD patients (p = 0.44, fig. 2b). No differences were also apparent in urinary MMP activity between DKD patients with (16.61 ± 5.46) and without (12.60 ± 4.72 Δ fl/h/mmol creatinine) cardiovascular complications (p = 0.3, fig. 2c). Furthermore, DKD patients with diabetic retinopathy had higher levels of MMP activity (17.25 ± 5.87) compared to those without (7.16 ± 1.49 Δ fl/h/mmol creatinine, p = 0.003, fig. 2d).

Urine MMP activity was subsequently broken down into that attributable to gelatinases (MMP-2 and -9) by using a DQ Collagen IV substrate and to interstitial collagenase (MMP-1, -8 and -13) by using a DQ Collagen I substrate. Gelatinase activity in DM controls was 3-fold higher than healthy volunteers, but only 25% greater in those with DKD. Interstitial collagenase activity in DKD was about 20% higher than in DM control which was itself almost 3 times higher than healthy subjects. The level of MMP activity attributable to gelatinases was almost the same in progressors and nonprogressors, whereas the interstitial collagenase:creatinine ratio was a 1.5-fold higher in the nonprogressive DKD patients, although the increase was not significant (p = 0.2).

Correlations

There was a series of positive correlations (Spearman's test correlation coefficient, r) between the total MMP activity and other study parameters (table 3). Total MMP activity correlated significantly with interstitial collagenase (r = 0.75, p < 0.0001, table 3) and gelatinase activity (r = 0.59, p = 0.001) as well as HbA1c (r = 0.33, p = 0.002). Urinary interstitial collagenase and gelatinase:creatinine ratios were very highly correlated with each other (r = 0.65, p < 0.0001). Negative correlation was observed with albuminuria (r = –0.23, p = 0.019) and serum creatinine (r = 0.24, p = 0.018). Among demographic parameters, urine MMP activity:creatinine ratio was correlated with female gender (r = 0.392, p < 0.001), whereas

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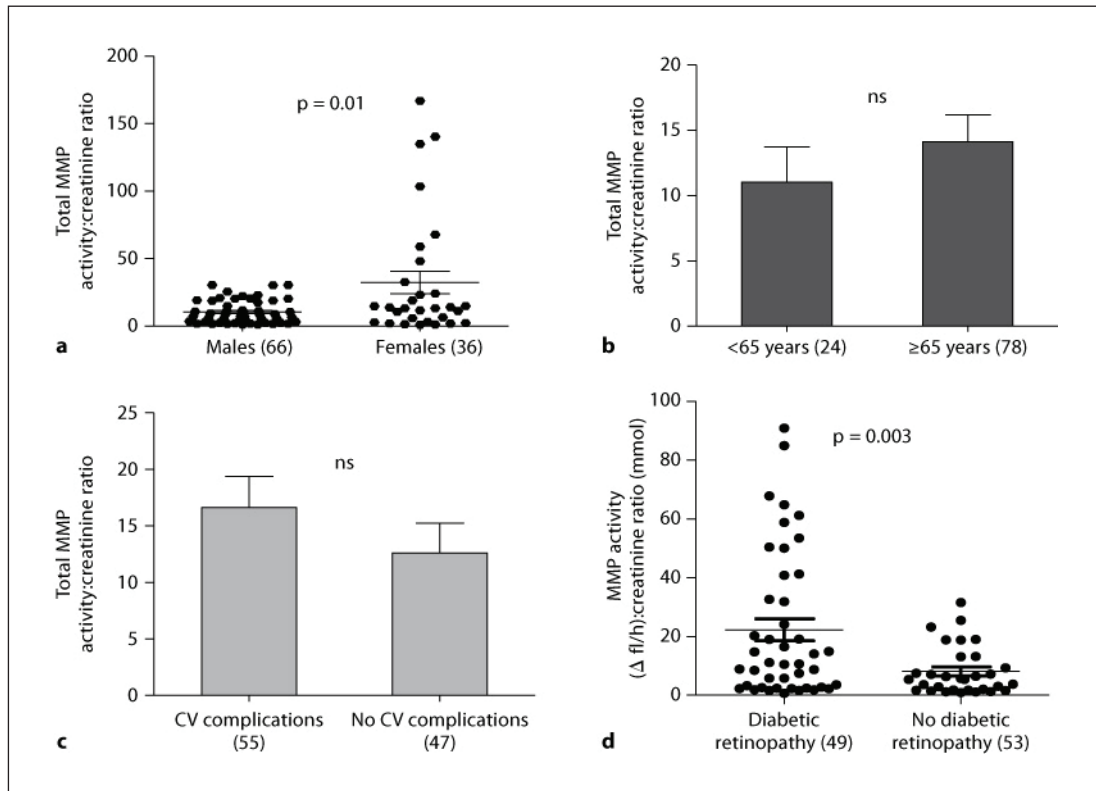
Fig. 1. MMP activity:creatinine ratio in diabetic kidney disease. MMP activity was measured in urine using the cleavage of DQ Gelatin and measuring the increase in fluorescence with time. **a** Mean total MMP activity:creatinine ratio in healthy volunteers, DM controls and DKD patients (p < 0.0001). **b** MMP activity:creatinine ratio by albuminuria in DKD patients. **c** Total MMP activity:creatinine ratio among DKD progressors and nonprogressors (p = 0.002). The data represents mean MMP activity:creatinine ratio ± standard error of the mean (SEM). ** p < 0.001; *** p < 0.0001; ns = nonsignificant.

there were no correlations found between total MMP activity and age, duration of the disease, body mass index, waist-to-hip ratio and smoking.

ROC Curve Analysis of Urine MMP Activity

To determine the selectivity and sensitivity of using MMP activity as a prognostic biomarker of DKD progression, a receiver operating characteristic (ROC) analysis was performed using albuminuria as a reference given it is the best current available predictor. The fraction of true positive results (sensitivity) and false positive results (1 – specificity) for urine total MMP activity (DQ Gelatine substrate), gelatinase MMP activity (DQ Collagen IV substrate) and interstitial collagenase MMP activity (DQ Collagen I substrate) was performed. A value of 0.5 is no better than expected by chance (null hypothesis), and a value of 1.0 reflects a prefect indictor. In comparison to albuminuria the ROC curve for total MMP activity was closer to that for an ideal predictor demonstrating improved characteristics at low sensitivity. The area under the ROC curve for urinary MMP activity was 77% (p = 0.0001; 95% CI = 65.7–88.2) and for albuminuria was 64% (p = 0.018; 95% CI = 52.6–75.5), demonstrating that MMP activity is better at predicting progressive CKD on a spot urine than albuminuria (fig. 3). Of note, the areas under the ROC curve for normoalbuminuric and microalbuminuric DKD were 74.2% (p = 0.002; 95% CI = 54.6–82.7) and 83.2% (p = 0.0001; 95% CI = 65.5–93.2), respectively, whereas the area under the ROC curve for macroalbuminuric DKD was 0.72 (p = 0.004; 95% CI = 51.3–78.6).

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Fig. 2. Total MMP activities by clinical parameters. MMP activity was measured in urine using the cleavage of DQ Gelatin. Total MMP:creatinine ratio was indicated by gender (**a**), age category (**b**), cardiovascular complications (**c**) and diabetic retinopathy (**d**). Assay reactions for all samples were carried out at room temperature. Data represents mean MMP activity:creatinine ratio ± SEM. ns = Nonsignificant.

Table 3. Correlation between MMP activity and other study parameters

Variables	Total MMPs: Cr	Gelatinases: Cr	Interstitial collagenases:Cr	Albuminuria	Serum creatinine	HbA1c
Total MMPs:Cr	r = 1					
Gelatinases:Cr	r = 0.59 p = 0.001	r = 1				
Interstitial collagenases:Cr	r = 0.75 p < 0.0001	r = 0.65 p < 0.0001	r = 1			
Albuminuria	r = −0.23 p = 0.019	r = −0.23 p = 0.022	r = −0.21 p = 0.038	r = 1		
Serum creatinine	r = −0.24 p = 0.018	r = −0.036 p = 0.72	r = −0.198 p = 0.047	r = 0.31 p = 0.002	r = 1	
HbA1c	r = 0.33 p = 0.001	r = 0.24 p = 0.032	r = 0.20 p = 0.041	r = 0.36 p = 0.001	r = 0.28 p = 0.031	r = 1

The correlations coefficient (r test) was calculated using Spearman's test for the nonparametric variables. A p value <0.05 was considered statistically significant. r = Correlation coefficient, p = probability value.

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Fig. 3. ROC curve analysis of predictors of progressive DKD. A ROC analysis was performed comparing the selectivity and sensitivity of total MMP activity:creatinine ratio vs. albumin:creatinine ratio in predicting the annual decline of kidney functions. The area under the curve represents the accuracy in prediction.
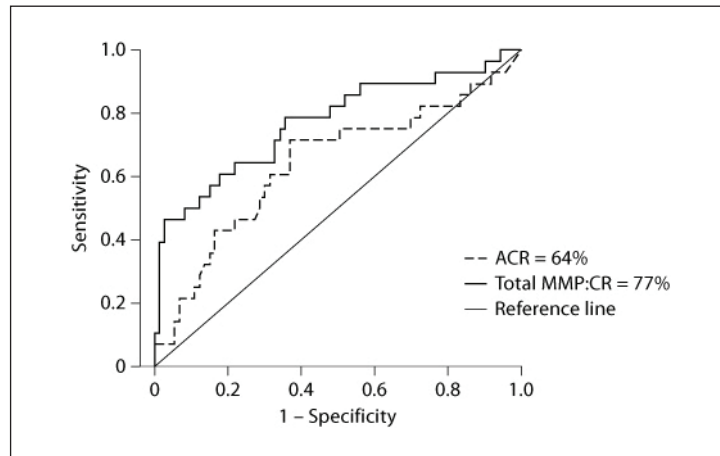
Discussion

The progression of DKD is assessed by the decline of GFR, which is often proceeded by an increased urine albumin excretion (UAE) [25]. However, there are some studies that showed that a significant proportion of patients might develop renal insufficiency in the presence of normal UAE. For instance, Caramori et al. (2003) reported more advanced diabetic lesions among normoalbuminuric diabetic patients [26]. Moreover, the UK Prospective Diabetes Study (UKPDS 74) demonstrated that 51% of patients who progressed to CKD had no preceding albuminuria [27]. Thus, normoalbuminuria does not protect against a decline in the renal function, in diabetic patients. Therefore, in addition to measurements of UAE, simultaneous estimation of GFR is beneficial in patients with DKD.

Among patients with T2DM who develop renal damage and CKD, several risk factors that influence disease progression have been identified. However, these known markers of DKD progression do not predict outcomes fully [28]. Therefore, additional biomarkers are needed to better define the nature, severity and outcome of DKD. Due to the fact that activity summarises the overall response from numerous MMPs and TIMPs it was felt this may provide a more robust and encompassing measurement. Therefore, the aim of this study was to test the hypothesis that MMP activity can be measured in human urine and can be used as a potential biomarker of the progression of DKD.

To the best of our knowledge, this data represents the first detailed analysis of total urine MMP activity in human DKD. We were able to measure the MMP activity in the urine from three different populations. Urinary MMP activity was detectable in 94.5% of patients with DKD plus 81 and 70% of diabetic controls and healthy volunteers, respectively. Indeed, what this approach surprisingly revealed was that urinary proteolytic activity was strongly up-regulated in patients with DKD compared to diabetic controls and especially to healthy control subjects. This contradicts reported results in kidney tissue, where low MMP activity is associated with kidney scarring and fibrosis of CKD [12, 29]. However, there was a significant reduction of MMP activity in DKD patients losing kidney function, which does correspond to previous work in kidney tissue [29]. Therefore, what appears to be reflected in the urine is that we observe an upregulation in MMP activity in those patients with nonprogressive DKD, but from this higher level compared to controls, the urine MMP activity declines in line with the loss of kidney function.

There was a notable increase in both interstitial collagenase (MMP-1, -8 and -13) and gelatinase (MMP-2 and -9) activities in the urine of DKD and DM controls compared to

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healthy volunteers indicating that both subgroups of MMP contribute towards the changes in MMP activity. The applications of 1,10-phenanthroline to reactions clearly indicates that a vast majority of substrate breakdown was due to MMP action as this specific MMP inhibitor has only minimal action against serine proteases.

These findings are in agreement with previous clinical studies carried out in human DKD. Firstly, a study conducted by Tashiro and colleagues (2004) in 47 T2DM patients with CKD and 7 healthy controls, showed that the levels of urinary MMP-9 and urinary type IV collagen in patients with DKD increased in accordance with the clinical stage of the disease [18]. They concluded that the overproduction and urinary excretion of type IV collagen, one of the main ECM components, coupled with the increase of urinary excretion of MMP-9 (important in collagen IV clearance) may occur in the early stages (either normoalbuminuric or microalbuminuric) of DKD. More recently, a cross-sectional study carried out by Van der Zijl and colleagues (2010) showed a significant elevation of urinary MMP-2, -8 and -9 levels in T2DM subjects compared to healthy controls [30]. Indeed, urinary MMP-9 showed the strongest association with clinical parameters related to the DKD. In addition, a marked increase in urinary excretion of MMP-2 was also demonstrated in another cross-sectional study of 91 patients with T1DM compared to healthy control subjects [31]. Significant correlations between urinary MMP-2 levels and many risk factors for nephropathy were reported in this study. These findings are in agreement with what we report here in that in nonprogressive DKD there is an elevated urine MMP activity (most likely due to glomerular hypertension) which subsequently drops as the renal MMP system is downregulated with progressive scarring of the kidney.

In respect to albuminuria, a marked increase in urinary MMP activity was clearly demonstrated in patients with either normoalbuminuria or microalbumnuria compared to those with macroalbuminuria. This affirms that changes in urinary MMP activity are already present in the initial development of DKD, and it seems to decrease sharply once the patient developed persistent proteinuria. A negative significant correlation between urinary MMP activity and albuminuria was observed. Importantly, increased MMP-9 in the plasma precedes the development of microalbuminuria in patients without changes in either MMP-1 or TIMP-1 levels [32]. This finding was supported by another observation demonstrating that urinary levels of MMP-9 are elevated in T2DM patients, and correlated with albuminuria [18].

The increase in the MMP activity among DKD and DM controls could be explainable in part to changes in the permeability and disruption of the filtration integrity of the GBM and changes in renal tubular handling of the MMP filtered load. Equally the increased production or secretion of MMP by renal tissue might be in response to hyperglycaemia [33]. Additionally, gelatinases are involved in the remodelling of type IV collagen which is usually accumulated in the ECM early in DKD patients. Therefore, the changes in their activity levels might reflect an early renal damage in diabetic patients even before the appearance of albuminuria [34].

The urinary MMP activity was greater in females than males with DKD. This is in keeping with a recent clinical study that found urine MMP-9 levels correlated with indicators of hyperglycaemia and albuminuria and were elevated in females [35]. In addition, DKD with diabetic retinopathy exhibited a markedly higher level of MMP activity. Jacqueminet et al. (2006) observed that diabetic patients with retinopathy had elevated systemic levels of MMP-9 as compared to levels in patients without retinopathy [16]. Gelatinase MMPs are also enhanced in retinal neovascularisation and main arteries of diabetic subjects.

The potential importance of MMP activity in the progression of renal scarring has been previously described [12, 36]. The majority of these studies reported that MMP activity progressively declines with advancement of CKD. In contrast, there are a limited number of reports that investigated the glomerular MMP expression in diabetic nephropathy owing to the shortage in renal biopsy specimens with variable outcomes. Del Prete et al. (1997) de-

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scribed downregulation of MMP-2 mRNA expression, and a peculiar pattern of MMP-2/TIMP-2 in glomeruli of 16 patients with T2DM irrespective of the level of albuminuria and of renal histology classification compared to 5 normal patients [17], suggesting that MMP-2/TIMP-2 imbalance might be crucial, but not sufficient for the pathogenesis of nephropathy in T2DM. On the other hand, upregulation of MMP-2 was detected in diabetic kidney tissue, and this increase was correlated with tubular atrophy [37]. This discrepancy in the results might be attributable to the confounding effects of diabetes treatment in humans.

Finally, it is tempting to link the early rise in MMPs in T2DM to the known association of this condition with vascular pathology and atherosclerosis. In this regard, it is worth mentioning that, of the 28 known MMPs, 11 MMPs have been explored in the context of atherothrombosis [38]. However, association studies of subclinical atherosclerosis have generated contradictory results in the role of MMP activities. In addition, circulating MMP levels as well as genetic variations within the genes encoding the different enzymes have been associated with both an increased and decreased cardiovascular risk. In fact, MMPs have been linked to the chronic inflammation, proliferation and plaque formation that characterise atherosclerosis [39]. Vascular pathology is an early manifestation of T2DM and is also likely to affect the kidney. Furthermore, MMPs are known to be produced by macrophages; cells known to infiltrate diabetic kidneys early in the course of diabetic nephropathy [40]. The subsequent fall in MMPs activity with DKD progression may simply reflect the reduction in intrarenal inflammation as kidney scarring evolves into a fibrotic process with less infiltration by macrophages and also a reduction in MMP producing intrinsic renal tubular cells. Further studies, however, are needed to address the specific role of individual MMP in atherothrombosis as well as to elucidate their interactions and overall actions during initiation and progression of atherosclerosis.

The study performed here is not without limitations, the first of which relates to the fact that MMP measurements have been made on only one urine sample. Multiple measures would have been preferred. Tissue MMP was not measured which is the principal limitation. It is unfortunate that age and gender distribution were different in the control groups, as these seem to influence MMP levels. Statistical analysis cannot correct fully for these differences. A propensity score analysis was not possible owing to the small number of controls. The CKD patients in our population study were mainly elderly patients making it more likely to include a significant percentage of patients suffering from ischemic nephropathy and hypertensive nephrosclerosis on a background of DM. Finally, there were no 'disease controls' (e.g. membranous nephropathy, FSGS, IgA nephropathy) included in this study; thus, specificity for DKD cannot be claimed. Despite these limitations, we believe these results are robust and valuable clinically.

In summary, the preliminary data presented here clearly show elevated urinary MMP activity in nonprogressive DKD patients with either normoalbuminuria or microalbuminuria (i.e. early stages) compared to those with macroalbumiuria.

The increase of urinary MMP activity indicates ongoing alteration in the turnover of glomerular and tubular ECM early in the DKD. The subsequent loss of urine MMP activity provides a highly sensitive and reliable biomarker of patients losing kidney function as described by ROC analysis data. It also determines the benefits of measuring overall MMP activity; the cumulative effects of all TIMPs and MMP on activity over individual TIMPs and MMP as others do. Measuring individual TIMPs or MMP may be misleading in the wider context of the final activity which determines ECM clearance. Importantly, the simplicity of the fluorescence-based activity assay makes it suitable for rapid, high-throughput analyses without specialised equipment and thus immediately applicable in most clinical chemistry laboratories. However, a larger and longer follow-up study for DKD patients is warranted to confirm the value of MMP activity to predict clinical outcomes.

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Disclosure Statement

No conflicts of interest declared.

References

1   Schernthaner G: Kidney disease in diabetology: lessons from 2009. Nephrol Dial Transplant 2010;25: 360–363.
2   Collins AJ, Foley RN, Herzog C: US Renal Data System 2010 annual data report. Am J Kidney Dis 2011;57:e1–e526.
3   Levey AS, Cattran D, Friedman A: Proteinuria as a surrogate outcome in CKD: report of a scientific workshop sponsored by the National Kidney Foundation and the US Food and Drug Administration. Am J Kidney Dis 2009;54:205–226.
4   Keane WF, Eknoyan G: Proteinuria, albuminuria, risk, assessment, detection, elimination (PA-RADE): a position paper of the National Kidney Foundation. Am J Kidney Dis 1999;33:1004–1010.
5   Mason RM, Wahab NA: Extracellular matrix metabolism in diabetic nephropathy. J Am Soc Nephrol 2003;14:1358–1373.
6   Lenz O, Elliot SJ, Stetler-Stevenson WG: Matrix metalloproteinases in renal development and disease. J Am Soc Nephrol 2000;11:574–581.
7   Visse R, Nagase H: Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res 2003;92:827–839.
8   Nagase H, Visse R, Murphy G: Structure and function of matrix metalloproteinases and TIMPs. Cardiovasc Res 2006;69:562–573.
9   Werb Z, Hembry RM, Murphy G: Commitment to expression of the metalloendopeptidases, collagenase and stromelysin: relationship of inducing events to changes in cytoskeletal architecture. J Cell Biol 1986;102:697–702.
10 Herman MP, Sukhova GK, Kisiel W: Tissue factor pathway inhibitor-2 is a novel inhibitor of matrix metalloproteinases with implications for atherosclerosis. J Clin Invest 1996;107:1117–1126.
11 Catania JM, Chen G, Parrish AR: Role of matrix metalloproteinases in renal pathophysiologies. Am J Physiol Renal Physiol 2007;292:F905–F911.
12 Johnson TS, Haylor JL, Thomas GL: Matrix metalloproteinases and their inhibitions in experimental renal scarring. Exp Nephrol 2002;10:182–195.
13 Bai Y, Wang L, Li Y: High ambient glucose levels modulates the production of MMP-9 and alpha5 (IV) collagen by cultured podocytes. Cell Physiol Biochem 2006;17:57–68.
14 McLennan SV, Fisher E, Martell SY: Effects of glucose on matrix metalloproteinase and plasmin activities in mesangial cells: possible role in diabetic nephropathy. Kidney Int Suppl 2000;77:S81–S87.
15 Signorelli SS, Malaponte G, Libra M: Plasma levels and zymographic activities of matrix metalloproteinases 2 and 9 in type II diabetics with peripheral arterial disease. Vasc Med 2005;10:1–6.
16 Jacqueminet S, Ben Abdesselam O, Chapman MJ: Elevated circulating levels of matrix metalloproteinase-9 in type 1 diabetic patients with and without retinopathy. Clin Chim Acta 2006;367:103–107.
17 Del Prete D, Anglani F, Forino M: Down-regulation of glomerular matrix metalloproteinase-2 gene in human NIDDM. Diabetologia 1997;40:1449–1454.
18 Tashiro K, Koyanagi I, Ohara I: Levels of urinary matrix metalloproteinase-9 (MMP-9) and renal injuries in patients with type 2 diabetic nephropathy. J Clin Lab Anal 2004;18:206–210.

Nephron Extra 2012;2:219–232

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A Potential Marker of Disease Progression

19 Diamant M, Hanemaaijer R, Verheijen JH: Elevated matrix metalloproteinase-2 and -9 in urine, but not in serum, are markers of type 1 diabetic nephropathy. Diabet Med 2001;18:423–424.

20 K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Am J Kidney Dis 2002;39:S1–S266.

21 Levey AS, Eckardt KU, Tsukamoto Y: Definition and classification of chronic kidney disease: a position statement from Kidney Disease: Improving Global Outcomes (KDIGO). Kidney Int 2005;67: 2089–2100.

22 Levey AS, Bosch JP, Lewis JB: A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of Diet in Renal Disease Study Group. Ann Intern Med 1999;130:461–470.

23 Jones C: Decline in kidney functions before and after nephrology referral and the effect on survival in moderate to advanced chronic kidney disease. Nephrol Dial Transplant 2006;21:2133–2143.

24 Davies DF, Shock NW: Age changes in glomerular filtration rate, effective renal plasma flow, and tubular excretory capacity in adult males. J Clin Invest 1950;29:496–507.

25 Yokoyama H, Kanno S, Takahashi S, Yamada D, Honjo J, Saito K, Sone H, Haneda M: Risks for glomerular filtration rate decline in association with progression of albuminuria in type 2 diabetes. Nephrol Dial Transplant 2011;26:2924–2930.

26 Caramori ML, Fioretto P, Mauer M: Low glomerular filtration rate in normoalbuminuric type 1 diabetic patients: an indicator of more advanced glomerular lesions. Diabetes 2003;52:1036–1040.

27 Retnakaran R, Cull CA, Thorne KI, Adler AI, Holman RR; UKPDS Study Group: Risk factors for renal dysfunction in type 2 diabetes: U.K. Prospective Diabetes Study 74. Diabetes 2006;55:1832–1839.

28 Altemtam N, Russell J, El Nahas M: A study of the natural history of diabetic kidney disease (DKD). Nephrol Dial Transplant 2012;27:1847–1854.

29 Ahmed AK, Haylor JL, El Nahas AM, Johnson TS: Localization of matrix metalloproteinases and their inhibitors in experimental progressive kidney scarring. Kidney Int 2007;71:755–763.

30 van der Zijl NJ, Hanemaaijer R, Tushuizen ME: Urinary matrix metalloproteinase-8 and -9 activities in type 2 diabetic subjects: A marker of incipient diabetic nephropathy? Clin Biochem 2010;43:635–639.

31 Thrailkill KM, Bunn RC, Moreau CS: Matrix metalloproteinase-2 dysregulation in type 1 diabetes. Diabetes Care 2007;30:2321–2326.

32 Ebihara I, Nakamura T, Shimada N: Increased plasma metalloproteinase-9 concentrations precede development of microalbuminuria in non-insulin-dependent diabetes mellitus. Am J Kidney Dis 1998;32:544–550.

33 Chung AW, Hsiang YN, Matzke LA: Reduced expression of vascular endothelial growth factor paralleled with the increased angiostatin expression resulting from the upregulated activities of matrix metalloproteinase-2 and -9 in human type 2 diabetic arterial vasculature. Circ Res 2006;99:140–148.

34 Lelongt B, Legallicier B, Piedagnel R: Do matrix metalloproteinases MMP-2 and MMP-9 (gelatinases) play a role in renal development, physiology and glomerular diseases? Curr Opin Nephrol Hypertens 2001;10:7–12.

35 Thrailkill KM, Moreau CS, Cockrell GE: Disease and gender-specific dysregulation of NGAL and MMP-9 in type 1 diabetes mellitus. Endocrine 2010;37:336–343.

36 Ahmed AK: Localization of matrix metalloproteinases and their inhibitors in experimental progressive kidney scarring. Kidney Int 2007;71:755–763.

37 Romanic AM, Burns-Kurtis CL, Ao Z: Upregulated expression of human membrane type-5 matrix metalloproteinase in kidneys from diabetic patients. Am J Physiol Renal Physiol 2001;281:F309–F317.

38 Back M, Ketelhuth DF, Agewall S: Matrix metalloproteinases in atherothrombosis. Prog Cardiovasc Dis 2010;52:410–428.

39 McKittrick IB: Urinary matrix metalloproteinase activities: biomarkers for plaque angiogenesis and nephropathy in diabetes. Am J Physiol Renal Physiol 2011;301:F1326–F1333.

40 Thrailkill KM, Clay BR, Fowlkes JL: Matrix metalloproteinases: their potential role in the pathogenesis of diabetic nephropathy. Endocrine 2009;35:1–10.