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ORIGINAL ARTICLE

Disease and gender-specific dysregulation of NGAL and MMP-9 in type 1 diabetes mellitus

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Abstract Neutrophil gelatinase-associated lipocalin (NGAL), a biomarker of renal injury, can bind matrix metalloproteinase-9 (MMP-9) and inhibit its degradation, thereby sustaining MMP-9 proteolytic activity. MMP-9 is produced by renal podocytes, and podocyte MMP production can be modified by high ambient glucose levels. Moreover, dysregulation of MMP-9 activity, gene expression, or urine concentrations has been demonstrated in T2DM-associated nephropathy and in non-diabetic proteinuric renal diseases. Our objective was to determine whether NGAL/MMP-9 dysregulation might contribute to or serve as a biomarker of diabetic nephropathy in type 1 DM (T1DM). Plasma MMP-9, and urine NGAL and MMP-9 concentrations were measured in 121 T1DM and 55 control subjects and examined relative to indicators of glycemia, renal function, and degree of albuminuria. T1DM was associated with a significant increase in urinary excretion of both NGAL and MMP-9, and urine NGAL:Cr (NGAL corrected to urine creatinine) and urine MMP-9:Cr concentrations were highly correlated with each other. Both were also positively correlated with measurements of glycemic control and with albuminuria. Plasma MMP-9, urine MMP-9, and urine NGAL concentrations were significantly higher in females compared to males, and urine MMP-9:Cr concentrations displayed a menstrual cycle specific pattern. Increased urinary excretion of NGAL and MMP-9 supports a role for NGAL/MMP-9 dysregulation in renal dysfunction; moreover, gender-specific differences could support a gender contribution to pathological mechanisms or susceptibility for the development of renal complications in diabetes mellitus.

Keywords Lipocalins · Metalloproteinases · Diabetic nephropathy · Renal function

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Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteinases whose activities are directed toward the degradation and turnover of extracellular matrix (ECM) proteins. The more than 20 known mammalian MMPs are categorized as collagenases (MMP-1, -8, -13, -18), gelatinases (MMP-2, -9), stromelysins (MMP-3, -10, -11), matrilysins (MMP-7, -26), membrane-type MMPs (MT-MMPs; MMP-14, -15, -16, -17, -24, -25), and "other MMPs" [1–3]. Because of their powerful degradative capacity, the activity of MMPs is tightly regulated by a family of tissue inhibitors of metalloproteinases (TIMPs 1–4), as well as other proteinase inhibitors, such as α2-macroglobulin and tissue factor pathway inhibitor-2 [1, 2, 4, 5].

Increased circulating concentrations of MMP-9 (gelatinase B) have been demonstrated in persons with obesity [6, 7], metabolic syndrome [8], and with type 2 diabetes mellitus (T2DM) [9]. Moreover, it has been postulated that plasma MMP-9 concentrations may be a biomarker for the

development of vascular complications in T2DM, because increased circulating concentrations of MMP-9 have been demonstrated in T2DM patients exhibiting acute coronary syndrome [10], peripheral arterial disease [11], or diabetic retinopathy [12]. These clinical observations are supported by in vitro data demonstrating that high glucose exposure specifically increases MMP-9 expression and activity in monocyte-derived macrophages [13].

With respect to renal complications, in the KKAy mouse (a rodent model of T2DM), expression of MMP-9 in the kidneys of mice that developed nephropathy is enhanced when compared to renal expression of this gelatinase in control animals [14]. In addition, increased urinary concentrations of MMP-9 have been detected in patients with T2DM and diabetic nephropathy, and the levels of MMP-9 were increased in accordance with the degree of albuminuria [15, 16]. Dysregulation of MMP-9 activity or expression has also been demonstrated in *non-diabetic* proteinuric renal diseases, both in rodents and humans, including focal segmental glomerulosclerosis [17], anti-GBM glomerulonephritis [18], and HIV-associated nephropathy [19].

Urinary neutrophil gelatinase-associated lipocalin (NGAL) is increasingly recognized as a sensitive biomarker of acute kidney injury in a variety of situations, including renal injury following cardiopulmonary bypass, kidney transplantation, or contrast-administration nephrotoxicity [20]. Recent reports suggest that urine NGAL may also be a sensitive biomarker for the progression of nephropathy in T2DM [21, 22]. As NGAL binds to MMP-9, inhibits its degradation, and thereby sustains MMP-9 proteolytic activity [23], we chose to investigate whether, collectively, NGAL and MMP-9 dysregulation could be demonstrated in *type 1 diabetes* and might be indicative of renal pathology by: (1) measuring concentrations of NGAL and MMP-9 in the urine of patients with T1DM and healthy control subjects and (2) analyzing a possible correlation between observed differences in NGAL/MMP-9 concentrations and various indices of glycemic control, T1DM disease severity, and renal function.

Materials and methods

Study design

Subjects with T1DM and age-matched healthy control subjects, ages 14–40 years, were recruited from clinics at the University of Arkansas for Medical Sciences (UAMS), Arkansas Children's Hospital (ACH), and surrounding communities. Approval was obtained from the Institutional Review Board of UAMS and informed consent or assent was obtained from all participants. Exclusion criteria included: (1) concurrent use of medications known to alter MMP activity or MMP-9 circulating concentrations (i.e., tetracyclines, glucocorticoids) [24]; (2) T2DM; (3) history of other chronic systemic inflammatory or autoimmune disease or malignancy; (4) pregnancy; and (5) concurrent ketonuria. Subjects were also excluded if the baseline examination revealed any site of active infection, or if baseline urinalysis was suggestive of possible urinary tract infection. Control subject data were excluded from final analyses if a subject was incidentally found to have albuminuria.

For all subjects, two-first-morning outpatient evaluations were conducted 3–5 days apart, so as to obtain: (1) medical history and demographic information; (2) duplicate fasting venipuncture laboratory measurements of plasma glucose (FPG), HbA1c, C-peptide, and serum creatinine (Cr); (3) a 3–5 day interval recording of continuous glucose monitor sensor data (using the Medtronic Minimed® CGMS, MMT-7102, Northridge, CA); and (4) a 24-h urine specimen, to be used for determination of albumin excretion rate, glomerular filtration rate (GFR; calculated using the MDRD study equation [25]), and for MMP-9 and NGAL measurements. Urine NGAL and MMP-9 concentrations were standardized to urine creatinine concentration in 24-h collections; consistent with this, abnormal urinary albumin excretion (UAE) was also defined according to the albumin-to-creatinine ratios (ACR), but using 24-h collections, as microalbuminuria for an ACR of 30–299 mg/g Cr or macroalbuminuria for an ACR of ≥300 mg/g Cr [26].

Clinical assays

Fasting plasma glucose (FPG) and C-peptide were measured by the UAMS General Clinical Research Center Core Laboratory [27]. HbA1c, serum creatinine (Cr), and urinary albumin and Cr concentrations (24-h urine specimen) were measured by LabCorp (Dallas, TX). In female subjects, serum estradiol was measured using a quantitative enzyme immunoassay (ALPCO Diagnostics, #11-ESTHU-E01, Salem, NH).

MMP-9, NGAL and TIMP-1 measurements

MMP-9 concentrations were measured in EDTA-plasma (0.5 µl, diluted 1:100) and 24-h urine collections (50 µl, undiluted) using the Fluorokine® MultiAnalyte Profiling (F-MAP) assay (R&D Systems, Inc., Minneapolis, MN). Specimens were analyzed in duplicate on a Luminex® 100™ Bioanalyzer (Luminex Corp. Austin, TX) according to the manufacturer's instructions (minimal detection limit, 7.4 pg/ml) and as we have previously described [27, 28]. Inter-assay and intra-assay precisions, respectively, for MMP-9 were 8.7 and 3.1% [28]. NGAL was measured in urine (50 µl, undiluted) using a Human Lipocalin-2/NGAL ELISA (R&D Systems; kit # DLCN20; minimal detection

Table 1 Subject demographics

Parameter	T1DM	Control	*P*-value (T vs. C)
N	121	55	NA
Age (years)	20.8 ± 7.6	24.3 ± 7.6	<0.001
Body Mass Index (kg/m^2)	24.9 ± 4.4	25.1 ± 4.7	NS
Fasting plasma glucose (mg/dl)	160.6 ± 65.7	81.4 ± 6.6	<0.001
Duration of T1DM (years)	9.7 ± 8.0	–	NA
CGMS average glucose (mg/dl)	171.6 ± 48.7	88.3 ± 9.9	<0.001
HbA1c (%)	8.3 ± 1.8	5.0 ± 0.3	<0.001
C-peptide (ng/ml)	0.15 ± 0.18	0.88 ± 0.53	<0.001
GFR (ml/min/1.73 m^2)	113.2 ± 31.0	91.1 ± 16.6	<0.001
UAE (mg/g Cr)[a]	41.7 ± 213.4	10.1 ± 5.7	0.001

NA not applicable, *NS* not significant

[a] For UAE and GFR, $n = 115$ for T1DM, $n = 54$ for control, due to seven incomplete urine collections

limit, 0.012 ng/ml). For specimens reading above the highest standard, urine samples were diluted 1:30 and reanalyzed.

The activity of MMPs is tightly regulated by a family of specific-tissue inhibitors of metalloproteinases (TIMPs 1–4); specifically, MMP-9 binds to TIMP-1 with high affinity [23]. In order to determine whether any change in plasma MMP-9 concentration was potentially offset by a simultaneous change in inhibitor concentration, plasma concentrations of TIMP-1 (50 µl, diluted 1:100) were also measured using a Human TIMP-1 ELISA (R&D Systems; kit #DTM100; minimal detection limit, 0.08 ng/ml).

Statistical analysis

Statistical analysis was performed using SAS software (version 9.2; SAS Institute Inc., Cary, NC) and SPSS Statistical software (version 17.0; SPSS Inc., Chicago, ILL). A minimal sample size of 50 per group was determined to provide approximately 80% power to detect a difference between groups for MMP-9 of 0.55 standard deviations. Results for FPG, HbA1c, C-peptide, and serum Cr obtained from the two study visits were averaged. Plasma MMP-9 and T1MP-1 concentrations were measured only once, at study visit 2. As MMP-9 and NGAL variables were not normally distributed, non-parametric statistical analyses (Mann–Whitney tests) were employed to compare T1DM and control groups with respect to enrollment and disease-specific variables. We also used non-parametric Kruskal–Wallis tests to compare more than two categories. Spearman's rank correlation coefficient was calculated as a measure of correlation between non-parametric variables. After investigation of the variables, we used a log transformation of the data to conduct the analysis of covariance, to assess the statistical significance of differences between groups. In the analysis of covariance models, demographic differences in age, sex, and other relevant variables were accounted for by including these variables as covariates in the model, either as a continuous or dichotomous variables as appropriate. Data throughout the text and in Table 1 are presented as mean ± standard deviation. Data in Table 2 are presented as median, minimum, and maximum values. Statistical significance was defined as $P < 0.05$.

Results

Baseline characteristics

Fifty-five control subjects and 121 subjects with T1DM were evaluated. Control and T1DM groups were comparable with respect to gender (56 vs. 51% female, respectively); racial distribution (86 vs. 93% Caucasian, respectively), baseline BMI (25.2 ± 4.7 vs. 24.9 ± 4.4 kg/m^2, respectively), and mean systolic and diastolic blood pressure (systolic: 119 ± 14 vs. 122 ± 13 mmHg; diastolic: 69 ± 9 vs. 71 ± 7 mmHg, respectively). The diabetic subgroup was slightly younger in age (Table 1). Therefore, additional analyses, as detailed below (see, *Relationship of NGAL and MMP-9 to age*) were conducted to examine any potential confounding effect of age on NGAL or MMP-9 results.

Expected differences between the control and T1DM subgroups were confirmed by baseline measurements of FPG, HbA1c, C-peptide, and 3–5 day average glucose by CGMS (Table 1). The mean HbA1c value for the T1DM subgroup was 8.3 ± 1.8%, suggesting sub-optimal glycemic control as defined by American Diabetes Association guidelines [29], but consistent with expectations for a T1DM population not otherwise selected on the basis of glycemic control [30].

Within the T1DM subgroup, a history of hypertension ($n = 10$), retinopathy ($n = 11$), or neuropathy ($n = 4$) was ascertained by self-report alone. Among T1DM subjects, the mean urinary albumin excretion (UAE) was 41.7 ± 213.4 mg/g Cr. T1DM subjects were further categorized as normoalbuminuric (NA: $n = 97$; UAE $\leq$ 29 mg/g Cr) or albuminuric (A: $n = 16$; $>$ 30 mg/g Cr) for between group

Table 2 MMP-9, NGAL, and TIMP-1

Parameter	All subjects median (min; max)	T1DM median (min; max)	Control median (min; max)	*P*-value (T1DM vs. cont)
Plasma MMP-9 (ng/ml)				
Total	76 (16; 1,824)	77 (23; 369)	76 (16; 1,824)	NS
Female	70 (16; 1,824)	69 (29; 308)	76 (16; 1,824)	NS
Male	85 (23; 369)	88 (23; 369)	74 (32; 140)	NS
Plasma TIMP-1 (ng/ml)	86 (41; 210)	85 (41; 210)	86 (52; 205)	NS
Urine MMP-9:Cr (ng/g)				
Total	50 (3, 83,968)	100 (5; 83,968)	27 (3; 1,409)	<0.001
Female	161 (4; 83,968)	298 (5; 83,968)	50 (4; 1,410)	<0.01
Male	27 (3; 8,632)	39 (5; 8,632)	16 (3; 101)	0.001
Urine NGAL:Cr (ng/g)				
Total	2,414 (17; 117,656)	3,784 (75; 117,656)	1,278 (17; 11,730)	<0.001
Female	9,364 (205; 117,656)	12,841 (238; 117,656)	2,054 (205; 11,730)	<0.001
Male	1,254 (17; 35,114)	1,824 (75; 35,114)	453 (17; 4,369)	<0.01
P-value: Plasma MMP-9 (female vs. male)	0.04	0.01	NS	–
P-value: Urine MMP-9:Cr (female vs. male)	<0.001	<0.001	<0.001	–
P-value: Urine NGAL:Cr (female vs. male)	<0.001	<0.001	<0.001	–

comparisons. Of the 16 albuminuric patients, UAE concentrations were within a microalbuminuria range in 14 subjects (UAE $>$ 30–299 mg/g Cr) and within a macroalbuminuria range (UAE $\geq$ 300 mg/g Cr) in 2 subjects. For the subset of albuminuric patients, the mean UAE was 226.5 $\pm$ 551.0 mg/g (median value, 50.8, minimum value, 30.4; maximum value, 2250.1 mg/g).

The use of angiotensin converting enzyme inhibitors (ACEIs) or ACE-receptor blockers (ARBs) was exclusionary for control subjects. Among the T1DM subgroup, 16 subjects were being treated with these drug classes (15 on ACEI drugs, 1 on an ARB); 14 of these 16 had a UAE $\leq$ 29 mg/g Cr (mean UAE: 11.2 $\pm$ 5.5 mg/g), and 2 of 16 had a UAE $>$ 30 mg/g Cr (subject-specific data: 30.4 and 443.0 mg/g). The mean UAE for T1DM subjects not on ACEI/ARB therapy ($n = 99$) was 42.1 $\pm$ 226.3 mg/g.

All female subjects had experienced menarche per inclusion criteria; all but two females were also premenopausal (one T1DM and one control, due to surgical total hysterectomy). A total of 21 females were receiving some from of oral contraceptive ($n = 19$) or hormone replacement ($n = 2$) therapy (12 T1DM and nine controls).

MMP-9 and TIMP-1 concentrations in biological fluids

Both NGAL and MMP-9 concentrations were significantly elevated in the urine of T1DM subjects, as compared with control subjects; the increase in urinary excretion of NGAL or MMP-9 was apparent whether analyzed as a urine NGAL or urine MMP-9 concentration corrected to the urine creatinine concentration (Table 2) or as total NGAL or total MMP-9 excretion per day (NGAL: 14,022 $\pm$ 2,085 vs. 2,792 $\pm$ 471 ng/day; $P < 0.001$. MMP-9: 2,580 $\pm$ 698 vs. 144 $\pm$ 47 ng/day; $P < 0.001$, respectively). Urine NGAL or MMP-9 concentrations were also significantly higher in female subjects compared to male subjects, whether examined across the entire study population or within the T1DM or control subgroups (Table 2; $P < 0.001$ for all analyses). Within each *gender* subgroup, the significant increase in urine NGAL or MMP-9 concentration in persons with type 1 diabetes compared with controls was maintained (Table 2).

In contrast, no differences were observed in plasma MMP-9 or plasma TIMP-1 concentrations between T1DM and control subjects (Table 2). Plasma MMP-9 concentration was, however, slightly lower in female subjects compared to male subjects, both for the entire study population and among the T1DM subgroup (Table 2).

In order to further evaluate gender differences in urine NGAL or MMP-9 concentrations, female subjects were subdivided into four groups, according to date from last menstrual period (LMP groups; 0–7; 8–14, 15–21, 22–34 days). Between group differences in urine MMP-9:Cr and urine NGAL:Cr concentrations were then examined among: (1) all female subjects ($n = 93$); (2) T1DM females alone ($n = 62$); or (3) T1DM females restricted to those not receiving any form of oral contraceptive (OC) medication ($n = 50$). Among female subjects (either all-inclusive or restricted to those with T1DM, whether on or not on an OC), a menstrual cycle specific pattern for the mean concentration of MMP-9 in urine was evident (Fig. 1). In contrast, urine concentrations of NGAL (Fig. 1)

did not vary significantly across the LMP groups. (The small sample size for the control/female subgroup alone ($n = 31$) rendered analysis is this subgroup insufficiently powered for evaluation.)

In order to determine whether the fluctuation in MMP-9 concentrations across the menstrual cycle was a function of concurrent glucose variation across the menstrual cycle, we examined the relationship between variations in mean CGMS glucose by LMP group. Among T1DM females, urinary excretion of MMP-9 was strongly associated with glucose ($P < 0.001$); however, after controlling for glucose, the significant differences in urine MMP-9 excretion persisted across LMP cycle groups ($P = 0.03$).

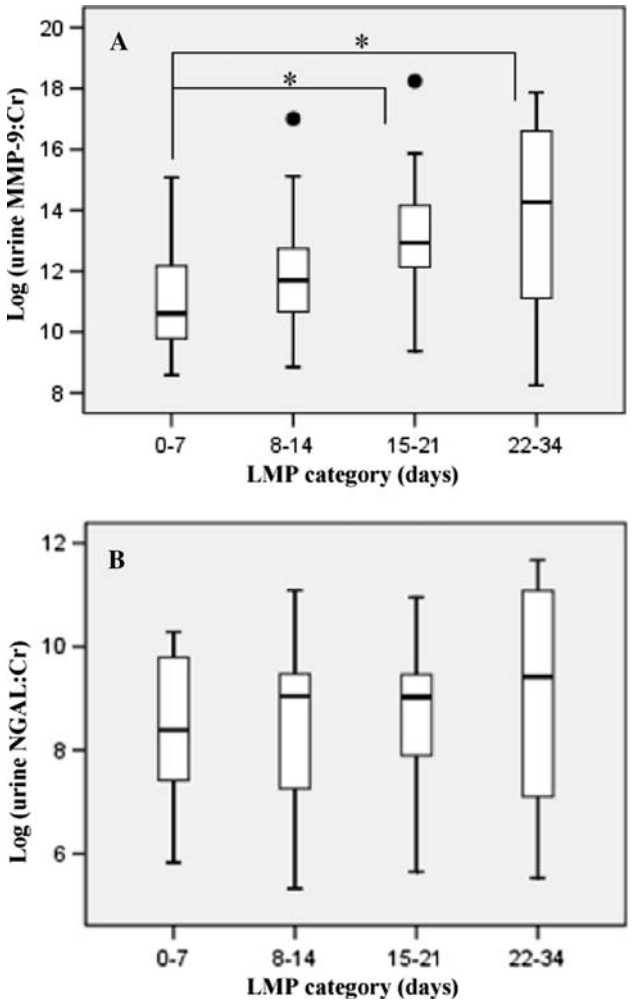

Fig. 1 Differences in urinary MMP-9 excretion across the menstrual cycle. The urinary excretion of MMP-9 (**a** MMP-9:Cr) and NGAL (**b** NGAL:Cr) among all female subjects, sub-grouped according to days from last menstrual period (LMP: 0–7, $n = 14$; 8–14, $n = 37$; 15–21, $n = 16$; 22–34, $n = 16$) is shown as log transformed data. The median (*transverse line*) is shown and the *box* represents the interquartile range. Outliers are depicted as *closed circles*. Urinary excretion of MMP-9, unlike NGAL, varied across the menstrual cycle. Using the Kruskal–Wallis analysis of variance, urinary MMP-9:Cr was statistically significantly different between LMP subgroups with $P = 0.03$. *Asterisks* designated specific between group differences with $P < 0.05$

In order to determine whether the fluctuation of MMP-9 concentrations across the menstrual cycle was related to variations in serum estradiol concentration across the month, we examined the relationship between serum estradiol and plasma or urine MMP-9 values. No correlation existed between serum estradiol concentration and plasma MMP-9, urine MMP-9:Cr, or total urine MMP-9 excretion per day, whether analyzed among all female subjects or restricted to T1DM females alone. However, median estradiol concentrations among the T1DM subgroup were higher than the control subgroup (144.7 vs. 74.4 pg/ml, $P = 0.001$).

No differences were apparent in urinary albumin excretion among female subjects when analyzed by menstrual cycle group ($P = 0.83$), suggesting that the gradual increase in urinary MMP-9 excretion proceeding across the menstrual cycle was not simply a consequence of menstrual cycle fluctuations in glomerular protein permeability.

Relationship of NGAL and MMP-9 to age

As noted in Table 1, the mean age of the T1DM subgroup was ~3.5 years younger than the control subgroup, a difference that was statistically significant, though seemingly not clinically significant. However, to account for possible confounding effects of age on our analyses, additional comparisons were made between the subset of T1DM and control subjects who were 18–40 years of age (adult subgroup: control, $n = 45$; age (mean ± SEM); 26.3 ± 6.9 years. T1DM, $n = 61$; age; 26.0 ± 7.6 years). A similar comparison was made between the subset of T1DM and control subjects who were <18 years of age (adolescent subgroup: control, $n = 10$; mean age, 15.1 ± 0.6 years. T1DM, $n = 60$; age, 15.4 ± 1.0 years). Consistent with differences reported for the entire study population, for subjects 18–40 years, urine MMP-9:Cr ($P = 0.001$), urine MMP-9 total excretion per day ($P = 0.002$), urine NGAL:Cr ($P < 0.001$), and urine NGAL total excretion per day ($P < 0.001$) were significantly higher among subjects with T1DM. For subjects 14 to ≤18 years of age, a trend toward higher values in the T1DM group was also evident, though these differences did not attain statistical significance, likely because of the much smaller sample size for control subjects ($n = 10$) who were under 18 years of age. In spite of this, using the analysis of covariance model, when controlling for age and gender, the increase in urinary excretion of MMP-9 and NGAL in T1DM subjects remained highly significant ($P < 0.001$ for both).

Table 3 Associations between urine NGAL or MMP-9 and clinical parameters

Parameter ($n = x$)	Urine NGAL:Cr		Urine MMP-9:Cr	
	R	*P*-value	*R*	*P*-value
Total urine NGAL/day ($n = 165$)	0.96	<0.0001	0.60	<0.0001
Total urine MMP-9/day ($n = 165$)	0.60	<0.001	0.97	<0.001
Fasting plasma glucose ($n = 166$)	0.37	<0.001	0.29	<0.001
CGMS glucose ($n = 166$)	0.42	<0.001	0.36	<0.001
HbA1c ($n = 167$)	0.41	<0.001	0.35	<0.001
C-peptide ($n = 167$)	−0.36	<0.001	−0.24	0.002
Duration of T1DM ($n = 114$)	0.25	0.006	–	NS
UAE ($n = 167$)	0.45	<0.001	0.31	<0.001
GFR ($n = 167$)	0.16	0.04	–	NS

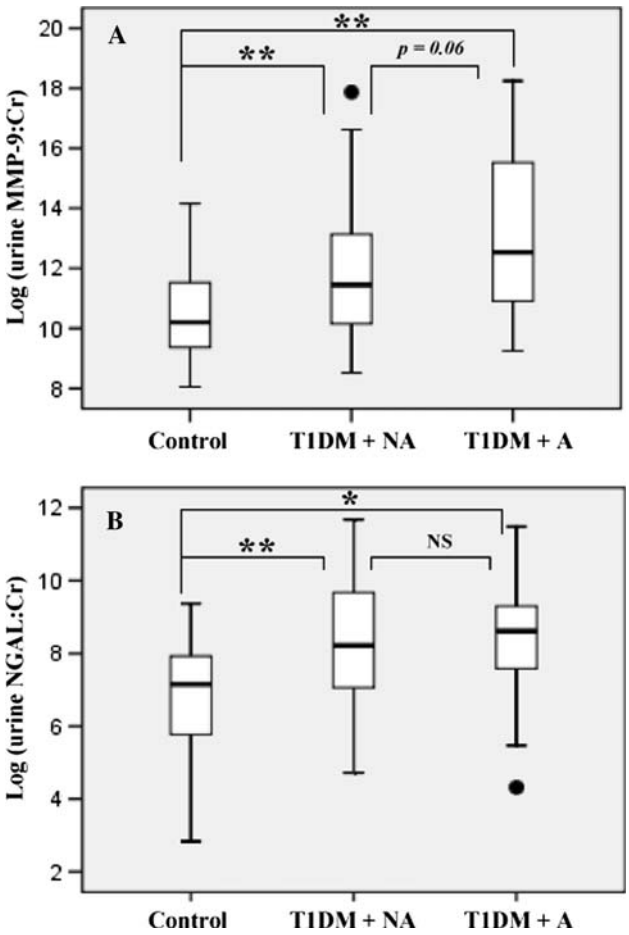

Fig. 2 Association of urinary MMP-9 and NGAL excretion with albuminuria. The urinary excretion of MMP-9 (**a** MMP-9:Cr) and NGAL (**b** NGAL:Cr) among all subjects, sub-grouped according to urinary albumin excretion, as controls, T1DM with normolalbuminuria (T1DM + NA) and T1DM + albuminuria (T1DM + A: see "Materials and methods" for details) is shown. The median (*transverse line*) is shown and the box represents the interquartile range. Outliers are depicted as closed circles. For MMP-9 (**a**), a step-wise increase in urine excretion was apparent among the subgroup of T1DM subjects with albuminuria. For NGAL (**b**), urine concentrations were elevated in all T1DM subjects, compared with control subjects. Using the Kruskal–Wallis analysis of variance, urinary MMP-9:Cr was statistically significantly different between subgroups with $P < 0.001$. *Asterisks* designated specific between group differences with $P < 0.05$ (*) or $P < 0.001$ (**)

Relationship of NGAL and MMP-9 to duration of disease

Among T1DM subjects, those with a duration of disease greater than 3 years had a significantly higher urinary excretion of both NGAL:Cr (14,781 ± 23,726 ng/g vs. 5,933 ± 10,656; $P = 0.003$) and MMP-9:Cr (3,390 ± 11,652 ng/g vs. 1,449 ± 4,728; $P = 0.01$), compared with those with disease duration of less than 3 years. When examined as a continuous variable, urine NGAL:Cr was also positively correlated with disease duration (Table 3).

Relationship of NGAL and MMP-9 to glycemic control

Urine NGAL:Cr and MMP-9:Cr values were both significantly positively correlated with multiple indices of glycemic control including FPG, CGMS average daily glucose, and HbA1c (Table 3). In addition, both urine NGAL:Cr and MMP-9:Cr were negatively correlated with fasting C-peptide concentration. In all cases, NGAL associations were stronger than MMP-9 associations (Table 3).

Relationship of NGAL and MMP-9 to renal function

Urine NGAL:Cr and MMP-9:Cr values were both significantly positively associated with urinary albumin excretion (UAE: Table 3). However, as shown in Fig. 2, comparing the urine concentration of MMP-9 across three groups [control subjects; T1DM subjects with normal UAE (<30 mg/g); and T1DM subjects with increased UAE (≥30 mg/g)], a statistically significant, step-wise increase in MMP-9:Cr values was apparent. When subdivided further, a significant increase in MMP-9:Cr concentrations was apparent for all T1DM subjects with UAE above a cut-point of 10 mg/g [median values: control ($n = 53$): 26,958 pg/g vs. T1DM + UAE < 10 mg/g ($n = 47$): 32,470 pg/g; comparison, not significant or control: 26,958 pg/g vs. T1DM + UAE ≥ 10 mg/g ($n = 67$): 346,223 pg/g. $P < 0.001$]. In contrast, urine NGAL excretion was comparably increased in both groups of T1DM subjects, *irrespective* of their UAE status (Fig. 2). Urine NGAL:Cr, but not MMP-9:Cr, was also weakly associated with GFR (Table 3).

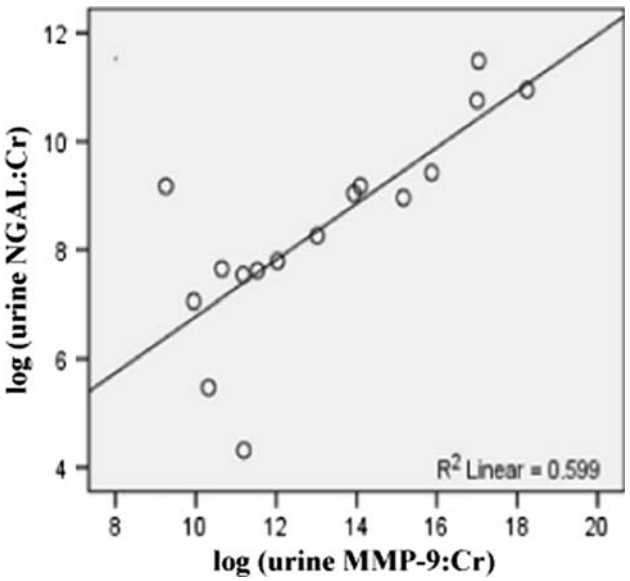

Fig. 3 Linear correlation between urine NGAL:Cr and MMP-9:Cr. Among the subgroup of T1DM subjects with albuminuria ($n = 16$) strong correlations between the urinary excretion of MMP-9 and NGAL were evident. Data are presented as the log transformation of protein concentration corrected to creatinine

Relationship between urine NGAL:Cr and MMP-9:Cr

Urinary concentrations of NGAL and MMP-9 were very highly associated with *each other* (NGAL:Cr vs. MMP-9:Cr, $R = 0.65$, $P < 0.001$). This association was notably stronger among the subgroup of 16 T1DM subjects with albuminuria [NGAL:Cr vs. MMP-9:Cr, $R = 0.78$, $P < 0.0001$; and Fig. 3 ($R^2 = 0.599$)].

Discussion

Herein, we have demonstrated that the urinary excretion of NGAL and MMP-9 is increased in subjects with T1DM. Mechanisms contributing to the exaggerated excretion of NGAL and MMP-9 are not known. Recognizing that: (1) plasma MMP-9 concentrations did not differ between those with or without diabetes and that (2) after controlling for GFR the increase in MMP-9 or NGAL urine excretion persisted among diabetic subjects ($P < 0.001$ for both), it would seem that the increase in urine NGAL/MMP-9 values is not simply a reflection of filtration of plasma constituents.

In fact, stronger relationships were seen between NGAL/MMP-9 and urinary albumin excretion than with GFR. We have recently reported that the endocytic, multiligand receptor, megalin (i.e., LRP2), known to be expressed on kidney proximal tubule cells and involved with the re-uptake of filtered small molecular weight proteins, is abnormally shed into the urine of albuminuric patients with type 1 diabetes [31]. Megalin is capable of binding to albumin [32], NGAL [33] and to the hemopexin domain of MMP-9 [34], inferring that diminished functioning or availability of this scavenger receptor in diabetes could contribute to proteinuria in general, and to the exaggerated urinary loss of NGAL and/or MMP-9, in particular. NGAL/MMP-9 complexes have been identified in normal urine [35], and the strong association between NGAL and MMP-9 in urine also suggests that these ligands may be co-excreted in T1DM.

Glycemic control and duration of disease are independent risk factors for the development of diabetic nephropathy [36]. This study demonstrated that urine NGAL and MMP-9 excretion are positively correlated with HbA1c, FPG, and CGMS glucose. Moreover, urine NGAL or MMP-9 excretion was higher in those T1DM subjects with a longer duration of disease.

Urinary excretion of both NGAL and MMP-9 also related to urinary albumin excretion (Table 3). However, as shown in Fig. 2, discrete differences between NGAL and MMP-9 were apparent when comparing normoalbuminuric and albuminuric T1DM subjects. Specifically, increased excretion of NGAL was apparent in all the subjects with diabetes. In contrast, a step-wise increase in urinary excretion of MMP-9 was coupled with a step-wise increased in UAE, such that a significant increase in urine MMP-9 *originated* at a UAE > 10 mg/g for T1DM subjects. As such, this data suggest that urinary concentrations of MMP-9 might function as a biomarker of latent nephropathy, prior to clinically defined microalbuminuria. However, this cross-sectional, observational study was designed only to identify proteins which might be indicative of renal pathology. Longitudinal prospective studies monitoring urine MMP-9 concentrations over time would be needed to confirm its utility as a predictive biomarker of DN.

A marked gender difference in the concentration of MMP-9 and NGAL in urine was also demonstrated, and to our knowledge it has not previously been reported. This was apparent both within the control subgroup and the T1DM subgroup; moreover, within the T1DM subgroup, there were no statistically significant differences between males and females with respect to age, duration of diabetes, or level of glycemic control to account for these gender differences. Explanations for these gender-specific findings may be multiple. NGAL mRNA expression in the endometrial epithelium of the mouse uterus has been shown to vary across the estrous cycle, with the increase in NGAL expression mirroring the estradiol surge in proestrus and estrus [37]. Gene expression profiles of estrogen responsive cells isolated from normal breast tissue have also identified NGAL as a potential estrogen target [38]. Estradiol supplementation of streptozotocin-induced diabetic rats has

been shown to increase MMP-9 expression in the kidney [39]. Since female subjects in this study (ages 14–40) were nearly all post-menarchal yet pre-menopausal, gender differences in the urinary excretion of NGAL and MMP-9 could reflect an estrogen-mediated difference in the expression of these proteins in renal or urinary tract tissues. These findings are also interesting in light of the observation that while female gender is protective against the development of ESRD in non-diabetic renal disease, this gender-protective effect is diminished in diabetes mellitus [40]. Hence, females may be particularly susceptible to the impact of hyperglycemia on renal function, and our findings could infer a gender-specific difference in renal susceptibility to NGAL or MMP-9 dysregulation.

In summary, urine NGAL has recently been shown to be a sensitive biomarker for the development of renal damage in a variety of conditions, including T2DM [20–22]. Herein, we provide evidence that NGAL excretion is increased also in T1DM, as is the excretion of its binding partner, MMP-9. Finally, very striking gender differences in the concentrations of these analytes in urine was evident, both in a control population and in a T1DM population, and this novel finding must be taken into account in their implementation as urinary biomarkers.

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