Urinary matrix metalloproteinase activities: biomarkers for plaque angiogenesis and nephropathy in diabetes

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McKittrick IB, Bogaert Y, Nadeau K, Snell-Bergeon J, Hull A, Jiang T, Wang X, Levi M, Moulton KS. Urinary matrix metalloproteinase activities: biomarkers for plaque angiogenesis and nephropathy in diabetes. Am J Physiol Renal Physiol 301:F1326–F1333, 2011. First published September 14, 2011; doi:10.1152/ajprenal.00267.2011.—Diabetic complications of nephropathy and accelerated atherosclerosis are associated with vascular remodeling and dysregulated angiogenesis. Matrix metalloproteinases (MMP) modify extracellular matrix during vascular remodeling and are excreted in urine of patients with vascular malformation or tumor angiogenesis. We hypothesized that urinary MMP activities would be sensitive biomarkers for vascular remodeling in diabetic complications. Activities of MMP-2, MMP-9, and its complex with neutrophil gelatinase-associated lipocalin (NGAL/MMP-9) were measured by substrate gel zymography in urine from nondiabetic (ND) and type 1 diabetic (T1D) rodents that were susceptible to both T1D-induced plaque angiogenesis and nephropathy, or nephropathy alone. Additionally, these urinary activities were measured in ND and T1D adolescents. Urinary MMP-9, MMP-2, and NGAL/MMP-9 activities were increased and more prevalent in T1D compared with ND controls. Urinary MMP-2 activity was detected in mice with T1D-induced plaque neovascularization. In nephropathy models, urinary NGAL/MMP-9 and MMP-9 activities appeared before onset of albuminuria, whereas MMP-2 was absent or delayed. Finally, urinary MMP activities were increased in adolescents with early stages of T1D. Urinary MMP activities may be sensitive, noninvasive, and clinically useful biomarkers for predicting vascular remodeling in diabetic renal and vascular complications.

atherosclerosis; diabetic nephropathy; albuminuria; neutrophil gelatinase-associated lipocalin; NGAL; diabetic microvascular complications; diabetic macrovascular complications

VASCULAR COMPLICATIONS ARE a major cause of death and morbidity in people with type 1 and type 2 diabetes (T1D and T2D), and diabetic kidney disease often heralds the rapid progression of atherosclerosis. Controlling hyperglycemia and dyslipidemia are important for diabetes management; however, HbA1c and lipid profiles do not sufficiently stratify risk for renal and vascular complications (1, 14). Furthermore, C-reactive protein has reduced prognostic significance in diabetes (19, 21). Thus new biomarkers for cardiovascular and renal complications would be clinically useful tools in diabetes care.

Matrix metalloproteinases (MMP) are activated during angiogenesis and vascular remodeling that can occur during both physiological processes and pathological diseases (2, 32). The MMP family includes over 20 zinc- and calcium-dependent endopeptidases that collectively degrade all forms of extracellular matrix and basement membrane proteins. MMP are secreted from various cell types as proenzymes, activated after proteolytic cleavage and further regulated by binding to specific tissue inhibitors of metalloproteases (TIMPs) (15). Some MMP are excreted in urine with preserved or latent activities, which renders their detection at high sensitivity (27). Even the large 125-kDa complex of MMP-9 and neutrophil gelatinase-associated lipocalin (NGAL; also known as lipocalin-2), which protects MMP-9 from autodegradation, can be excreted in urine. MMP-2, MMP-9, and NGAL/MMP-9 are nearly absent in urine of unaffected adults but are excreted by individuals with various types of cancer or invasive vascular malformations and have been shown to predict both clinical stage and prognosis (10, 11, 23).

Several studies have described altered MMP expression in diabetes, but few have correlated excretion patterns of MMP activities with types of diabetic complications. In diabetic nephropathy, macrophages infiltrating glomeruli and tubular interstitia elaborate various MMP (18, 30). MMP-2 and MMP-9 proteins are also enhanced in retinal neovascularization and major arteries of diabetic subjects (4, 5). Hyperglycemia induces expression of MMP-9 by endothelial cells (34). Plaque angiogenesis is more abundant in atherosclerotic plaques from diabetic patients compared with nondiabetic subjects (25, 26). These combined observations support the hypothesis that MMP activities might correlate with vascular or renal complications in diabetes.

In this report, urinary MMP activities were monitored in a novel mouse model for T1D-induced plaque angiogenesis and temporally correlated with plaque angiogenesis or nephropathy end points. The results demonstrated that urinary MMP-2 activities correlated with a stage of atherosclerosis when plaque angiogenesis was accelerated by T1D, whereas NGAL/MMP-9 preceded albuminuria and correlated with signs of nephropathy in wild-type mice that do not acquire atherosclerosis. Finally, urinary MMP activities were detected in a pilot study of adolescents with early T1D, which demonstrates feasibility for future clinical validation of urinary MMP activity biomarkers in diabetes complications.

MATERIALS AND METHODS

Mouse model of T1D-enhanced plaque angiogenesis. T1D accelerates atherosclerosis in ApoE-null mice (3). This study used a highly angiogenic strain of ApoE-null mice [ApoE−/−:Col18a1−/−, designated as ApoE-hemagglutinin (HA)] that lacks an angiogenesis inhibitor type XVIII collagen in the aorta and develops twofold more plaque angiogenesis compared with ApoE-null littermates (28). Age 8- to 12-wk-old male ApoE-HA mice were injected intraperitoneally
with streptozotocin [50 mg/kg for 5 days. Animal Models of Diabetic Complications Consortium (www.AMDCC.org)]. Nondiabetic (ND) control mice received 0.1 M citrate buffer, pH 4.5. T1D was confirmed by elevated blood glucose (>300 mg/dl) within 2 wk after injection. Mice were fed standard rodent chow, and NPH insulin was given three times per week if needed to maintain body weights. Urine samples were collected weekly and frozen at −80°C. After 12 wk, mice were euthanized for terminal harvest of blood, aorta, and kidneys. Plaque morphometry and in situ CD31 staining of vasa vasorum were performed as described (29). Diabetes and atherosclerosis animal studies were approved by Institutional Animal Care and Use Committee at the University of Colorado Anschutz Medical Campus.

Rodent models of diabetic nephropathy. T1D was induced by streptozotocin in 8-wk-old male C57BL6J mice and in Sprague-Dawley male rats, which develop diabetic nephropathy, but not atherosclerosis (37). Urinary MMP activities and albumin-to-creatinine ratios were measured weekly over 16 wk. Random blood glucose and body weights were monitored weekly. Kidney sections were stained with periodic acid-Schiff (PAS) staining to detect glomerular changes characteristic of diabetic nephropathy. Albumin, creatinine, and protein levels in urine were measured using Albuwell ELISA (Exocell), QuantiChrom Creatinine (BioAssay Systems), and DC protein (Bio-Rad) assays, respectively. Serum glucose and HbA1c were measured at the Barbara Davis Center for Childhood Diabetes.

Substrate zymography. Gelatin substrate zymography was performed on 10% SDS-PAGE gels containing 1 mg/ml gelatin (161–1167, Bio-Rad) according to reported methods (1a). Urines were screened for any MMP-2 (72 kDa), MMP-9 (92 kDa), and NGAL/MMP-9 (125 kDa) activities, which were detected at their predicted migration distances as lucent bands in Coomassie blue-stained gelatin gels (27). Activity-dose curves showed a linear range for quantification of MMP-9 protein standards (R&D Systems). MMP activities were quantified by densitometry relative to the band intensity of a 100-pg MMP-9 protein standard (Scion Image) and normalized for equal creatinine content.

Immunohistochemistry. Paraffin sections of kidneys were stained with PAS to detect proteoglycans in glomeruli exposed to diabetes. MMP were detected in kidney and aorta tissues using primary antibody directed to MMP (0.4 µg/ml, sc-50351; Santa Cruz Biotechnology), MMP-2 (1 µg/ml, AB19167, Millipore), and MMP-9 (1 µg/ml, ab38898, Abcam). Antigen-antibody complexes were detected after sequential incubations with biotinylated goat anti-rabbit IgG, avidin-horseradish peroxidase, and substrate Vector NovaRed (all reagents from Vector Labs). Image analysis was performed with IP Lab software.

Human subjects. Urine MMP activities were measured in T1D and ND adolescents aged 12–19 yr under a protocol approved by the University of Colorado Denver Institutional Review Board. T1D was defined by American Diabetes Association criteria and insulin requirements, along with the presence of one or more diabetes-associated autoantibodies. Subjects were excluded if they had an initial HbA1c >12%, recent diabetic ketoacidosis, or other conditions of pregnancy, breast feeding, smoking, and regular physical activity of >3 h/wk. Urine samples were collected in the late afternoon following 3 days of restricted physical activity and a standardized weight maintenance diet provided by the pediatric Clinical Translation Research Center. Urine samples with positive leukocyte esterase, blood, or large amounts of ketones were excluded.

Statistical analysis. GraphPad and SAS software were used to compare urinary MMP activities between T1D and ND cohorts (J. Snell-Bergeon). Fisher’s exact t-tests compared urinary MMP activities based on binary values (presence or absence). Relative MMP activity data are expressed as means ± SD and compared for statistical significance between ND and T1D groups using an unpaired t-test. MMP activities in urine samples of T1D and ND adolescents (ages 12–19 yr) were compared using the Kruskal-Wallis test.

RESULTS

Urinary MMP-2 activity is increased in T1D-accelerated plaque angiogenesis. ND and T1D male cohorts of the highly angiogenic ApoE−/−:Col18a1−/− strain (ApoE- HA; n = 13/group) were fed a chow diet for 12 wk to develop T1D-accelerated atherosclerosis and plaque angiogenesis (28). T1D ApoE-HA mice had increased plasma cholesterol and triglyceride levels compared with ND controls fed the same chow diet (cholesterol 806 ± 75 for T1D vs. 370 ± 21, P < 0.0001; triglycerides 82 ± 15 for T1D vs. 64 ± 15 mg/dl, P = 0.0054, unpaired t-test). Sudan IV-stained atheromas occupied 40 ± 5.7% area of the descending aortas in T1D mice compared with 4.5 ± 1% in ND (P < 0.0001, unpaired t-test). Plaque-associated vasa vasorum detected by CD31-IgG staining were found in 46% of the T1D-ApoE-HA aortas (Fig. 1, A and B).

Urines were sampled at 2-wk intervals by gelatin substrate zymography to determine the temporal excretion of MMP activities. The gelatinase activities for MMP-2 (72 kDa), the proenzyme form of MMP-9 (92 kDa), and the NGAL/MMP-9 complex (125 kDa) produced proteolytic bands on the gel (Fig. 1C) at the indicated migration distances (38).

After 12 wk of T1D, all ApoE-HA mice excreted MMP-9, 46% (6 of 13) excreted MMP-2, and 23% (3 of 13) excreted NGAL/MMP-9 activities, whereas 1 ND mouse excreted MMP-9 (Fig. 1D). Urinary MMP-9 appeared first at 6 wk and MMP-2 emerged at 10 wk. In contrast, NGAL/MMP-9 activity appeared late in only a few mice that had already excreted MMP-2 and MMP-9. Both the prevalence (Table 1) and mean activities of MMP-2 and MMP-9 (Fig. 1E, MMP-2 P = 0.0325, MMP-9 P = 0.0459) were increased in T1D ApoE-HA mice compared with ND.

Interestingly, only those T1D-ApoE-HA mice that had formed plaque vasa vasorum elaborated urinary MMP-2 activity. To distinguish whether urinary MMP-2 activity was a marker for extensive atherosclerosis or T1D conditions, urinary MMP activities were monitored in a separate group of ND ApoE-HA mice fed a 0.15% cholesterol diet for 24 wk to acquire the same extent of atherosclerosis as the T1D group (Table 1, ND-high cholesterol diet vs. T1D). The cholesterol-fed ND mice developed more extensive atherosclerosis, but 8 of the 15 (53%) mice excreted only MMP-9 activity. Thus MMP-2 was more specific to T1D mice.

Urinary MMP activities in diabetic nephropathy. Since the kidneys from T1D ApoE-HA mice showed some areas of increased PAS-positive staining in glomeruli (data not shown), the observed urinary MMP profiles could correlate with either plaque angiogenesis or nephropathy end points. To identify MMP activities that correlate with nephropathy alone, urinary MMP activities were monitored weekly in ND and T1D C57BL6J mice (n = 14/group) that are susceptible to diabetic nephropathy but lack the ApoE-null genotype required to develop atherosclerosis. NGAL/MMP-9 and MMP-9 showed an early rise that ultimately affected 86 and 57% of T1D mice after 16 wk of diabetes, respectively (Fig. 1F). After 16 wk of T1D, mean urinary NGAL/MMP-9 and MMP-9 activities were significantly elevated in T1D mice compared with ND (Fig. 1G), while MMP-2 activities remained low (T1D vs. ND, P = 0.0008 for NGAL/MMP-9; P = 0.0016 for MMP-9, P = 0.2006 for MMP-2). Both NGAL complex and MMP-9 activ-
Fig. 1. Urinary matrix metalloproteinase (MMP) activities in mice with type 1 diabetes (T1D)-induced plaque angiogenesis or nephropathy. A: highly angiogenic ApoE<sup>−/−</sup>;Col5a1<sup>−/−</sup> mice (ApoE-HA) develop plaque neovascularization after 12 wk of T1D. Extensive adventitial CD31+ neovascularization (arrow) around atheroma from ApoE-HA aorta is shown. B: whole mount CD31-IgG staining demonstrates vasa vasorum (arrow) around a sadan IV-stained atheroma in the aortic arch of a T1D ApoE-HA mouse. C: gelatin substrate zymography shows MMP activities in urine from chow-fed ApoE-HA mice after 4 and 12 wk of T1D. Proteolytic bands corresponding to MMP-2 (72 kDa), MMP-9 (92 kDa), and neutrophil gelatinase-associated lipocalin (NGAL)/MMP-9 complex (125 kDa) are shown at their migration distances. D: temporal progression of positive MMP activities in T1D ApoE-HA mice that develop increased plaque neovascularization (NV) after induction of T1D. E: urinary MMP-2 (black bar, means ± SD, n = 13), MMP-9 (white bar), and NGAL/MMP-9 (gray bar) activities were compared between T1D and nondiabetic (ND) ApoE-HA mice after 12 wk of T1D. MMP activity was measured by densitometry relative to the lucent band produced by a 100-pg MMP-9 protein standard and normalized for equal creatinine content. MMP-2 and MMP-9 activities were increased in T1D ApoE-HA mice (P = 0.0325 for MMP-2 and P = 0.0459 for MMP-9, unpaired t-test). F: urinary MMP activities were monitored in ND and T1D wild-type C57BL6/J mice that are prone to diabetic nephropathy but not atherosclerosis. The percentages of T1D mice (n = 14) with positive MMP activities are shown after successive weeks of T1D. NGAL/MMP-9 and MMP-9 arise earliest after T1D induction and are more prevalent in the nephropathy model compared with ApoE-HA mice that develop plaque neovascularization. G: relative urinary MMP-2 (black bar, means ± SD), MMP-9 (white bar), and NGAL/MMP-9 complex activities (gray bar) after 16 wk of T1D were compared between ND and T1D groups. NGAL/MMP-9 and MMP-9 monomer activities were increased in T1D wild-type mice that develop nephropathy (P = 0.0016 for NGAL and P = 0.0008 for MMP-9, unpaired t-test). H: albumin-to-creatinine ratio (ACR; μg protein/mg creatinine) in T1D mice was measured over time after onset of diabetes. NGAL/MMP-9 and MMP-9 activities emerged before ACR exceeded 100 μg/mg. The transient elevation of ACR 4 wk after streptozotocin was due to reduced urine creatinine, not an absolute rise in albuminuria.

Table 1. Urinary MMP activities in atherosclerosis-prone mice with T1D

<table>
<thead>
<tr>
<th></th>
<th>T1D (n = 13)</th>
<th>ND (n = 13)</th>
<th>P Value*</th>
<th>ND-hc (n = 15)</th>
<th>P Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-9</td>
<td>13</td>
<td>1</td>
<td>&lt;0.0001</td>
<td>8</td>
<td>0.0069</td>
</tr>
<tr>
<td>MMP-2</td>
<td>6</td>
<td>0</td>
<td>0.0149</td>
<td>0</td>
<td>0.0046</td>
</tr>
<tr>
<td>NGAL/MMP9</td>
<td>3</td>
<td>0</td>
<td>0.220</td>
<td>0</td>
<td>0.087</td>
</tr>
</tbody>
</table>

MMP, metalloproteinase; T1D, type 1 diabetes; ND, nondiabetic NGAL-neutrophil gelatinase-associated lipocalin. *P value, 2-sided Fisher’s exact test, TID vs. ND. †P value, 2-sided Fisher’s exact test, T1D vs. high-cholesterol diet-fed ND mice (ND-hc).
macrophages and endothelium, and NGAL in intimal macrophages and smooth muscle cells, which was consistent with published studies (13, 30). The cell distributions of MMP proteins were also examined in T1D and ND kidneys. Although NGAL is expressed by tubule cells and is a predictive marker for acute renal failure following surgery, NGAL showed a greater abundance in glomeruli of T1D and ND kidneys (Fig. 4). In contrast, MMP-9 and MMP-2 were more abundant in blood vessels and tubules. The glomeruli of T1D kidneys contained increased numbers of infiltrated MMP-9-producing mononuclear cells compared with ND (Fig. 4B). This glomerular abundance of MMP-9 and NGAL could potentially facilitate release into urine during diabetic nephropathy.

**Urinary MMP activities in human subjects with T1D.** The above animal studies demonstrated that urinary MMP-2 activity correlated with angiogenic stages of atherosclerosis and NGAL/MMP-9 activities preceded microalbuminuria in diabetic nephropathy. Next, urinary MMP profiles were investigated in a pilot study of human adolescents with early stages of T1D and minimal renal impairment or albuminuria. Table 2 summarizes the clinical characteristics of these subjects and the percentage of positive urinary MMP activities in each group. The median duration of diabetes was 66 mo (range 7–187 mo), and the average HbA1c was 8.1 ± 1.3%. Only one subject had microalbuminuria (albumin-to-creatinine ratio = 39.99 μg/mg) in this young T1D cohort, but urinary MMP-2 and NGAL/MMP-9 activities were significantly increased in T1D subjects relative to ND (Fig. 5). Mean MMP-2 was 0.075 (range 0–0.247) relative units (RU) for T1D vs. 0.005 (0–0.07), \( P = 0.0013 \); NGAL/MMP-9 was 0.044 RU (0–0.246) vs. 0.003 (0–0.038), \( P = 0.0479 \); and MMP-9 was 0.155 RU (0–0.687) vs. 0.0255 (0–0.253), \( P = 0.1346 \) (Kruskal-Wallis). These same T1D urines were also evaluated for immunoreactive MMP proteins by Western blot analysis in parallel (Fig. 6). Results indicated that protein immune-detection methods were 10-fold less sensitive, did not distinguish active from inactive MMP-9 proteins, and correlated poorly with MMP activity assays.

**DISCUSSION**

These results in preclinical models of T1D vascular and renal complications demonstrated that urinary MMP activities were increased in T1D and correlated with nephropathy and
plaque angiogenesis end points. Urinary MMP-2 activity correlated more specifically with angiogenic stages of atherosclerosis, whereas NGAL/MMP-9 and MMP-9 were significantly elevated in diabetic nephropathy and emerged before albuminuria in nephropathy models without concurrent atherosclerosis. Although MMP-9 was the most ubiquitous activity in T1D, it was also elevated with advanced atherosclerosis in ND ApoE-HA mice and was not specific for diabetic-associated vascular remodeling. The temporal appearances of NGAL/MMP-9 and MMP-2 activities in nephropathy and T1D-induced plaque angiogenesis were consistent with their respective distributions in glomeruli and blood vessels.

The observed correlations between urinary MMP activity profiles and vascular and renal complications corroborate reports of higher plasma MMP-9 or MMP-2 protein levels in diabetic rodents (40). Plasma MMP-2 levels were also increased in T2D and T1D adults compared with ND controls (7, 19, 24). A separate study found plasma MMP-9 levels were increased 3 yr before microalbuminuria (9). The major limitation for use of plasma MMP levels as biomarkers is their overlapping range of values between diabetic and ND individuals, which reduces the prognostic performance of the test for intermediate values. In contrast, urinary MMP activities are either absent or marginally detectable in ND subjects.

Table 2. Prevalence of MMP activities in adolescents with TID

<table>
<thead>
<tr>
<th>Activity</th>
<th>ND (n = 14)</th>
<th>T1D (n = 17)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2, % positive</td>
<td>1 (7%)</td>
<td>11 (63%)</td>
<td>0.002*</td>
</tr>
<tr>
<td>MMP-9, % positive</td>
<td>2 (14%)</td>
<td>6 (35%)</td>
<td>0.240*</td>
</tr>
<tr>
<td>NGAL/MMP-9, % positive</td>
<td>1 (7%)</td>
<td>6 (35%)</td>
<td>0.094*</td>
</tr>
<tr>
<td>Age, years</td>
<td>15 ± 2.4</td>
<td>16 ± 1.9</td>
<td>0.214*</td>
</tr>
<tr>
<td>M/F</td>
<td>6/8</td>
<td>7/10</td>
<td>1.0*</td>
</tr>
<tr>
<td>Duration, mo</td>
<td>0</td>
<td>66 (7-187)</td>
<td>&gt;0.0001</td>
</tr>
<tr>
<td>ACR &gt;30 µg/mg</td>
<td>0</td>
<td>1 (6%)</td>
<td>1.0*</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>5.2 ± 0.38</td>
<td>8.1 ± 1.35</td>
<td>&gt;0.0001†</td>
</tr>
</tbody>
</table>

Values are means ± SD. ACR, albumin-to-creatinine ratio. *Chi-square tests with Fisher’s exact P values, binary values, 1 or 0 for presence or absence of MMP activity or albuminuria. †Unpaired t-test P values, T1D vs. ND.

The early rise of
urinary MMP activities enhances their utility as a screening tool for high-risk T1D patients, because biomarker-guided preventions may be more effective when given at earlier stages of complications.

The present study also differed by its method of measuring activities by gelatin-substrate zymography compared with Diamant et al. (12), who measured MMP-2 and MMP-9 activities of antibody-captured proteins in urine of T1D subjects. Unlike the former method, zymography is able to distinguish the activities of MMP-9 monomers from the NGAL/MMP-9 complex, which was a more specific marker in nephropathy models. Also, MMP protein contents in urine can vary significantly from their corresponding activities; hence, excess inert MMP proteins could compete with active proteins for binding to the antibody-coated beads and interfere with activity results.

Protein assays have advantages for rapid quantification in biological fluids, but these methods cannot distinguish active from inactive molecules, and processed MMP products may remain in circulation with different clearance rates compared with the parent proteins. Active MMP proteins are tightly regulated in vivo. Thus activity assays may detect the biologically relevant molecules that are more direct and immediate correlates of disease.

A recent clinical study of T1D and ND subjects (ages 20.8 ± 7.6 yr) found that urine MMP-9 and NGAL proteins correlated with indicators of hyperglycemia and albuminuria and were greater in females (33). Interestingly, urine MMP-9 proteins increased over time after the day of the last menstrual period. It is not yet known whether gender-related differences in urinary MMP activity have prognostic significance for the development of renal and vascular complications in female and male diabetic patients. Such studies should exclude any urine samples with trace amounts of blood or leukocytes that cause falsely elevated MMP activities.

One potential confounding factor for the validation of urine MMP activity biomarkers is the presence of low urinary MMP-2 and MMP-9 activities in growing children. These activities diminish with age and were less common in the age range of subjects investigated for this study (23). Furthermore, urinary MMP activities associated with physiological growth tend to be lower than the activities associated with pathological conditions of vascular remodeling and usually do not contain the high-molecular-weight complexes of NGAL/MMP-9 and MMP-9 multimers (22).

Other limitations of this study should be acknowledged. The direct source of urinary MMP excreted in urine cannot be determined; however, this limitation does not refute the potential use of urinary MMP activities as surrogate biomarkers if they correlate with defined end points. Single urine specimens were analyzed in the T1D individuals. Activity concentrations in 24-h collections may be less variable; however, active MMP products may degrade after collection. Collections for single-void specimens should be controlled to avoid the first morning void, times after physical exertion, and samples that contain blood or infection. Each MMP activity was compared separately; however, combinations of diverse MMP profiles could enhance the specificity and predictive value of these biomark-

![Fig. 5. Urinary MMP activities in T1D adolescents. Urinary MMP-2 (A), MMP-9 (B), and NGAL/MMP-9 (C) were compared between T1D and ND adolescents. Relative activity units for MMP-2 and NGAL/MMP-9 were increased in T1D (P = 0.002 and P = 0.048, respectively; Kruskal-Wallis test).](image)

![Fig. 6. Paired analysis of MMP-9 activities vs. protein levels in T1D urine samples. A: gelatin substrate zymography of T1D urine samples (lanes 1–7) show zones that represent MMP-2 (72 kDa), MMP-9 (92 kDa), and NGAL/MMP-9 complexes (125 kDa) relative to 100-pg MMP-9 standard. B: the same volumes of urine assayed by gelatin-substrate zymography in A were also analyzed for immunoreactive MMP-9 proteins by Western blot analysis. The zymogram loaded with 100 pg of MMP-9 (92 kDa) protein standard shows a strong lysis band that is not detected by immunoblotting methods. Lanes 1, 3, 4, and 5 show abundant MMP-9-immunoreactive proteins that are not associated with gelatinase activity at the size range expected for the proenzyme form of MMP-9. MMP-9 proteins that migrate at 180 kDa represent MMP-9 homodimers.](image)
ers. Finally, assays could be broadened to include other classes of extracellular matrix proteases that may be involved in the susceptibility of T1D nephropathy and vascular complications. For instance, genome-wide association studies recently identified several variations in the MMP-3/MMP-12 loci that influence susceptibility to diabetic nephropathy (17).

The preclinical studies described temporal patterns and correlations of urinary MMP-2 and NGAL/MMP-9 profiles with respective end points of diabetes-accelerated atherosclerosis and nephropathy in animal models. Future studies are needed to validate serial measurements of urinary MMP profiles in larger numbers of individuals with T1D or T2D with renal biopsies and/or measures of vascular dysfunction to determine their predictive value for renal and vascular complications in humans with diabetes. In addition, it is not yet known whether urinary MMP profiles correlate with hyperglycemia control, insulin resistance, or other markers of renal impairment besides albuminuria (31). Ultimately, urinary MMP activity biomarkers will need to show that they add prognostic significance beyond standard monitors of diabetic control. These questions could be investigated in clinical studies that have serial urine samples and monitored T1D or T2D individuals for vascular or renal complications. Given that MMP-9 and MMP-2 genes are transcriptional targets of the peroxisome proliferator-activated receptor-γ and thiazolidinedione agonists inhibit both MMP proteins and NGAL (20, 36, 39), it would be interesting to monitor urinary MMP profiles before and after these treatments to determine whether they predict treatment response or alter subsequent risks for vascular or renal complications (16, 37).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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