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Original Article

Urine Levels of Matrix Metalloproteinases and Tissue Inhibitor of Metalloproteinases in Children with Type 1 Diabetes Mellitus

Yürük Yıldırım et al. Urine MMPs and TIMPs in Type 1 Diabetes Mellitus

Zeynep Yürük Yıldırım[1], Alev Yılmaz[1], Cemile Pehlivanoğlu[1], Asuman Gedikbaşı[2], Mehmet Yıldız[3], Ahmet Dirican[4], Rüveyde Bundak[5], Feyza Darendeliler[5], Sevinç Emre[1], Ahmet Nayır[1]
[1]Istanbul University, Istanbul Faculty of Medicine, Pediatric Nephrology Department, Istanbul, Turkey
[2]Bakirkoy Dr. Sadi Konuk Training and Research Hospital, Biochemistry Department, Istanbul, Turkey
[3]Istanbul University, Istanbul Faculty of Medicine, Department of Pediatrics, Istanbul, Turkey
[4]Istanbul University, Istanbul Faculty of Medicine, Istanbul, University, Biostatistics Department, Istanbul, Turkey
[5]Istanbul University, Istanbul Faculty of Medicine, Pediatric Endocrinology Department, Istanbul, Turkey

Zeynep Yürük Yıldırım MD, Istanbul University, Istanbul Faculty of Medicine, Pediatric Nephrology Department, Istanbul, Turkey
+90 532 378 42 69
znyuruk@gmail.com
Submitted: 14-Sep-2018
Accept: 06-Nov-2018
https://orcid.org/0000-0003-2891-2231

What is already known on this topic?
It has been demonstrated that mesangial expansion in diabetic nephropathy begins before microalbuminuria occurs. Only a few studies reported to have some alteration of urine levels of Matrix Metalloproteinases and Tissue Inhibitor of Metalloproteinases levels in patients with type 1 diabetes mellitus and these studies have conflicting results.

What this study adds?
Our results demonstrated that the indicators of fibrosis in urine do not increase in early stage of the disease. This finding suggests that the chronic changes in the kidney occurs over a long time.

Abstract
Objective: Histopathological changes of the kidney in type 1 diabetes mellitus (T1DM) begin before the microalbuminuria. Therefore, efforts are focused on finding a biomarker for the detection of early diabetic kidney injury. The aim of the study is to determine whether urine levels of indicators of fibrosis alter in diabetic children and if they may predict a progressive renal injury in T1DM.
Methods: Urinary matrix metalloproteinase 2 and 9 (MMP2 and MMP9), tissue inhibitor of metalloproteinase 1 and 2 (TIMP1 and TIMP2), Transforming growth factor βeta-1 (TGF-β1) were assessed in 33 patients with T1DM with normal renal functions as well as in 24 healthy children. Microalbuminuria was not present in the patient group except three. The results were adjusted to urine creatinine and the comparison between patients and controls was evaluated. Also, same measurements were repeated after one year and were compared to the first year results.
Results: Urine MMP2/Creatinine (Cr), MMP9/Cr, TIMP1/Cr, TIMP2/Cr, TGF-β1/Cr were not different between the patient and control groups ($p>0.05$). There was also no significant difference between the results of first and second year according to these biomarkers ($p>0.05$). All these parameters were not correlated to HbA1c, body mass index (BMI) and duration of T1DM. Interestingly, all parameters were negatively correlated to the age of onset of T1DM ($p<0.05$)
Conclusion: Our findings suggest that urinary biomarkers of fibrosis do not increase in diabetic children without microalbuminuria. As the age of onset of T1DM decreases, the risk of early fibrosis may increase according to our results.
Keywords: Type 1 Diabetes Mellitus, Diabetic Nephropathy, Children, Biomarker, MMP, TIMP

Introduction

Type 1 Diabetes Mellitus (T1DM) is one of the most common chronic diseases of childhood (1,2). T1DM causes many macro and microvascular complications. Diabetic nephropathy (DN) is one of the microvascular complications of T1DM (3,4). If T1DM is not under control, it leads to end-stage renal disease (ESRD) which is resulted from renal fibrosis (5,6,7). It has been known that development of renal fibrosis is a result of excessive accumulation of extracellular matrix components due to increased production and decreased degradation of matrix (8). Matrix components are regulated by matrix metalloproteinases (MMPs) such as MMP2, MMP9 (9). They cleave denatured collagens, laminin and some cell adhesion molecules and growth factors such as TGF-β. TIMPs are known as regulator of MMPs. TIMPs can either inhibit or sometimes activate MMPs' activity (10).
The prominent characteristic of DN is extracellular matrix (ECM) accumulation and consequently development of mesangial expansion (8). These changes begin at the second stage of DN and become more prominent in later stages (11). Since MMPs regulate remodeling of ECM, they are important for tissue development (9). MMP2 and MMP9 have crucial role on the degradation and regulation of ECM in the glomeruli (8). Therefore, MMPs may be involved in the pathophysiology of DN (8). Transforming growth factor βeta-1 (TGF-β1) is an important growth factor involved in kidney fibrosis and DN by various pathways.

Diabetic nephropathy usually manifests in adulthood and microalbuminuria is considered as the first laboratory sign of nephropathy (11). However, microalbuminuria usually occurs 6-15 years after diagnosis of T1DM. Early biomarkers predicting DN are needed to prevent ESRD. We hypothesized that the biomarkers of renal fibrosis may increase before microalbuminuria occurs because microalbuminuria is not a beginning of the disease, it is a result of ongoing renal damage in DN (11).

The aim of the study is to determine whether urine levels of MMP2, MMP9, TIMP1, TIMP2, TGF-β1 increase in children with T1DM and may predict a progressive renal injury.

Patients and Methods

Thirty-three consecutive patients (18 male, 15 female) with T1DM who applied to the outpatient clinic of the Pediatric Endocrinology Department of the Istanbul University Istanbul Faculty of Medicine were enrolled in the study. Patients' characteristics were given in Table 1. To our knowledge, there are no any standard normative data of urine levels of MMP2, MMP9, TIMP1, TIMP2, TGF- β1 in children according to age. For this reason, 24 healthy children (15 male, 9 female) were enrolled in the study as control group. This study was approved by the local ethical committee (No:2013/108) and written informed consent was obtained from children's parents.

Standard physical examination of the patients was performed and blood samples were drawn for the biochemical examination. Height and weight measurements of the patients were taken by the same auxologist according to standard methods. Body mass index (BMI) kg/m^2 was evaluated according to percentile curves of Turkish children and patients with a BMI above 95th percentile were considered as having obesity (12). Standard deviation score (SDS) of BMI were calculated according to national data (12). Hypertension was defined as the systolic and/or diastolic blood pressure to be higher than the 95th percentile for age and gender (13).

The HbA1c values assessed within the previous 3 months were collected from the patient files. Estimated GFR values were calculated by using Schwartz formula (14). A urinalysis and urine culture were performed to exclude urinary tract infection for each patient. None of the patients had urinary tract infection. Also, there was no patient with a record of urinary tract infection, urolithiasis and nephrotoxic drug usage in last three months. Patients who have urine microalbumin/ creatinine ratio (uMA/Cr) greater than 30 mg/g in at least two of the three urine specimens were considered microalbuminuric (15). Urine samples were obtained to measure urine levels of MMP2, MMP9, TIMP1, TIMP2, TGF- β1, microalbumin, and creatinine. The samples were centrifuged at 4º C for 15 minutes at 4,000xg. Until analyzed, the supernatants were stored at -80º C. All processes were done under the same conditions and time for urine of all children. The Abbott Architect c16000 analyzer was used to measure uCr and uMA, with uMA expressed in mg/L and uMA/Cr expressed in mg/g. Urine levels of MMP-2, MMP-9, TIMP-1, TIMP-2, TGFβ1 were assessed by enzyme-linked immunosorbent assay (ELISA) technique. Urine MMP2, MMP9, TIMP1 and TIMP2 levels were analysed using Human MMP-2 ELISA Kit (Cat no: YHB1973Hu), Human MMP-9 ELISA Kit (Cat no:YHB1982Hu), Human TIMP-1 ELISA Kit (Cat no: YHB3003Hu), Human TIMP-2 ELISA Kit (Cat no: YHB3004Hu) and Human TGF-B1 ELISA Kit (Cat no: YHB3051Hu) purchased from YH Biosearch Laboratory following the manufacturer's instructions. The intra-assay and the inter-assay coefficient of variations (CV) of MMP2, MMP9, TIMP1, TIMP2 and TGF-β were < 10%, and 12%, respectively. MMP2 and TIMP2 levels were expressed as ng/mL, MMP9 and TGFβ-1 levels were expressed as ng/L. TIMP1 levels were expressed as pg/mL. The results were adjusted to urine creatinine. Results of TGF-β1/Creatinine (/Cr), MMP2/Cr, MMP9/Cr and TIMP2/Cr were expressed as ng/mg, and TIMP1/Cr was expressed as pg/mg. The same measurements were repeated after one year to determine whether urine levels of these markers alterated in diabetic children with time.

Statistical analysis

Statistical calculations were performed with IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp. Besides standard descriptive statistical calculations (mean, standard deviation, median, and IQR), T-test was employed in the comparison of two groups and in the assessment of first and second year's values. Kruskal-Wallis test was used to compare subgroups. Pearson correlation test was used in the correlations between variables. Statistical significance level was established at $p<0.05$.

Results

Mean age was 11.73 ± 3.82 (4.5–17.8) years in the T1DM group and 11.6 ± 3.0 in the control groups. There was no statistical difference between the two groups regarding age and gender distribution ($p > 0.05$). The mean follow-up duration was 40.6 ± 25.5 (6.4–93.9) months. All patients were on intensive insulin treatment. The mean BMI of the patients was 19.32 ± 3.49 (13.72–26.65). Mean SDS of BMI was 0.08±1.01 (-1.15-2.32). Normal blood pressure was observed in all patients. The mean estimated GFR was 157.46 ± 34.61 mL/min/1.73 m2 (107.25–303.32). Mean HbA1c was 9.11 ± 2.17 % (5.7–15.5). Mean uMA/Cr was 20.17 ± 47.51 (1.28–239.41) mg/g Cr. Microalbuminuria was present in only 3 patients.

Urine MMP2/Cr, MMP9/Cr, TIMP1/Cr, TIMP2/Cr, TGF-β1/Cr were not different between the patient and control groups ($p>0.05$) (Table 2). There was no significant difference between the results of first and second year according to these biomarkers ($p>0.05$) (Table 2). All these parameters were not correlated to age, HbA1c, BMI and duration of T1DM. Interestingly, all parameters were negatively correlated to the age of onset of T1DM ($p<0.05$) (Table 3). Positive correlation was found between all of the parameters to each other ($p<0.05$).

The patients were divided into 2 subgroups as 0-5 years (n=19) and over 5 (n=14) years according to the diabetes duration. There was no difference between the two groups according to urine MMP2/Cr, MMP9/Cr, TIMP1/Cr, TIMP2/Cr, TGF-β1/Cr (Table 4). Also, the patients were divided into three groups as good (n=8), moderate (n=14) and poor glycemic control (n=11) based on HbA1c levels (Table 5). These urine biomarkers were not different between the good, moderate or poor glycemic control (Table 5).

Discussion

Since ECM changes are the major factor of DN, we hypothesized that the alteration of urine MMP2, MMP9 and TIMP1, TIMP2 may begin before microalbuminuria. Also, we expected this alteration to become more prominent with time because kidney injury of DN is a progressive process. In this study, our results did not support our hypothesis. Urine MMP2/Cr,

MMP9/Cr, TIMP1/Cr, TIMP2/Cr were not different between patients and controls, also they did not change in one year. It seems that chronic changes in DN do not begin in early stages of the disease.

The role of MMPs in the pathogenesis of DN is not fully understood. Although it has been demonstrated that dysregulation of MMPs in DN, the results in literature are contradictory (8). Decreased expression of MMP2 and MMP9 was reported several experimental studies on DN, while other studies reported increased expression of MMPs (9,16,17). Additionally, it has been notified that MMP2 knock-out mice have exacerbation of DN, however MMP9 knock-out mice have attenuation of DN (18, 19). Expression of TIMP1 and TIPM2 are increases in DN (8,9,20,21).

There are only a few studies evaluated urinary MMPs in patients with diabetes. McKittrick et al (22) evaluated urine activities of MMP2 and MMP9 in patients with T1DM and they found that urinary MMP9 did not differ between the patients and controls supporting our results. Activities of MMP2 increased in the patients group unlike our study (22). Lahio et al (23) demonstrated elevation of urinary activity of MMP9 in adult patients with type 2 DM. However, their study group is quite different from our group. Most of their patients had macroalbuminuria and had diabetes duration longer than 10 years. Tashiro et al (24) evaluated urinary MMP9 in adult patients with type 2 DM. They did not find any differences in urinary MMP9 between normo/microalbuminuric patients and healthy controls similar to our results, however urinary MMP9 was higher in macroalbuminuric patients. J.van der Zijl et al (25) evaluated urinary MMP2 and MMP9 levels in adult patients with type 2 DM. Urinary MMP9 levels were higher in microalbuminuric group than in the controls yet there was no difference according to MMP2 activity. Elevation of urinary MMP9 activities were found to be related to older age, longer duration of diabetes, high levels of HbA1c, increased blood pressure. Thrailkill et al (26) evaluated MMP2 in patients of T1DM and they found that MMP2 increased in the plasma and urine although they did not find any differences between patients and controls according to TIMP1 and TIMP2. Supporting our results, when they evaluated the younger groups (<18) and compared amongst themselves, they did not find any differences according to urine MMP2/Cr and total urine MMP2 concentrations (26). Thrailkill et al (27) found that elevation of urinary MMP9 in normoalbuminuric patients with T1DM. The diabetes duration of the patients was 9 years which was longer than our study group. These studies suggest that the role of the clinical use of urinary MMPs is not fully understood. These differences between the studies may be due to the fact that patient groups are quite different to each other as well as the evaluation method of urine MMPs. Diabetes duration has a significant role on the alteration of urinary MMP2 and MMP9 in diabetic patients according to these studies. Also it seems this alteration becomes more prominent in the later stage of DN. The mean duration of diabetes was only 3.5 years in our patients. ECM accumulation and mesangial expansion began at second stage of DN (11). Also, our patients did not have microalbuminuria except three. We could not demonstrate difference according to these biomarkers probably due to diabetes duration of our patients have not yet reached the second and/or later stage of DN. Based on a few previous studies which demonstrated higher values of urine MMP2/Cr and MMP9/Cr in adult diabetic patients, we thought that these markers may increase with time as diabetic injury progresses (24-27). However, we could not find differences between first year and second year regarding urine MMP2/Cr, MMP9/Cr, TIMP/Cr and TIMP2/Cr. These results show that urine levels of these markers did not change early phases of DN and cannot predict early progression of DN since they did not change with one year. Some comorbid conditions may affect urine MMP2 and MMP9, TIMP1 and TIMP2, TGF-β1levels such as renal scar, nephrotic syndrome, focal segmental glomerulosclerosis, pancreatic cancer, chronic kidney failure other than diabetes mellitus (28-33). However, our patients did not have any known comorbid disorders except diabetes mellitus.

It has been considered TGF-β1 is the most important cytokine in glomerular and tubulointerstitial fibrosis (34). Additionally, expression of TGF-β1 is increased with hyperglysemia, thus TGF-β1 is involved in the DN pathogenesis with various pathways (34). Furthermore, MMPs not only cleave ECM proteins but also target some non-ECM proteins including TGF-β1 and activation of TGF-β/Smad signal pathway is accompanied by the MMP2 and MMP9 upregulation (9,10). Therefore, we evaluated urinary TGF-β1 besides the urinary MMPs in our patients. TGF-β1 also was not increased in our patients. In fact, this result was consistent with our results for MMP2 and MMP9. These results may suggest that chronic fibrotic changes may not become apparent in our patients and these markers do not increase in urine in the early phases of diabetic kidney injury. Poor metabolic control, higher BMI, duration of disease and onset of diabetes at puberty have been identified as risk factors for DN. Therefore, we evaluated correlation of these biomarkers and HbA1c, BMI, duration of T1DM and age of onset of T1DM. Only age of onset was negatively correlated to all these biomarker of renal fibrosis. This finding suggest that among the poor prognosis indicators of T1DM, the most important determinant seems to be the age of onset of the disease regarding renal damage.

The limitations of our study are the relatively small sample size, that we have only three microalbuminuric patients. Thus, we could not compare microalbuminuric and normoalbuminuric patients according to these markers. Also we did not perform the kidney biopsy therefore we did not demonstrate the pathological DN stage of our patients. Despite these limitations our study has important results. In fact, there were no differences between patients and controls according to these biomarkers and they did not change after one year of follow-up. These findings weaken the role of these biomarkers to detect early diabetic kidney injury. In this respect, future studies with longer follow-up and large population are needed to highlighting this issue.

In conclusion, our findings suggest that urinary biomarker of fibrosis have not increased in diabetic children without microalbuminuria yet. As the age of onset of T1DM decreases, the risk of early fibrosis may increase according to our results.

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Authorship Contribution: ***Concept:*** Zeynep Yuruk Yildirim, Alev Yilmaz, Sevinc Emre, Ahmet Nayir. ***Design:*** Zeynep Yuruk Yildirim, Alev Yilmaz, Sevinc Emre, Ahmet Nayir ***Data Collection or Processing***: Zeynep Yuruk Yildirim, Alev Yilmaz, Cemile Pehlivanoglu, Feyza Darendeliler, Ruveyde Bundak, Asuman Gedikbasi. ***Analysis or Interpretation:*** Zeynep Yuruk Yildirim, Ahmet Nayir, Alev Yilmaz, Cemile Pehlivanoglu, Mehmet Yildiz, Asuman Gedikbasi, Feyza Darendeliler,

Ruveyde Bundak, Ahmet Dirican. ***Literature Search:*** Zeynep Yuruk Yildirim, Alev Yılmaz, Mehmet Yildiz. ***Writing:*** Zeynep Yuruk Yildirim, Alev Yilmaz, Mehmet Yildiz, Feyza Darendeliler, Ruveyde Bundak, Ahmet Nayir.

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Table 1. Patients' characteristic

	Mean±SD (Min-Max)
Age (years)	11.73±3.82 (4.5-17.8)
Gender (Female/Male)	15/18
DM duration (months)	40.60±25.5 (6.4-93.9)
HbA1c (%)	9.11±2.17 (5.7-15.5)
Microalbuminuria (mg/g creatinine)	20.17±47.51 (1.28-239.41)
Body mass index	19.32±3.49 (13.72-26.65)
Standard deviation score of body mass index	0.08±1.01 (-1.15-2.32)
Estimated glomerular filtration rate (ml/min/1.73m^2)	157.46±34.61 (107.25-303.32)
	n
Gender (Female/Male)	15/18
Pubertal Status at first year	
Pubertal/prepubertal	22/11
The status of metabolic control	
Good glycemic control (HbA1c: 6.5-7.5%)	8
Moderate glycemic control (HbA1c: 7.5-9%)	14
Poor glycemic control (HbA1c: >9%)	11

Table 2: Urinary biomarkers of patients and control groups in first and second year

	Control Group (Mean±SD)	T1DM Group First year (Mean±SD)	T1DM Group Second year (Mean±SD)	Controls vs T1DM First year p	Controls vs T1DM Second year p	T1DM First vs second year p
MMP2/Cr ng/mg	0.403± 0.321	0.737± 1.125	0.539±0.367	0.123	0.152	0.250
MMP9/Cr ng/mg	1.386±1.041	2.418±3.698	1.911±1.317	0.147	0.110	0.340
TIMP1/Cr pg/mg	0.286±0.237	0.478±0.688	0.359±0.252	0.182	0.276	0.245
TIPMP2/Cr ng/mg	0.035±0.029	0.066±0.097	0.072±0.132	0.098	0.167	0.872
TGF-B/Cr ng/mg	0.795±0.608	1.145±1.705	0.893±0.628	0.324	0.561	0.309

MMP2/Cr: Matrix metalloproteinase 2/Creatinine, **MMP9/Cr**: Matrix metalloproteinase 9/Creatinine, **TIMP1/Cr:** Tissue inhibitor of metalloproteinase1/Creatinine, **TIMP2/Cr:** Tissue inhibitor of metalloproteinase 2/Creatinine, **TGF-B/Cr:** Transforming growth factor βeta-1/Creatinine, **T1DM**: Type 1 Diabetes Mellitus

Table 3: Correlations of Urine Matrix Metalloproteinase/Creatinine and Tissue Inhibitor of Metalloproteinases/Creatinine with age of onset Diabetes and HbA1c and BMI and Diabetes Duration.

		Age of onset of Diabetes	HbA1C	BMI	Diabetes Duratation
	r	-0.461	-0.063	0.219	0.199
MMP2/Cr (pg/mg)	**p**	**0.012**	0.749	0.254	0.300
	r	-0.461	-0.043	0.245	0.222
MMP9/Cr (pg/mg)	**p**	**0.012**	0.826	0.199	0.246
	r	-0.484	-0.076	0.214	0.205
TIMP1/Cr (pg/mg)	**p**	**0.008**	0.699	0.266	0.287
	r	-0.422	-0.070	0.262	0.211
TIPMP2/Cr (pg/mg)	**p**	**0.023**	0.724	0.170	0.272
	r	-0.462	-0.025	0.217	0.199
TGF-B/Cr (pg/mg)	**p**	**0.012**	0.898	0.258	0.301

MMP2/Cr: Matrix metalloproteinase 2/Creatinine, **MMP9/Cr**: Matrix metalloproteinase 9/Creatinine, **TIMP1/Cr:** Tissue inhibitor of metalloproteinase 1/Creatinine, **TIMP2/Cr:** Tissue inhibitor of metalloproteinase 2/Creatinine, **TGF-B/Cr:** Transforming growth factor βeta-1/Creatinine, **HbA1c:** Hemoglobine A1c **, BMI:** Body Mass Index

.

Table 4- The relationships between urine biomarkers and diabetes duration

	Control Group (n=24) (Median)	**Diabetes duration 0-5 Years (n=19) (Median)**	**Diabetes duration >5 Years (n=14) (Median)**	**p**
MMP2/Cr (pg/mg)	0.34	0.39	0.40	0.193
MMP9/Cr (pg/mg)	1.12	1.17	1.45	0.147
TIMP1/Cr (pg/mg)	0.21	0.28	0.28	0.187
TIPMP2/Cr (pg/mg)	0.03	0.04	0.03	0.120
TGF-B/Cr (pg/mg)	0.63	0.61	0.71	0.315

MMP2/Cr: Matrix metalloproteinase 2/Creatinine, **MMP9/Cr**: Matrix metalloproteinase 9/Creatinine, **TIMP1/Cr:** Tissue inhibitor of metalloproteinase 1/Creatinine, **TIMP2/Cr:** Tissue inhibitor of metalloproteinase 2/Creatinine, **TGF-B/Cr:** Transforming growth factor βeta-1/Creatinine

Table 5- The relationships between urine biomarkers and diabetes duration

	Control Group (n=24) (Median)	**Good glycemic control HbA1c: 6.5-7.5% (n=8) (Median)**	**Modarate glycemic control HbA1c: 7.5-9% (n=14) (Median)**	**Poor glycemic control HbA1c: >9% (n=11) (Median)**	**p**
MMP2/Cr (pg/mg)	0.34	0.52	0.43	0.35	0.319
MMP9/Cr (pg/mg)	1.12	1.53	1.43	1.33	0.531
TIMP1/Cr (pg/mg)	0.21	0.34	0.31	0.25	0.458
TIPMP2/Cr (pg/mg)	0.03	0.04	0.04	0.03	0.275
TGF-B/Cr (pg/mg)	0.63	0.75	0.65	0.62	0.934

MMP2/Cr: Matrix metalloproteinase 2/Creatinine, **MMP9/Cr**: Matrix metalloproteinase 9/Creatinine, **TIMP1/Cr:** Tissue inhibitor of metalloproteinase 1/Creatinine, **TIMP2/Cr:** Tissue inhibitor of metalloproteinase 2/Creatinine, **TGF-B/Cr:** Transforming growth factor βeta-1/Creatinine