

# Serum Matrix Metalloproteinase-9, Tissue Inhibitor of Metalloproteinase-1 and Matrix Metalloproteinase-9/Neutrophil Gelatinase-associated Lipocalin Complex Levels in Patients with Early-stage Diabetic Nephropathy

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**Keywords.** diabetic nephropathy, matrix metalloproteinase-9, tissue inhibitor-1, MMP-9/NGAL complex, extracellular matrix

**Introduction.** This study was aimed to investigate the efficacy of matrix metalloproteinase-9 (MMP-9), tissue inhibitor of metalloproteinase-1 (TIMP-1), and the neutrophil gelatinase-associated lipocalin (NGAL)-bound form of MMP-9 (MMP-9/NGAL complex) markers in the determination of early nephropathy in patients with type 2 diabetes mellitus.

**Materials and Methods.** Twenty-five type 2 diabetic patients with normoalbuminuria, 27 type 2 diabetic patients with microalbuminuria, and 23 healthy controls were recruited. Serum levels of MMP-9, TIMP-1, and MMP9/NGAL complex were measured in all participants.

**Results.** The MMP-9 level and MMP-9/TIMP-1 ratio were higher in patients with microalbuminuria when compared to the controls, while TIMP-1 level was lower ( $P = .005$ ,  $P = .006$ , and  $P = .02$ , respectively). The MMP-9 level and MMP-9/TIMP-1 ratio were significantly higher in the patients with normoalbuminuria when compared to the controls ( $P = .005$  and  $P = .02$ , respectively). In the normoalbuminuric patients, MMP-9 levels were negatively correlated with estimated GFR ( $r = -0.553$ ,  $P = .008$ ) and positively correlated with glucose ( $r = 0.449$ ,  $P = .04$ ), creatinine ( $r = 0.454$ ,  $P = .03$ ), and MMP9/NGAL complex ( $r = 0.575$ ,  $P = .005$ ). In the microalbuminuric patients, MMP-9 levels were positively correlated with total cholesterol ( $r = 0.430$ ,  $P = .03$ ), and MMP9/NGAL complex ( $r = 0.650$ ,  $P = .001$ ).

**Conclusions.** The levels of MMP-9, TIMP-1, and MMP9/NGAL complex were similar in microalbuminuric and normoalbuminuric patients with type 2 diabetes mellitus. However, there was a significant MMP-9/TIMP-1 imbalance in both groups which may reflect impaired extracellular matrix homeostasis.

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## INTRODUCTION

Diabetic nephropathy (DN) is one of the most important microvascular complications related to diabetes mellitus (DM), the prevalence of which is increasing around the world.<sup>1</sup> Structural

abnormalities, such as changes in the composition and thickness of the glomerular basal membrane, abnormal extracellular matrix (ECM) depositions, mesangial enlargements, glomerulosclerosis, tubulointerstitial atrophy and fibrosis are seen

in DN.<sup>2-4</sup>

Matrix metalloproteinases (MMPs), known also as the zinc-dependent endopeptidase family, and their tissue inhibitors (TIMPs) provide homeostasis between the synthesis and degradation of the ECM to preserve the structure and functional integrity of the glomerulus.<sup>5</sup> In particular, MMP-9 and its inhibitor, TIMP-1, can play an important role in the development of DN.<sup>6,7</sup>

In previous studies, neutrophil gelatinase-associated lipocalin (NGAL) has been reported to be associated with such traditional kidney function markers as serum creatinine, cystatin C, and estimated glomerular filtration rate (GFR), and it has been identified as a potential biological marker for the determination of early-stage nephropathy.<sup>8,9</sup> Neutrophil gelatinase-associated lipocalin can form the MMP-9/NGAL complex by binding to MMP-9, and so may ensure the maintenance of MMP-9 activity by protecting MMP-9 from degradation to a certain degree.<sup>10</sup>

To the best of our knowledge, there have been no studies to date evaluating MMP-9/NGAL complex levels in DN. We aimed in this study to investigate the efficacy of MMP-9, TIMP-1, and MMP-9/NGAL complex markers in the early determination of nephropathy in patients with type 2 DM.

## MATERIALS AND METHODS

### Participants

This cross-sectional study was conducted in the Department of Internal Medicine of Mustafa Kemal University Hospital in Turkey between March 2016 and August 2016. Participants in the study were 52 patients with type 2 DM and 23 healthy individuals who applied to the hospital for a checkup with no specific clinical or laboratory problems. Diabetic patients were categorized according to their urine albumin-creatinine ratio (UACR) as normoalbuminuric (UACR < 30 mg/g; n = 25) or microalbuminuric (UACR, 30 mg/g to 300 mg/g; n = 27).<sup>11</sup> Patients with nondiabetic kidney disease, cancer, infection, other endocrine diseases, thyroid disorder, and liver and heart diseases were excluded from the study. Approval of the local ethics committee of Mustafa Kemal University was obtained for the study.

### Measurements

Detailed clinical data of the study population,

including age, sex, duration of DM, blood pressure levels, and cigarette smoking habits, were recorded. Hypertension was defined as blood pressure levels of 140/90 mm Hg and higher or the use of antihypertensive drugs by the individual. The Modification of Diet in Renal Disease equation was used to estimate GFR.<sup>12</sup>

Fasting venous blood and urine samples were collected from the participants, and the collected specimens were centrifuged for 10 minutes at 2000 g after a waiting period of 30 minutes. The separated sera were then stored at -80°C until they were tested for MMP-9, TIMP-1, and MMP9/NGAL complex levels.

Glucose, creatinine, lipid profile, C-reactive protein, and urinary albumin and creatinine levels were analyzed using an Architect c8000 analyzer (Abbott Laboratories, Lake Bluff, IL, USA) with classical methods. Glycated hemoglobin levels were detected with ion-exchange high performance liquid chromatography using a Bio-Rad Variant II analyzer (Bio-Rad, Hercules, CA, USA).

Serum MMP-9 and TIMP-1 levels were detected using a human enzyme-linked immunosorbent assay kit (RayBiotech, Inc), and the inter-assay and intra-assay coefficient of variation for both were less than 12% and 10%, respectively. The serum MMP9/NGAL complex was detected using a human enzyme-linked immunosorbent assay kit (Cusabio Inc), and the inter-assay and intra-assay coefficient of variation for the MMP9/NGAL complex were less than 10% and 8%, respectively.

### Statistical Analyses

The SPSS software (Statistical Package for the Social Sciences, version 21.0, IBM Corp, New York, NY, USA) was used for the statistical evaluation of the data. The normal distribution of variables was evaluated using a Kolmogorov-Smirnov test. Categorical variables were compared with the chi-square test and continuous variables were compared with the Mann-Whitney U test. A Spearman test was used for the correlation analysis. The study data was expressed as median (minimum to maximum) and number (percentage). A *P* value less than .05 was considered significant.

## RESULTS

This study was performed on 52 type 2 diabetic patients (25 normoalbuminuric and

27 microalbuminuric) and 23 healthy controls. The demographic characteristics and laboratory results of all groups are presented in Table 1. No significant differences were identified in terms of age, sex, cigarette smoking, blood pressure levels, or the ratio of total cholesterol to high-density lipoprotein cholesterol.

The levels of glucose, glycated hemoglobin and C-reactive protein were significantly higher in the normoalbuminuric group than in the control group. Glucose, glycated hemoglobin, urea, creatinine and C-reactive protein levels were higher in the microalbuminuric group than in the control group, while estimated GFR was lower. Duration of DM, UACR, and creatinine levels were higher in the microalbuminuric patients than in the normoalbuminuric patients, while estimated

GFR was lower.

Serum levels of MMP-9, TIMP-1, MMP-9/NGAL complex, and MMP-9/TIMP-1 ratio are presented in Table 2. Serum MMP-9 level and MMP-9/TIMP-1 ratio were significantly higher in both normoalbuminuric and microalbuminuric group than in the control group, while no difference was found in those parameters between the two diabetic groups. The TIMP-1 level was lower only in microalbuminuric group than in the control group. The MMP9/NGAL complex levels were similar in the three groups.

Correlations between the MMP-9, TIMP-1, MMP9/NGAL complex levels, and MMP-9/TIMP ratio in diabetic groups and other parameters were also analyzed. In the normoalbuminuric group, MMP-9 levels were negatively correlated with

**Table 1.** Demographic Data and Laboratory Results of the Study Groups\*

Parameter	Control (n = 23)	Normoalbuminuria (n = 25)	Microalbuminuria (n = 27)	P†	P‡	P¶
Age, y	52 (40 to 63)	53 (40 to 65)	55 (42 to 69)	.27	.11	.49
Male sex	11 (47.8)	15 (60)	17 (60.7)	.40	.28	.83
Current smoker	3 (13)	4 (16)	4 (14.8)	.77	.86	.91
Systolic blood pressure, mm Hg	130 (110 to 140)	130 (110 to 160)	130 (90 to 180)	.38	.36	.82
Diastolic blood pressure, mm Hg	80 (70 to 90)	80 (60 to 110)	80 (60 to 100)	.36	.11	.51
Hypertension	...	11 (44)	16 (59.3)	...	...	.27
Duration of diabetes, y	...	4 (1 to 20)	10 (2 to 26)	...	...	.03
Urine albumin-creatinine ratio, mg/g	...	10.2 (5.3 to 26.4)	87.4 (32.8 to 282.2)	...	...	<.001
Glomerular filtration rate, mL/min/1.73 m <sup>2</sup>	101 (81 to 115)	100 (70 to 113)	90 (60 to 117)	.98	.048	.04
Glucose, mg/dL	95.1 (83 to 122.7)	162.6 (99.2 to 331.7)	159.4 (101.1 to 458)	<.001	<.001	.93
Glycated hemoglobin, %	5.7 (5 to 6.3)	8.8 (5.7 to 15)	10.5 (6.2 to 16.6)	<.001	<.001	.06
Urea, mg/dL	11.3 (5.9 to 18.8)	11.9 (6.9 to 30)	15.9 (6.9 to 29.9)	.57	.02	.07
Creatinine, mg/dL	0.72 (0.59 to 1.03)	0.73 (0.53 to 1)	0.81 (0.53 to 1.36)	.97	.02	.04
C-reactive protein, mg/L	3.3 (3.16 to 3.56)	3.3 (3.27 to 31.8)	3.9 (3.16 to 11.3)	.004	.001	.93
Total-high-density lipoprotein cholesterol ratio	5.13 (3.79 to 9.08)	5.42 (3.64 to 9.20)	5.48 (0.72 to 9.28)	.70	.29	.59

\*Values are mean (range) or frequency (percentage).

†Comparison between control and normoalbuminuria groups

‡Comparison between control and microalbuminuria groups

¶Comparison between normoalbuminuria and microalbuminuria groups

**Table 2.** Mean Marker Levels of the Study Groups\*

Marker	Control (n = 23)	Normoalbuminuria (n = 25)	Microalbuminuria (n = 27)	P†	P‡	P¶
MMP-9, pg/mL	2084 (502 to 5038)	3264 (1369 to 4927)	3111 (781 to 5676)	.005	.005	.65
TIMP-1, pg/mL	14980 (947 to 24610)	11840 (2520 to 21500)	10505 (1415 to 19640)	.36	.02	.17
MMP-9/TIMP-1	0.16 (0.03 to 0.31)	0.29 (0.10 to 0.86)	0.35 (0.06 to 1.12)	.02	.006	.40
MMP-9/NGAL complex, ng/mL	2.76 (0.36 to 19.53)	3.30 (0.92 to 17.92)	2.48 (0.34 to 13.73)	.63	.85	.44

\*MMP-9 indicates matrix metalloproteinase-9; TIMP-1, tissue inhibitors of metalloproteinase-1; and MMP-9/NGAL complex, matrix metalloproteinase-9 to neutrophil gelatinase-associated lipocalin complex.

†Comparison between control and normoalbuminuria groups

‡Comparison between control and microalbuminuria groups

¶Comparison between normoalbuminuria and microalbuminuria groups

estimated GFR ( $r = -0.553$ ,  $P = .008$ ) and positively correlated with glucose ( $r = 0.449$ ,  $P = .04$ ), creatinine ( $r = 0.454$ ,  $P = .03$ ), and MMP9/NGAL complex ( $r = 0.575$ ,  $P = .005$ ). In the microalbuminuric group, MMP-9 levels were positively correlated with total cholesterol ( $r = 0.430$ ,  $P = .03$ ) and MMP9/NGAL complex ( $r = 0.650$ ,  $P = .001$ ). A positive correlation was present between MMP-9/NGAL complex levels and systolic blood pressure ( $r = 0.454$ ,  $P = .02$ ). Positive correlations were found between the MMP-9/NGAL complex levels and the MMP-9 ( $r = 0.676$ ,  $P < .001$ ), systolic blood pressure ( $r = 0.406$ ,  $P = .004$ ), and diastolic blood pressure ( $r = 0.314$ ,  $P = .03$ ), considering all diabetic patients.

## DISCUSSION

The course of DN, as one of the most common complications in type 2 DM, involves the progressive accumulation of ECM in some components of the kidney.<sup>13</sup> Matrix metalloproteinases are the proteolytic enzymes that are responsible for protein degradation/turnover in the ECM, and so abnormalities in MMP expression or activity are important elements in the development and progression of DN.<sup>5</sup> Tissue inhibitor of metalloproteinases play a critical role in the control of MMPs activity, and their association may play a key role in the remodeling of ECM.<sup>14</sup>

Prolonged hyperglycemia has been observed to be effective on the expression and activity of MMPs.<sup>15,16</sup> Hyperglycemia may induce formation of advanced glycation end products, reactive oxygen species, and transforming growth factor  $\beta$ , as well as the activation of such transcription factors as nuclear factor-kappa B and activator protein-1, which result in the dysregulation of MMPs expression, leading to the progress of DN.<sup>17</sup>

Plasma MMP-9 and TIMP-1 levels in patients with type 2 DM have been reported to be higher than the control group in several studies.<sup>18,19</sup> Li and colleagues<sup>6</sup> noted an increased production of MMP-9 through the stimulation of various cytokines in the kidneys of diabetic rats in their study. Increased levels of MMP-9 have been reported to play a role in the development of DN by changing the podocyte structure and function, as well as ECM composition. Increased urinary MMP-9 levels have been observed to be associated with the degree of albuminuria in patients with DN.<sup>20,21</sup> In a study by Rysz et al. and coworkers,<sup>7</sup> MMP-9/TIMP-1 ratio was found to be

higher and TIMP-1 level to be lower in patients with DN than in the control group. Similarly, we found that the MMP-9 levels and MMP-9/TIMP-1 ratios were higher in both normoalbuminuric and microalbuminuric patients than in the controls. In addition, a positive correlation was found between MMP-9 and glucose in the normoalbuminuric group. These results suggest that hyperglycemia may cause an MMP-9/TIMP-1 imbalance by inducing the upregulation of MMP-9. Changes in the balance of MMP-9/TIMP-1, which plays an important role in ECM homeostasis, results in the development of diabetic renal lesions and increased glomerular membrane permeability, thus leading to diabetic albuminuria.<sup>3</sup> Serum MMP-9 levels correlated with creatinine and estimated GFR that reflect kidney function in this study, and this supports the theory stated above.

Contrary to the above observations, some investigators found that MMP-9 levels were lower in type 2 diabetic patients than in the controls.<sup>22,23</sup> In another study performed on streptozotocin-induced diabetic rats, MMP-9 expression and activity were observed to be decreased.<sup>24</sup> Papazafiropoulou and colleagues<sup>25</sup> demonstrated that MMP-9 levels were similar in diabetic patients and control group, while TIMP-1 was found to be lower in diabetic patients. The results of the published studies that are reviewed above demonstrate that current data on this subject is contradictory, and these discrepancies may arise based on many factors, such as whether the diabetic patients included in the study had any comorbidities like hyperlipidemia, hypertension or cardiovascular disease, and also the effects of the medications they use. Medications, such as aspirin, calcium channel blockers, lipid-lowering statins, angiotensin-converting enzyme inhibitors, and angiotensin receptor blockers, have been reported to change the activity and level of MMP-9 in previous studies.<sup>26-29</sup> Another factor is that some variables, such as age, sex, exercise habits, and ethnic origin of the participants included in the study, may affect the MMP-9 or TIMP-1 levels.<sup>30,31</sup> Studies have reported biological variations of ECM, such as MMPs and TIMPs,<sup>32,33</sup> and so conflicting results may arise from the within-subject and between-subject biological variations. It is notable that differences in the methods used to determine the MMP-9 and TIMP-1 levels may also cause variations regarding the results.



Accepted as a marker of acute renal damage,<sup>34</sup> NGAL may protect MMP-9 from degradation and thus increase MMP-9 activity in the ECM when it forms a complex with MMP-9.<sup>10</sup> The MMP-9/NGAL complex level was similar in all three groups in this present study. The similarity between the groups might be due to the fact that a small percentage of MMP-9 takes part in the NGAL complex and the study population was small.<sup>10</sup>

This study has some limitations. First, our findings should be interpreted with caution, given the small number of subjects included in the study. Second, since the data related to the medications used by the diabetic patients was missing, the effects of those drugs on the MMP-9, TIMP-1 and MMP-9/NGAL complex could not be evaluated. Third, there was an absence of patients with overt DN (macroalbuminuria) in this study.

## CONCLUSIONS

A significant imbalance was observed in the MMP-9/TIMP-1 ratios of both the normoalbuminuric and microalbuminuric diabetic patients. Increased MMP-9 levels in diabetic patients suggest that an MMP-9 inhibitor may be a therapeutic target in this disease.

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## CONFLICT OF INTEREST

None declared.

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