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Study of MMP-2 and MMP-9 in Type 2 Diabetic Patients with and without Microalbuminuria

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ABSTRACT:

Diabetes nephropathy (DN) is characterized by accumulation of extracellular matrix (ECM) in the kidney. Matrix metalloproteinases (MMPs) are types of enzymes which are mostly involved in ECM homeostasis. Gelatinase A (MMP-2) and gelatinase B (MMP-9) are the most important MMPs in normal kidneys, so they may be studied as earlier and more specific markers for DN. This study aimed to evaluate the diagnostic value of MMP-2 and MMP-9 as new markers in type 2 diabetic patients with and without microalbuminuria (MA). Subjects were 77 classified into 3 main groups according to their albumin creatinine ratio (ACR in mg/g). Group1: control group: includes 20 healthy subjects. Group2: 30 Normoalbuminuric (NA) subjects (ACR < 30 mg/g). Group3: includes 27 subjects with MA (ACR 30-300 mg/g). MMP-2 and MMP-9 were increased in diabetic groups when they were compared with control group. MMP-2 and MMP-9 showed significant positive correlations with MA, ACR and fasting plasma glucose in both diabetic groups. The cutoff value for MMP-2 was 73.23ng/ml (59.3% sensitivity and 90% specificity); the cutoff value for MMP-9 was 31.65ng/ml (77.8% sensitivity and 55% specificity). MMP-9 and -2 were significantly increased in MA and NA diabetic patients than normal volunteers.

Keywords: *Diabetes nephropathy, Microalbuminuria, MMP-2, MMP-9.*

[I] INTRODUCTION

Diabetic kidney disease or DN is a well described complication of diabetes that results from glomerular (as well as tubular and interstitial) damage secondary to continued hyperglycemia [9]. Up to 30% of people with newly diagnosed T2DM will already have abnormally high urine albumin levels; about 75% of these people will have MA and about 25% will have overt DN [25]**.** MMPs are zinc-containing endopeptidases that are involved in remodeling the ECM and are crucial for tissue development and homeostasis [2]. A typical MMP consists of a propeptide of

about 80 amino acids, a catalytic MMP domain of about 170 amino acids, a linker peptide of variable lengths (also called the Fhinge region) and a hemopexin domain of about 200 amino acids [17]. Under normal physiological conditions, the proteolytic activity of the MMPs is controlled at any of the following three known stages: Gene expression (transcription), activation of the zymogens and inhibition of the active forms by various tissue inhibitors of MMPs (TIMPs). In pathological conditions this equilibrium is shifted toward increased MMPs activity leading to tissue degradation [27].

The gelatinases, MMP-2 and MMP-9, are critical for normal vascular development, functioning, and remodeling. These particular MMPs regulate inflammation, with MMP-2 having anti-inflammatory actions and MMP-9 thought to be proinflammatory [29]. MMP-2 is expressed in the collecting duct in the rabbit [19], the glomerulus and proximal tubules of rats [10], and in the proximal and distal tubules in the monkey [2]. The expression of MMP-9 appears to be mainly confined to the glomerulus [10]; although there are reports of expression in the rabbit collecting duct [19]. Gelatinase A (MMP-2) and gelatinase B (MMP-9) are the most important MMPs in normal kidneys and are therefore assumed to play major roles in basement membrane homeostasis [7].

[II] MATERIALS AND METHODS

2.1. Materials

Diabetic patients were recruited from the outpatient clinic of "National Institute for diabetes and Endocrinology" El -Kasr El-Einy Hospital, Cairo, Egypt. Diabetic patients were chosen according to criteria of ***WHO, 2006*** [28]. Control subjects were relatives of patients and some of them were technicians and coworkers in the Faculty of Pharmacy (girls), Al Azhar University. Subjects were classified into 3 main groups: Group1: Control subjects: included 20 (9 males, 11 females) healthy subjects with matched sex and socioeconomic state. Their age was ranged from (45-69 yrs), mean ±SE was (55±1.7). BMI was ranged from (24-29 Kg/m^2), mean ±SE was (26±0.48). They were with comparable age, sex, BMI range to the diabetic groups. They weren't suffering from any current or chronic disease and weren't receiving any regular medications. Group2: NA subjects: included 30 (14 males, 16 females) recently diagnosed diabetics (1-5 yrs) duration, mean ±SE was (2±0.19). Their age was ranged from (46-65 yrs), mean ±SE was (50±1.4). BMI was ranged from (25-29 Kg/m^2), mean ±SE was (26±0.5). Group3: MA subjects: included 27 (13 males, 14 females) diagnosed diabetics (5-10 yrs) duration mean ±SE was (6.8±0.3). Their age was ranged from (45-66 yrs) mean ±SE was (54±2). BMI was ranged from (25-29 Kg/m^2), mean ±SE was (26±0.64). Smokers, alcoholics, hypertensive and obese were excluded from the study. Diabetic patients weren't receiving any medications other than oral antidiabetic drugs. None of them were complaining of any acute or chronic illness or neoplastic disease as confirmed by history taking from them.

2.2. Methods

10 ml venous blood sample was withdrawn from each subject after an overnight fasting (≥12 hours). The venous blood sample was divided into three tubes. EDTA tube (whole blood to measure HbA1c), fluoride tube for FPG measurement and the remainders were allowed to clot at room temperature then centrifugated. Serum was separated and divided into three aliquots; two of them were stored at temperature ≤ -20□ C till the time of assay of MMP-2 and MMP-9 using ELISA technique. The other aliquot was used to measure the following parameters: lipid profile (total cholesterol (T-C), triacylglycerol (TAG), HDL-C and LDL-C, urea, creatinine using the Dimension® RxL Max® Integrated Chemistry System (DADE BEHRING instruments Inc, USA). Fresh morning urine

samples were collected from each subject for measurement of urinary creatinine, microalbumin and (ACR) using ARCHITECT c8000™ clinical chemistry system (Abbott, USA).

[III] RESULTS

Table (1) shows that the level of each of FPG, HbA1c, MMP-2 and MMP-9 was significantly increased in NA and MA diabetic groups when they were compared with control group (p value <0.05). Each of FPG and MMP-9 was significantly increased in MA diabetic patients in comparison with NA diabetics. There was a statistically significant difference between groups regarding the T-C, TAG and LDL-C. HDL-C was significantly deceased in diabetic groups in comparison with control. Concerning MA, urine creatinine and ACR, their levels were significantly increased in diabetic group with MA when they were compared with NA diabetic and control groups. Regarding serum creatinine and BUN, there wasn't a significant difference between groups in their levels.

Group / Variable	Control	NA	MA	P value
FPG (mg/dl)	96±5.3	200±13.2[a]	259±16.5 [a,b]	0.000
HbA1c (%)	5±0.29	8.5±0.35 [a]	9.9±0.45 a	0.000
MMP-2 (ng/ml)	65±1.5	74±1.4 [a]	81±2.6 a	0.000
MMP-9 (ng/ml)	35±3.6	50±2 [a]	60±2.5 [a,b]	0.000
T-C (mg/dl)	166±5.6	202±6.9 [a]	201±8 a	0.001
TAG (mg/dl)	96±9.2	158±13 [a]	196±29 [a,b]	0.010
LDL (mg/dl)	116±6.1	130±5.5 [a]	131±6 a	0.000
HDL (mg/dl)	47±2.3	37±0.85 [a]	38±1 a	0.031
Creatinine (mg/dl)	0.68±0.02	0.74±0.02	0.72±0.02	0.228
BUN (mg/dl)	10.6±0.51	12.3±0.52	11±0.71	0.515
MA (mg/l)	15±1.9	15.5±1.5	88±10.7 [a,b]	0.000
Urine creatinine (g/l)	105.7±9.1	116±11.9 [a]	162.5±11 [a,b]	0.003
ACR (mg/g)	10.9±0.92	12.5±0.93	110±13 [a,b]	0.000

Table: 1. Descriptive statistics and comparison between all groups of all laboratory tests.
(a: p<0.05 in comparison to control, b: p<0.05 in comparison to diabetic group without MA).

MMP-2 correlations with	NA diabetics		MA diabetics	
	Pearson coefficient (r)	P value	Pearson coefficient (r)	P value
FPG (mg/dl)	0.497	0.008	0.460	0.031
MA (mg/l)	0.449	0.019	0.496	0.016
ACR (mg/g)	0.422	0.045	0.480	0.011

Table: 2. Correlation coefficient between MMP-2 and each of FPG, MA and ACR in NA and MA diabetic groups.

MMP-9 correlations with	NA diabetics		MA diabetics	
	Pearson coefficient (r)	P value	Pearson coefficient (r)	P value
FPG (mg/dl)	0.473	0.026	0.514	0.041
MA (mg/l)	0.408	0.048	0.505	0.046
ACR (mg/g)	0.417	0.034	0.550	0.027

Table: 3. Correlation coefficient between MMP-9 and each of FPG, MA and ACR in NA and MA diabetic groups.

Variable	Cutoff value	Sensitivity	Specificity	PPV	NPV

MMP-2	>73.23	59.3%	90%	88.9%	62.1%
MMP-9	>31.65	77.8%	55%	70%	64.7%

Table: 4. The output data of ROC curves for MMP-2, MMP-9

Variables	ACR& MMP2	ACR& MMP9	MMP2& MMP9
Difference between areas	0.250	0.394	0.144
Standard error	0.071	0.083	0.106
95% Confidence interval	0.112 to 0.388	0.232 to 0.557	0.063 to 0.352
Significance level	P = 0.000	P = 0.000	P = 0.173

Table:5. Comparison between MMP-2, MMP-9 and ACR.

Table (2) represents the positive correlations between MMP-2 and each of FPG, MA and ACR in NA and MA diabetic groups. Table (3) shows the significant positive correlations between MMP-9 and each of FPG, MA and ACR in NA and MA diabetic groups. The output data for receiver operating characteristic (ROC) curves for MMP-2, MMP-9 are illustrated in table (4). Table (5) illustrates the differences between MMP-2, MMP-9 and ACR regarding the diagnostic performance of each of them.

[IV] DISCUSSION

The mean of age in control, NA and MA diabetic groups was (55±1.7, 50±1.4 and 54±2) respectively. The duration was (2±0.19 and 6.8±0.3) for NA and MA diabetic groups respectively. This is in agreement with ***Shet and his colleagues*** [22] who studied the level of MMP-9 in diabetic subjects with and without MA and they found that the mean age of the subjects with MA was (55.28±8.100 years) and the mean age in subjects without MA was (49.47±10.21) years. The mean duration of diabetes in subjects with MA was (8.81±5.61) years whereas the duration of diabetes in subjects without MA was (2.17±1.81) years which showed a significant long duration of diabetes in subjects with MA compared to subjects without MA.

There was a significant increase in FPG (P=0.000) and HbA1c (p=0.000) in type 2 diabetics with or without MA in comparison with control group. These results were in agreement with ***Kundu and his colleagues*** [13] who studied MA levels in type 2 diabetics and correlated changes in MA levels to HbA1c level and duration of diabetes and they found a significant increase in FPG and HbA1c in MA group more than control.

Glycated hemoglobin is a blood glucose control marker in diabetic patients. It results from posttranslational changes in the hemoglobin molecule, and its level correlates well with glycemic levels over the previous six to ten weeks. Higher levels of HbA1c were associated with increased risk for development of microangiopathy in diabetes. This may be due to the fact that HbA1c has special affinity for oxygen thereby causing tissue anoxia and plays a role in causation of micro and macroangiopathy [13].

The current study revealed a significant positive correlation between MMP-2 and -9 and FPG in both diabetic groups. Those findings were in compatible with ***Lewandowski and his colleagues*** [14] who found that there was a significant positive correlation between MMP-9 and HbA1c and they found a significant positive correlation between MMP-2 and glucose level.

There was a significant increase in the level of each of T-C (P = 0.001), TAG (P = 0.010) and LDL (P=0.000) in diabetic group with and without MA than control group, but HDL showed a significant decrease in its level in diabetics when they were compared to control (P=0.031). These findings were in agreement with ***Dwivedi and Sarkar*** [5] who studied the effect of oxidative stress on DN progression and they found that the level of each of T-C, TAG and LDL in DN group was significantly increased

than the control group. Also they found a significant decrease in HDL level in DN group in comparison with control.

Altered metabolism of TAG-rich lipoproteins is crucial in the pathophysiology of the atherogenic dyslipidemia of diabetes. Alterations include both increased hepatic secretion of VLDL and impaired clearance of VLDL and intestinally derived chylomicrons. Increased hepatic production and/or retarded clearance from plasma of large VLDL also results in increased production of precursors of small dense LDL particles. The reductions in HDL associated with T2DM and insulin resistance are due to many factors, but a major factor appears to be increased transfer of cholesterol from HDL to TAG-rich lipoproteins, with reciprocal transfer of TAG to HDL [12].

Microalbumin, urine creatinine and ACR were found to be higher in MA group when they were compared with NA and controls and found to be statistically significant (P=0.000, 0.003 and 0.000 respectively). These results were in agreement with ***Kundu and his colleagues*** [13]**.**

The current study revealed that there were significant positive correlations between MMP-2 and each of MA and ACR in both of diabetic groups. Also there were significant positive correlations between MMP-9 and each of MA and ACR in diabetic groups. This was in agreement with ***Chang and his colleagues*** [3] who concluded that MMP-2 and MMP-9 might contribute in the pathogenesis of CKD as there were correlations between MMP-2, -9 and serum creatinine in CKD patients. Another study found a positive correlation between MMP-2 and -9 and MA in CKD patients and their study suggested that there may be a connection between an inflammatory state, biochemical response and the MMP levels in uremic and dialysis patients [18].

It has been hypothesized that the early changes in glomerular basement membrane thickness and content ultimately affect its filtration properties, leading first to increased urinary albumin excretion and eventually to proteinuria [24].

The current study revealed that the levels of MMP-2 and MMP-9 were significantly increased in diabetic groups with and without MA with respect to control group. These findings were in compatible with ***Derosa and his colleagues*** [4] who measured MMP-2 and -9 in children and adolescents with T1DM with and without macroangiopathy and they found that their levels was significantly increased in diabetic groups than the control. ***Shet and his colleagues*** [22] studied the level of MMP-9 in diabetic subjects with and without MA and they found that its level was significantly increased in diabetic subjects in comparison with control.

In agreement with the current study, ***Signorelli and his colleagues*** [23] measured the plasma levels of MMP-2 and MMP-9 in T2DM with or without peripheral artery disease and in normal volunteers and they found that their levels were higher in type 2 diabetics with or without peripheral artery disease in comparison with normal volunteers. In addition, ***Gharagozlian and his colleagues*** [7] investigated the serum level of MMP-2 and MMP-9 in type 1 diabetics with nephropathy and there level were significantly higher than that in normal volunteers. In agreement with the current study, ***Caseiro and his colleagues*** [1] studied the proteolytic events underlying T1DM and related complication through protease profiling in the body fluids serum, urine and saliva and they found that only MMP-2 and MMP-9 were observed in serum with significantly increased levels and activity observed in diabetic patients.

The current study results is supported by ***Rysz and his colleagues*** [21] as they measured the serum level of MMP-2, MMP-9, TIMP-2 and TIMP-1 in patients with T2DM, DN and healthy controls and there was an approximately 2-fold increase of MMP-2/TIMP-2 and MMP-9/TIMP-1 ratio was found in DN patients when they were compared with diabetes with normal renal

function. ***van der Zijl and his colleagues*** [26] determined MMP-2, -8 and -9 activity in 24-h urine collections in relation to risk factors for DN in T2DM patients with and without albuminuria and controls and they found that their levels were the highest in albuminuria patients.

In contrast to the current study, ***Lewandowski and his colleagues*** [15] concluded that MMP-2 and MMP-9 concentrations were lower in subjects with T2DM than in non-diabetic controls. These results can be explained by a another study which revealed that high glucose concentrations have been shown to act on mesangial cells directly to decrease the activities of MMPs, the group of enzymes responsible for mesangium matrix degradation [16]. ***Lewandowski and his colleagues*** [15] stated that their data support a possibility that prolonged hyperglycaemia might be related to increased activity of some MMPs, given the positive correlation between serum MMP-9 concentrations and HbA1c. In addition, this hypothesis was supported by their findings that there was a decline in serum MMP-9 concentrations at the time of discharge from hospital (when glycaemic control was better) as well as by a significant decrease in MMP-9 at three months post-discharge, accompanied by a fall of HbA1c.

A pivotal study using a rodent model of hereditary kidney disease demonstrated that genetic ablation of any one of three key MMPs (MMP-2, MMP-3, or MMP-9) involved in the progression of renal dysfunction led to the compensatory upregulation of other MMPs, suggesting some redundancy and compensation within this family of proteinases. This study also demonstrated that the preservation of GBM or ECM integrity before the onset of proteinuria lead to disease protection, whereas MMP-inhibition in later stages of disease resulted in accelerated glomerular and interstitial fibrosis [30].

The hypertrophy of the glomerulus and increased volume of the kidney is observed in the early stage of the DN. Long duration of this glucose metabolism disturbance results in a characteristic progressive thickening of basement membrane with increased mesangial volume. Increased activity of MMPs has been already observed in the early phase of DN without features of structural damages of kidneys. Proteolytic enzymes secreted by neutrophils digest proteins and glycosaminoglycans of the basement membrane of glomeruli [20].

Diabetic nephropathy is characterized by accumulation of ECM in the kidney. Glomerular mesangial expansion and tubulo-interstitial fibrosis eventually leads to renal failure [14].

In order to evaluate the diagnostic performance of MMP-2 and -9 and study the sensitivity and specificity of each of them ROC curves were carried out for each of them and we found that MMP-2 (specificity =90%) is more specific than MMP-9 (specificity =55%) in detection of MA, but MMP-9 is more sensitive than MMP-2 (sensitivity was 77.8% and 59.3% respectively). The cutoff points of MMP-2 and MMP-9 were (> 73.23 ng/ml and > 31.65 ng/ml) respectively. The post ANOVA (Tukey) test was performed for MMP-2 and -9 to investigate the difference between their levels between each two group. Regarding MMP-9 there was a significant increase in its level in MA group when they were compared to NA and control group. In contrast, there was a non significant increase in MMP-2 level in MA group when they were compared to NA group, but there is significant increase in MMP-2 level in both of NA and MA groups when they were compared with control group.

This data suggests that MMP-9 is more sensitive and earlier marker than MMP-2 in differentiation of DN. These suggestions are supported by the ROC curve results which showed that MMP-9 was more sensitive and increase earlier than MMP-2. These findings were consistent with ***Ebihara and his colleagues*** [6] who found that

MMP-9 increase in the urine earlier than albumin in type 2 diabetics.

One of the early pathophysiological steps in DN is the deposition of type IV collagen in the basal membrane. Since MMP-2 and -9 are involved in the degradation of type IV collagen, alterations in their expression or activity might reflect early renal damage in diabetics, even before the evidence of clinical and laboratory changes [8].

Hyperglycemia-mediated alterations in MMPs secretion or activation contribute to the pathophysiology of other diabetic co-morbidities. The impact and contribution of MMPs to the onset and progression of DN may be most critical in the earlier phases of the disease process, at a time in which enhanced matrix turnover, release of pro-fibrotic growth factors and altered cell motility may damage the glomerular apparatus and tubular architecture. It was stated that several MMPs have been shown to play a role in renal pathology, enlargement of the kidney mesangium due to ECM over-accumulation is a major characteristic of DN, preceding the onset of albuminuria. If similar mechanisms are involved in progression of DN, then very early intervention (prior to the onset of MA) or even prophylaxis with anti-MMPs therapies in genetically and metabolically susceptible individuals with diabetes could prevent or significantly retard the development of DN in at-risk individuals [24].

[V] CONCLUSION

MMP-2 and -9 were significantly increased in MA and NA diabetic patients than normal volunteers, so they may be considered as diagnostic markers for DN. MMP-2 and -9 are involved in regulation of ECM turnover especially in kidneys and there increase in diabetic groups suggests that deregulation of ECM which may be induced by hyperglycemia could be considered as a hypothesis of how DN happens. MMP-9 is more sensitive and increase earlier, but less specific than MMP-2 in detection of DN.

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