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URINARY NEPHRIN AND URINARY MCP-1 BIOMARKERS PREDICTS THE PROGRESSION OF DIABETIC NEPHROPATHY IN TYPE 2 DIABETIC PATIENT

Dalia Mahmood Khalaf [1], Refif Sabih Jamal Al-Shawk[2], Isam Noori Salman Al-Karawi[3]
Department of Microbiology/ College of Medicine / Al-Mustansiriya University [1 2]
Consultant Physician/ Diabetes Center/ Al-Mustansiriya University [3]
*Corresponding author's email: dalymahmood@yahoo.com

ABSTRACT
Diabetic nephropathy (DN) is the leading cause of chronic renal disease. It is associated with increased cardiovascular mortality. The diabetic nephropathy has been defined as increased protein excretion in urine. Early stage is characterized by a small rise in urinary albumin excretion (UAE), also called microalbuminuria. More advanced disease is defined by the presence of macroalbuminuria. The latter is named overt diabetic nephropathy. The role of urinary Nephrin and urinary monocyte chemoatractant protein MCP-1 in the onset and progression of diabetic nephropathy. Sixty Iraqi type 2 diabetes mellitus T2DM patients and 20 control subjects matched for age, gender and ethnic background. The patients and controls were characterized in family history of diabetes, diabetic nephropathy groups divide according to A: C ratio. The patients were also assessed for duration of disease, fasting serum glucose, serum creatinine and blood urea. Both urinary Nephrin and MCP-1 levels were measured by enzyme linked immunosorbant assay the sandwich method. The mean urinary level of MCP-1 was (15.381 $\pm$ 6.105 pg/ml) and Nephrin (100.017 $\pm$ 52.230 ng/ml) were significantly higher in T2DM patients as compared to controls. The mean levels of uMCP-1 and Nephrin in macroalbuminurea group of patients were significantly higher than those in normoalbuminurea (p <0.008 and p<0.030) respectively, and only of the Nephrin in the normoalbuminurea group of patients showed significant increase in level as compared to the controls (52.230±19.619 *vs.*75.050±37.802 ng/ml) (p<0.022). For early diagnosis and detection of DN revealed that the cut-off value of uMCP-1 was 6.23 pg/mg with 70% sensitivity and 75% specificity; whereas, Nephrin was 49.5 ng/ml with 78% sensitivity and 55% specificity. The linear correlation revealed a significant positive correlation between urinary MCP-1, Nephrin and A: C ratio, also o showed a significant negative linear correlation between urinary MCP-1 and glumerular filtration rate. Nephrin may be considered as potential predictor and prognostic biomarkers for the early detection and progression of diabetic nephropathy while the uMCP-1 may be considered as potential prognostic biomarkers for the diabetic nephropathy.

KEYWORDS: diabetic nephropathy, Monocyte chemoattractant protein-1, Nephrin.

INTRODUCTION

Diabetic nephropathy is one of the most important long-term complications regarding morbidity and mortality in diabetic patient. The clinical syndrome of this disease is characterized by continual albuminuria, developmental reduction in the glomerular filtration rate (GFR) and increased arterial blood pressure[1]. Characterizes the most common cause of end stage renal disease (ESRD) worldwide, and patients with DN are at a greater risk of mortality, mostly from cardiovascular complications, than other patients with diabetes [2]. Nephrin forms an integral part of podocytes, which together with endothelial cells and the basement—form the glomerular filtration barrier [3]. Podocytes can become injured in human and experimental glomerular diseases and conditions that cause podocyte injuries; these are collectively known as podocytopathies [4]. diabetic nephropathy, is one of the disease that may induse podocyte damage and dysfunction [5]. Chronic low-grade inflammation and elevated oxidative stress, have been considered to play a vital role in the pathogenesis and progression of DN, suggesting that microinflammation is a common mechanism in the development of diabetic vascular complications. Expression of cell adhesion molecules, chemokines and pro- inflammatory cytokines are increased in the renal tissues of diabetic patients and animals [6]. The renal expression of Nephrin might be impaired in diabetic nephropathy. Patients with diabetic nephropathy have markedly reduced renal Nephrin expression and smaller amount electron-dense slit diaphragms compared with patients without diabetes and minimal nephropathic changes or controls [7]. Furthermore, Nephrin excretion is elevated 17-30% in patients with diabetes (with and without albuminuria) compared with that in individuals without diabetes. Thus, Nephrin excretion could be an early finding of podocyte injury, even before the onset of albuminuria [8].

The MCP-1 Is a member of CC class of chemokine which binds to CCR_2, a chemokine receptor. The CCL_2 is thought to play a key role in recruitment of monocytes into different renal compartments. It is secreted by mononuclear and various non-leukocytic cells including renal resident cells [9]. In patients with DN, urinary CCL_2 levels were significantly raised at different stages of DN, and were correlated with the number of infiltrating

macrophages in the interstitium[10,11] and linked to a pathway that may be important in DN pathogenesis [12].

MATERIALS & METHODS

This study was conducted at National Diabetes Center for Treatment and Research /Al-Mustansiriya University between October 2015-March 2016. The study included 60 Iraqi type 2 diabetes mellitus (T2DM) patients, 26 females and 34 males, the age range within 40-60 years randomly selected. Patients were free of acute illness or infection at time of study also those with known diseases, which are associated with disordered glucose metabolism; such as Cushing's disease, acromegaly, chronic pancreatitis and pancreatactomy were excluded, as well as, other chronic kidney diseases and pregnant women . They had no history of smoking or alcohol drinking. The diagnosis of T2DM was made on the basis of the recommended criteria by ADA[13]. For the purpose of comparisons, 20 Iraqi control subjects comparable to diabetes mellitus patients were selected among subjects who were healthy in terms of non-diabetic, non-hypertensive, no other endocrine disorders or metabolic kidney diseases and had no kidney dysfunction diseases and were free of acute illness or infection at time of sampling. Also, they had no history of smoking or alcohol drinking. Estimation of urine microalbuminuria concentration was done by using an auto analyzer device (complyzer 13) and its disposable reagent strips (combina13) as supplied by Human Company, Germany. The reagent strip has sulfonephthalein as indicator which is highly sensitive for albumin. The diabetic patients were classified into three groups according to their A:C ratio (with or without microalbuminuria), 20 diabetic patients without microalbuminuria (urinary albumin to creatinine ratio (A:C) < 30 mg/g Cr (<3.4 mg/mol), 20 diabetic with microalbuminuria (A:C) 30-300 mg/g Cr (3.4–33.9 mg/mol) and 20 patients with macroalbuminuria (A:C) of ≥ 300mg /g Cr (> 33.9 mg/mol).

Urine collection and ELISA

The freshly voided urine samples were performed by giving each subject a befittingly disposable container. The sample collected was divided into three parts, the first part for immediate measurement for microalbuminuria, Also the general physical, chemical and microscopic examination of urine were performed on the second part of urine specimen. Then the third were centrifuged at 2000 rpm/min for 10 min. Two milliliters supernatant was taken and divided into two parts for the estimation of uNephrin and u MCP-1 levels in Eppendorff tube and stored in deep freeze (-20ºC) until use. Urinary level for Nephrin (mybioscource inc., USA) and MCP-1 (biovision company, USA) were were quantitatively determined in patients and control subjects by means of sandwich ELISA test using commercially available kits. The absorbances were read in a microplate reader (Human, Germany) and were calculated by interpolation from a standard curve that was performed in the same assay as that for the sample (Figure 1 and 2), using a curve fitting equation.

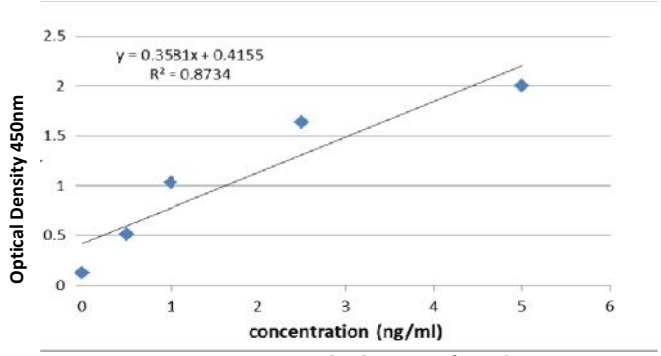

FIGURE 1: Standard curve of Nephrin

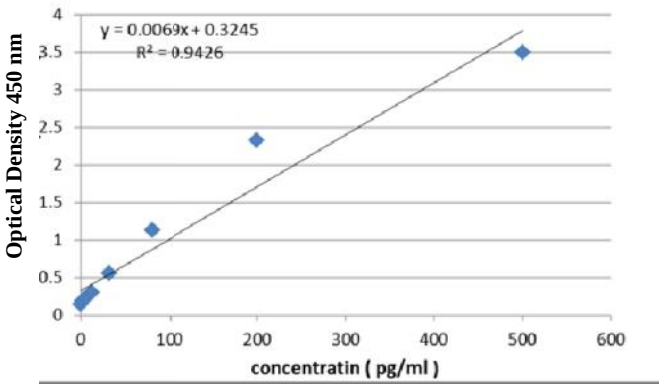

FIGURE 2: Standard curve of MCP-1

Statistical analysis

Analysis of data was carried out using the available statistical package of SPSS-22 (Statistical Packages for Social Sciences- version 22).

RESULTS

Patients-Control Differences in investigated parameters

The FSG showed a significant (P = 0.0001) increased mean in patients as compared to controls (236.07 *vs* 98.40 mg/dL). The T2DM patients showed increased mean of blood creatinine in comparison with controsl (0.96 *vs.* 0.65 mg/dl) and the difference was highly significant (P ≤ 0.0001). The blood urea also increased significantly (P ≤ 0.008) in diabetic patient as in compared with control (31.83 *vs.* 24.97 mol/l). The T2DM patients showed increased mean of A:C ratio in comparison with controls (42.60 *vs.* 1.929) and the difference was highly significant (P= 0.006). The eGFR showed a highly significant (P = 0.0001) increased mean in the control as compared to patient (109.15 *vs.* 86.65) and the means of urinary Monocyte chemo-attractant protein-1(MCP-1) and Nephrin were increased in T2DM patients as compared to control group (15.381 vs. 6.105 pg/ml), (100.017 vs. 52.230 ng/ml) respectively. The differences were significant for MCP-1 and Nephrin (P < 0.012, P < 0.020) respectively (Table 1).

TABLE 1: Means of FSG, serum creatinine, serum urea, A: C ratio, e GFR, urinary MCP-1 and Nephrin in T2DM and controls

Parameters	Mean ± SE (Range)		P value ≤
	Patients (n =60)	Controls (n =20)	
Fasting serum glucose (mg/dL)	236.07 ±87.73 (127.0-578.0)	98.40 ±16.76 (75.0-131.0)	0.0001
serum creatinine (mg/dl)	0.96 ±0.02 (0.50-1.60)	0.65 ±0.01 (0.50-0.80)	0.0001
serum urea (mol/l)	31.83 ±10.77 (9.70-66.0)	24.97 ±5.26 (16.0-35.0)	0.008
Urinary albumin to creatinine ratio (A:C ratio) (mg/mol)	42.60 ±63.52 (0.56-166.60)	1.929 ±0.601 (0.56-2.20)	0.006
eGlomerular filtration rate (eGFR) (ml/min/1.73m2)	86.65 ±16.93 (43.0-118.0)	112.70 ±3.93 (99.0-182.0)	0.0001
uMCP-1 (pg/ml)	15.381 ±15.502 (0.068-80.850)	6.105 ±7.238 (0.06-30.0)	0.012*
Nephrin (ng/ml)	100.017 ±88.668 (15.0-640.0)	52.230 ±19.619 (13.0-89.0)	0.020*

Urinary albumin to creatinine ratio (A: C ratio) Impact on MCP-1 and Nephrin

To investigate the impact of urinary albumin to creatinine ratio, as defined by A: C ratio, on the investigated parameters (uMCP-1 and uNephrine), statistical differences between means of these parameters were assessed among the three groups of urinary albumin to creatinine ratio in diabetic patients; normoalbuminuria group (<3.4 mg/mol), microalbuminuria (3.4 – 33.9 mg/mol) and macroalbuminuria (> 33.9 mg/mol) Levels of uMCP-1 in the three groups of patients were much higher than those in the control group and among the three diabetes groups, these levels increased consistently with A: C ratio. Level of uMCP-1 in macroalbuminurea was significantly higher than those in normo ($p < 0.008$) and micro ($P < 0.016$), and the level in microalbuminurea was elevated compared to that in control group ($P < 0.043$).The level in normoalbuminurea group was higher than controls and lower than microalbuminurea but with no significant differences (Table 2).

TABLE 2: Urinary MCP-1 level in three groups of diabetic patients defined by A:C ratio and with controls.

uMCP-1 (pg/ml)	Control	Normo albuminurea	Micro Albuminurea	Macro Albuminurea
Number	20	20	20	20
Mean ± S.E.	6.105±7.238	10.116±8.461	11.459±8.826	24.568±21.506
Range	(0.06-30.0)	(0.068-28.78)	(0.182-26.55)	(0.798-80.85)
Compared to Control	-	0.115	0.043*	0.001*
Compared to Normoalbuminurea	-	-	0.626	0.008*
Compared to Microalbuminurea	-	-	-	0.016*
Comparing ALL (ANOVA)	-	-	-	0.0001*

*Significant difference

The Levels of Nephrin in the three groups of patients were higher than that in the control group These levels increased consistently with A:C ratio .level of Nephrin in macroalbuminurea, microalbuminurea and normoal buminurea were significantly higher than those of controls ($p < 0.004$, $p < 0.016$ and $p < 0.022$ respectively). Macroalbuminurea group of patients showed significant increase in urinary Nephrin level as compared to micro and normoalbuminurea group of patients ($p<0.046$ and $p < 0.030$ respectively) (Table 3).

TABLE 3: Nephrin level in three groups of diabetic patients defined by A:C ratio and controls.

Nephrin (ng/ml)	Control	Normo albuminurea	Micro albuminurea	Macro Albuminurea
Number	20	20	20	20
Mean ± S.E.	52.230±19.619	75.050±37.802	80.000±45.204	145.000±133.166
Range	(13.0-89.0)	(15.0-143.0)	(25.0-164.0)	(27.0-(640.0)
Compared to Control	-	0.022*	0.016*	0.004*
Compared to Normal albuminurea	-	-	0.709	0.030*
Compared to Microalbuminurea	-	-	-	0.046*
Comparing ALL (ANOVA)	-	-	-	0.001*

*Significant difference

Impact duration of disease on the urinary level of MCP-1 and Nephrin

MCP-1

The <10y, 10-14y and =>20y duration groups of T2DM showed significant difference as compared to control, although the =>20y group of patients was higher than the others but with no significant differences. Comparing all the four groups shows a significant differences (p= 0.047) (table 4).

TABLE 4: Urinary MCP-1 level in four groups of diabetic patients defined by duration of disease.

uMCP-1 (pg/ml)	Control	<10 years	10-14 years	15-19 years	=>20 years
Number					
Mean ± S.E.	6.105±7.238	17.01±18.09	14.59±11.39	10.50±8.78	19.76±21.30
range	(0.06-30.0)	(0.18-72.62)	(0.36-46.25)	(0.07-26.55)	(0.18-80.85)
Compared to control	-	0.019*	0.010*	0.114	0.012*
Compared to >10 years	-	-	0.657	0.220	0.711
Compared to 10-14 years	-	-	-	0.274	0.406
Compared to 15-19 years	-	-	-	-	0.133
Comparing ALL (ANOVA)	-	-	-	-	0.047*

*Significant difference

Nephrin

In table (5) there was significant deference between T2DM patient groups (10-14y, 15-19y and =>20y) as compared to control, although the deferences between diabetic patients groups wasn't significant. Interestingly the =>20y group of patient showed obvious decrease in its level as compared to 15-19y group but the decrease was not significant (p=0.112). Comparing all the four groups shows significant differences (p=0.006).

TABLE 5: Urinary Nephrin level in four groups of diabetic patients defined by duration of disease.

U Nephrin (ng/ml)	Control	<10 years	10-14 years	15-19 years	=>20 years
Number					
Mean ± S.E.	52.230±19.619	99.40±48.40	72.75±39.07	149.13±153.2	79.21±44.23
range	(13.0-89.0)	(25.0-164.0)	(17.0-170.0)	(15.0-640.0)	(25.0-146.0)
Compared to control	-	0.0001*	0.048*	0.008*	0.021*
Compared to <10 years	-	-	0.101	0.241	0.252
Compared to 10-14 years	-	-	-	0.063	0.674
Compared to 15-19 years	-	-	-	-	0.112
Comparing ALL (ANOVA)	-	-	-	-	0.006*

*Significant difference

Receiver Operator Curve (ROC) Analysis for the investigated parameters

As shown in tables (6) and (7), the ROC analysis revealed the descending order (Nephrin=0.725 with sensitivity 78% and specificity 55%) and (uMCP-1=0.70 with sensitivity 78% and specificity 75%) of parameters that showed a significant variation.(table 6) (table 7).

TABLE 6: Area under the curve (AUC) for urinary MCP-1 and urinary Nephrine of T2DM group of patients.

Parameters	AUC	P value
uMCP-1	0.725	0.003*
uNiphren	0.720	0.003*

TABLE 7: cut off value, sensitivity and specificity for Nephrin and uMCP-1 in T2DM patient

	u MCP-1	uNephrin
Cut-off value	6.23	49.5
Sensitivity	70%	78%
Specificity	75%	55%

Linear Correlation of urinary Nephrin and **uMCP-1**
The A:C ratio showed a significant positive linear correlation with Nephrin in T2DM patient. And the eGFR showed a significant negative linear correlation with MCP-1 in T2DM patient, and positive linear correlation of A:C ratio with MCP-1.(figure3) (figure 4) (figure 5).

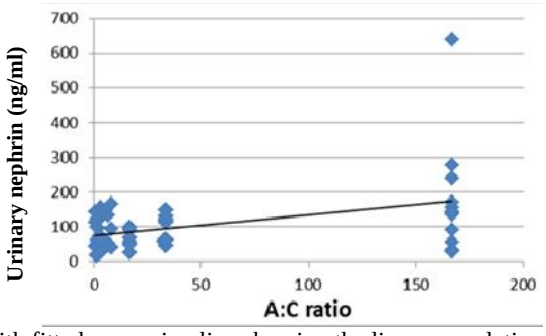

FIGURE 3: Scatter diagram with fitted regression line showing the linear correlation between A:C ratio and urinary Nephrin for T2DM patient

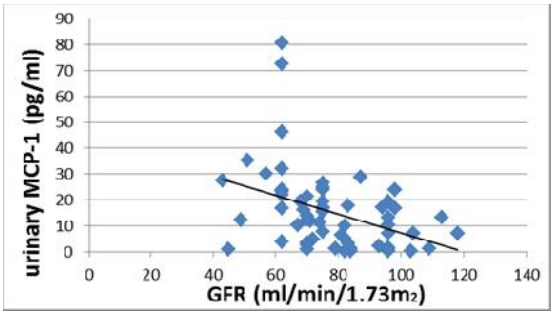

FIGURE 4: Scatter diagram with fitted regression line showing the linear correlation between GFR and urinary MCP-1 for T2DM patient.

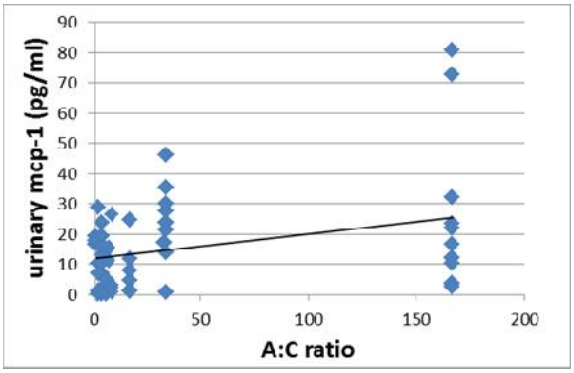

Figure 4: Scatter diagram with fitted regression line showing the linear correlation between A: C ratio and urinary MCP-1 for T2DM patient

DISCUSSION
The results of this study showed that urinary MCP-1 concentration is increased in diabetic patients as compared to controls and increased in macroalbuminurea as compared to normoalbuminurea, microalbuminurea groups and control subjects, an increased level of uMCP-1 although not significant in normoalbuminurea as compared to control group and the microalbuminurea which showed significant increase as compared to control this findings suggested that some changes would have occurred early in the pathogenesis of DN and MCP-1 may play an important role in the progression and development of DN, This raise of uMCP-1 levels with the progression of diabetic nephropathy suggest that inflammation can be considered as one of the major cause and the increased level indicates the extent of renal tubular damage., such finding is in agreement with BANBA *et al*.; and Tilak *et al*.; study who found that urinary MCP-1 levels were

elevated in diabetic patients with macroalbuminurea compared to normoalbuminurea, microalbuminurea and control subjects and they showed the mean value of duration increased in patients with advanced nephropathy [14,15]. Other study conducted by Shoukry *et al*., showed that the uMCP-1 levels was significantly elevated in macroalbuminurea and microalbuminurea diabetic patients compared to that in normoalbuminurea diabetic patients and control subjects and In a study conducted by Giunti *et al.* how found Found the MCP-1 in mesangial cells, binding to CCR2 induced the increase in fibronectin protein levels that induce production of TGF-β1- and NF-κB-dependent and its loss improve mesangial cells. Suggesting the role of MCP-1/CCR2 system in the diabetic glomerulus. These data suggest that the increasing level of MCP-1may predict in the progression of DN and its effect on podocyte [16, 17] .

This study showed that there was significant increase of urinary Nephrin in diabetic patients as compared to controls and this significant increase of Nephrin in normoalbuminurea group of diabetic nephropathy patient as compared to control as well as significant deference of Nephrin in micro and macroalbuminurea groups as compared to control which could investigate that Nephrinuria is a reliable biomarker of preclinical (onset) diabetic nephropathy as well as progression, such finding is in agreement with Jim *et al*. and Kandasamy *et al.* who concluded that Nephrin in urine can be used as an early biomarker of diabetic nephropathy, urinary Nephrin was higher in diabetics than in non diabetics and correlated with increasing albuminuria [18,19].

This study investigated the impact of T2DM duration on Nephrin, the mean value of urinary Nephrin level in patients group with lowest duration was higher than that with longest duration, such finding is in agreement with Pätäri *et al.* and Alter *et al.* who found that urinary Nephrin levels decline with the increasing duration of hyperglycemia and proteinuria this could be explained that this protein is a marker of early stage of disease [20, 21]. Pagtalunan *et al*. how described broadening of foot processes associated with a reduction in podocyte number and density per glomerulus in DM, suggesting that podocytopenia and podocyte damage may contribute to the progression of diabetic nephropathy and long duration [22].

Elevated urinary Nephrin appears to be characteristic for early stage of diabetic nephropathy rather than for advanced stages. Moreover, urinary Nephrin excretion increases directly which reflects the glomerular damage even before albuminuria emerges, these findings were confirmed by Pätäri *et al*. and Wang *et al* when they investigate the gene expression of podocyte-associated molecules in the urinary sediment of patients with diabetic nephropathy as compared to healthy controls [23]. The investigators found that urinary Nephrin levels (measured by mRNA expression) correlated with proteinuria but not with eGFR and this could explain the result of this study which failed to give a significant negative correlation between Nephrin and eGFR but there was a significant positive correlation with A:C ratio.

This study also investigate the impact of T2DM duration on urinary level of MCP-1and the mean value of this marker in patients with longest duration of T2DM was higher than that with the lowest duration, this explained the long standing type 2 diabetes is frequently accompanied by subclinical inflammation (slow inflammation) so most of bio markers increase and precede the nephropathy so become a good predictor for detection of disease.

A strong positive correlation between uMCP-1 and A:C ratio was observed in diabetics which is in consistence with Shoukry *et al.* who revealed that uMCP-1 was significantly positively correlated to urinary albumin/ creatinine ratio[16]. In contrast a significant negative correlation between uMCP-1 levels in diabetic patients and eGFR was seen which is in consistence with Ibraheem and Rashed study that also found an inverse correlation between uMCP-1 and eGFR and defined the use of MCP-1 as marker reflecting the degree of kidney damage as estimated by glomerular filtration rate (24).

The (ROC) curve analysis for uMCP-1 and Nephrin levels (MCP-1 = 0.720and Nephrin =0.725) as an early diagnosis and detection of DN revealed that the cut-off value of uMCP-1 was 6.23 pg/mg with 70% sensitivity and 75% specificity; whereas, the cut-off value of u Nephrin was 49.5 ng/ml with 78% sensitivity and 55% specificity , so these results suggest that Nephrin may be considered as potential predictor and diagnostic biomarkers for the early detection of diabetic nephropathy. While the uMCP-1 may be considered as potential prognostic biomarkers for the diabetic nephropathy.

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