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Urinary podocin level as a predictor of diabetic kidney disease

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ABSTRACT

Background: Albuminuria showed to be a deteriorating condition in diabetic kidney disease (DKD) associated with high morbidity and mortality. A need for a novel marker for early detection of DKD development and progression becomes mandating.
Objective: To study the clinical value of urinary podocin as an early marker of diabetic kidney disease and its association with severity of the disease.
Patients and Methods: This study included 45 individuals with type 2 DM whose GFR >60 mL/min/1.73 m^2, recruited from Ain Shams University Hospital, Cairo, Egypt. Patients were further divided into three groups according to urinary albumin/creatinine ratio (ACR). In addition to, ten healthy volunteers serving as the control group was enrolled in the study. Routine chemistry including serum creatinine, fasting blood glucose (FBG), HbA1c, albumin, lipid profile, urine analysis, ACR and urinary podocin quantification were conducted for all participants (by ELISA method).
Results: Podocin was higher in patients with ACR <30 mg/g, ACR 30-299 mg/g and ACR ≥ 300 mg/g versus healthy controls, respectively ($P < 0.001$). Both GFR and serum albumin showed highly significant negative correlations with urinary podocin. Significant positive correlations were detected between urinary podocin with blood urea nitrogen (BUN), serum creatinine, FBG, HbA1c, cholesterol, and triglyceride levels.
Conclusions: Urinary podocin is assumed to be a promising marker for early DKD detection in type 2 DM patients.

Original Article

Implication for health policy/practice/research/medical education:
The development of diabetic kidney disease (DKD) needs early detection and management aiming to decrease the incidence of end-stage kidney failure. We believe that our study has implication for the diabetic patients. This study included 45 patients with types 2 diabetes mellitus (DM). We found urinary podocin is a promising marker for early DKD detection in types 2 DM.
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1. Background

Diabetic kidney disease (DKD) is a major public health problem that is associated with enhanced rates of cardiovascular morbidity and mortality. Forty percent of diabetic patients are predicted to develop end-stage renal disease (ESRD) (1).

Diabetic nephropathy was classified according to Joint Committee into; stage 1: pre-nephropathy with normoalbuminuria (albumin/creatinine ratio [ACR] <30 mg/g and glomerular filtration rate [GFR] ≥30 mL/min/1.73 m^2), stage 2: incipient nephropathy with microalbuminuria (ACR 30–299 mg/g and GFR ≥ 30 mL/min/1.73 m^2), stage 3: macroalbuminuria (ACR ≥300 mg/g or persistent proteinuria ≥0.5 g and GFR ≥ 30 mL/min/1.73 m^2), and stage 4: kidney failure with any albuminuria/proteinuria status and GFR <30 mL/

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min/1.73 m^2 (2).

Podocyte is the largest highly differentiated epithelial cell in the glomerulus, present in the outer surface of the glomerular capillary loop. Podocytes consist of large cell body, cell processes and foot processes which are the most characteristic structure of podocytes. The foot processes of adjacent podocytes interdigitate, leaving between them filtration openings. Slit diaphragm proteins (nephrin, podocin), transcriptional factor (WT1), adapter proteins (Nck, Crk), cytoskeletal proteins (F-actin-aactinin4-synaptopodin-NMHCIIA), podocalyxin (PCX) detached from glomerular basement membrane (GBM) and lost by various stress, resulting in podocytopenia**,** leading to glomerulosclerosis and also will trigger tubulointerstitial damage and fibrosis, progressive decline of GFR up to ESRD in different glomerular diseases (3-5).

Proteinuria appears when podocyturia exceeds its normal range. Therefore, the diagnosis of proteinuria is a late event. Podocyturia could be used as an early indicator of glomerular pathology (6,7). Podocyturia points to a dynamic injury, on the other hand, proteinuria, cannot differentiate between active and chronic glomerular damaging process (8).

Quantifications of podocyturia using immunofluorescent staining, urinary podocyte culture, flow cytometry, and cytology still have technical problems. Urinary podocyte molecules are emerging noninvasive methods for detecting podocyte damage, as podocytes can be identified by detection of specific markers as podocalyxin, WT1, synaptopodin, nephrin and others (9). However, different and difficult techniques in assay mean that more work is required to evaluate and determine a sound marker of clinical value for detecting podocyte injury and loss.

Podocin is a membrane protein of the filtration slits of podocytes that binds to intracellular domain of nephrin via its C-terminus, with CD2AP. This interaction is important to oligomerize nephrin in lipid rafts to stabilize the slit diaphragm which has a role in permselectivity function of glomerulous (10).

2. Objectives

The present study aimed to examine the urinary podocin level as an early marker of DKD in patients with type 2 diabetes mellitus (DM). Furthermore, to evaluate its relationship with the seriousness of the disease.

3. Patients and Methods

3.1. Patients

This is a case-control study, including 45 individuals recruited from Ain Shams University Hospital, having type 2 DM and a GFR >60 mL/min/1.73 m^2. They were divided into three groups: 15 patients with ACR: <30 mg/g, 15 patients with ACR: 30-300 mg/g and 15 patients with ACR: >300 mg/g. Furthermore, 10 healthy subjects age- and gender-matched were included, serving as control group. Patients with fever, urinary tract infection, uncontrolled hypertension, congestive heart failure, malignancy or menstruation were excluded. An informed written consent was obtained from all participants. A full history was taken from all participants, in addition to clinical examination with special concern on age, gender, body mass index (BMI), blood pressure, duration of DM and co-morbidities.

3.2. Samples

A total of 7 mL venous blood was withdrawn from each subject after 10 hours of fasting. Five milliliters were collected in sterile vacutainers with a Z Serum Sep Clot Activator (Greiner Bio-One). After clotting, serum was obtained by centrifugation at 1500 × g for 15 minutes. Serum was used for immediate measurement of fasting blood glucose (FBG), urea, creatinine, albumin and lipids (cholesterol and triglycerides). The remaining two milliliters were put in a test tube with ethylene diamine tetra-acetate (EDTA) (1.2 mg/mL) as an anticoagulant, to be used for performing complete blood count and HbA1c.

Fifteen milliliters of morning urine samples were collected aseptically from all participants, 1 mL was used for ACR, 10 mL were used for urine analysis and rest of the sample was centrifuged for 20 minutes at 3000 rpm then supernatant was collected aliquoted and stored at -20°C for podocin levels detection.

3.3. Routine laboratory investigations

Routine chemistry was assayed on Beckman autoanalyzer (Beckman Instruments Inc. Scientific Instrument Division, Fullerton, USA). CBC was assayed on Coulter Beckman Cell counter (Beckman Instruments Inc. Scientific Instrument Division, Fullerton, USA). HbA1c levels were determined by an ion-exchange HPLC method (Bio-Rad D10 HbA1c, BioRad Laboratories, Hercules, CA, USA).

3.4. GFR calculation

GFR was calculated by an equation developed by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) (11).

GFR (mL/min/1.73 m^2) = 141 × min (serum creatinine/κ, 1)$^{\alpha}$ × max (serum creatinine/κ, 1)$^{-1.209}$ × 0.993^{Age} × 1.018 (if female) × 1.159 (if black), where

k is 0.7 for females and 0.9 for males, α is −0.329 for females and −0.411 for males, min shows minimum serum creatinine/k or 1, and max shows maximum serum creatinine/k or 1 (11).

3.5. Assay of urinary podocin

Urinary podocin was assayed by human podocin enzyme-linked immunosorbent assay (ELISA) kit supplied by SunLong Biotech Co., LTD (Gongzhu District, Hangzhou, Zhejiang, China, Catalogue Number: SL1430Hu). Sandwich ELISA technique was applied. The concentration of human podocin was calculated using a standard curve.

3.6. Ethical issues

The study was in accordance with the Declaration of Helsinki. All participants gave their informed consent to enter the study. The study has been approved by the ethical committee of Faculty of Medicine, Ain Shams University.

3.7. Statistical methods

For statistical analysis, SPSS (version 22.0, IBM Corp.) was used. Qualitative data were presented as numbers and percentages. While mean and standard deviation were used in case of quantitative data were. For comparison between different groups of categorical variables, Chi-square test was used. Fisher's exact test or Monte Carlo correction was used for correction for chi-square test when more than 20% of the cells have expected count less than 5. In the case of normally distributed quantitative variables, student t-test was applied, while ANOVA (F-test) was used to compare between more than two groups. As regards skewed data, Mann-Whitney U test was used for comparison between two groups and Kruskal-Wallis test was applied for more than two groups. Spearman's rank correlation coefficient (rs) was used to assess the degree of correlation between two sets of variables if one or both of them showed a skewed distribution. The diagnostic performance of podocin was evaluated in terms of its diagnostic sensitivity and specificity. The area under the curve (AUC) was used to describe the overall test performance. Forward multiple linear regression analysis was applied to detect independent variables that correlate to urinary podocin or GFR.

4. Results

Forty-five patients' group included 51.1% females and 48.9% males, with mean age (SD) of 49.8 (7.28) year. The mean (SD) duration of DM 58.63 (26.79) months, GFR mean (SD) 89.59 (13.66) mL/min/1.73 m^2. Twenty patients (44.4%) were hypertensive. Descriptive and comparative statistics of the clinical characteristics and laboratory parameters of the different studied groups are shown in Tables 1 and 2.

Urinary podocin levels showed a highly statistically significant difference between the patients' group and the control group, between normoalbuminuria and microalbuminuria, between normoalbuminuria and macroalbuminuria, and between microalbuminuria and macroalbuminuria, respectively ($P<0.001$).

The duration of DM among patients' group was variable. Twenty-five patients were having DM for

Table 1. Descriptive and comparative statistics of the clinical characteristics of the different studied groups

	Type 2 DM patients			Healthy Controls (n=10)	*P* value
	Normo (n=15)	Micro (n=15)	Macro (n=15)		
Sex, No. (%)					
Male	10 (66.7)	8 (53.3)	5 (33.3)	5 (50.0)	χ2, $P = 0.336$
Female	5 (33.3)	7 (46.7)	10 (66.7)	5 (50.0)	
Age (y), mean ± SD	46.73 ± 5.81	50.40 ± 7.42	52.27 ± 7.78	48.10 ± 5.63	F, $P = 0.146$
BMI (kg/m^2), mean ± SD	25.29 ± 2.10	26.95 ± 1.57	26.18 ± 2.43	25.41 ± 1.77	F, $P = 0.250$
Duration of DM (mon), mean ± SD	25.087 ± 22.48	49.20 ± 26.85	101.60 ± 31.05		K-W χ2, $P<0.001$
P value between groups	$P1 = 0.004$*, $P2 < 0.001$*, $P3 < 0.001$*				
Systolic BP (mm Hg)	115.67 ± 7.04	123.0 ± 17.20	125.67 ± 14.38	110.0 ± 10.80	
$P_{control}$	0.716	0.085	0.026*		0.019
P value between groups	$P1 = 0.427$, $P2 = 0.171$, $P3 = 0.944$				
Diastolic BP (mm Hg), mean ± SD	75.0 ± 8.66	77.67 ± 9.04	82.67 ± 8.63	75.0 ± 8.82	0.080

χ^2: Chi square test, **F:** F test (ANOVA), *P* value between groups was done using Tukey post hoc test, $P_{control}$**:** *P* value for comparing between control and each other groups, P_1: *P* value for comparing between normo and microalbuminuria, P_2: *P* value for comparing between normo- and macroalbuminuria, P_3: *P* value for comparing between micro- and macroalbuminuria.

Table 2. Descriptive and comparative statistics of the different studied groups as regards the laboratory parameters

	Type 2 DM patients			Healthy Controls (n=10)	*P*
	Normo (n=15)	Micro (n=15)	Macro (n=15)		
S. albumin (g/dL), Mean ± SD	4.55 ± 0.55	3.33 ± 0.21	2.71 ± 0.27	4.14 ± 0.54	
$P_{control}$	0.080	<0.001*	<0.001*		<0.001*
P value between groups	$P_1 < 0.001^*$, $P_2 < 0.001^*$, $P_3 = 0.001^*$				
BUN (mg/dL), Mean ± SD	10.13 ± 3.83	11.80 ± 2.04	16.07 ± 3.69	10.10 ± 2.47	
$P_{control}$	1.000	0.557	<0.001*		<0.001*
P value between groups	$P_1 = 0.479$, $P_2 < 0.001^*$, $P_3 = 0.003^*$				
Creatinine (mg/dL), Mean ± SD	0.90 ± 0.14	1.0 ± 0.13	1.13 ± 0.14	0.81 ± 0.12	
$P_{control}$	0.315	0.006*	<0.001*		<0.001*
P value between groups	$P_1 = 0.242$, $P_2 \leq 0.001^*$, $P_3 = 0.045^*$				
Hemoglobin (g/dL), Mean ± SD	15.26 ± 1.07	13.41 ± 1.62	12.23 ± 1.87	14.30 ± 1.51	
$P_{control}$	0.435	0.498	0.010*		<0.001*
P value between groups	$P_1 = 0.010^*$, $P_2 < 0.001^*$, $P_3 = 0.172$				
FBS (mg/dL), Mean ± SD	92.67 ± 8.79	106.47 ± 11.56	116.87 ± 10.02	83.0 ± 5.73	
$P_{control}$	0.075	<0.001*	<0.001*		<0.001*
P value between groups	$P_1 = 0.001^*$, $P_2 < 0.001^*$, $P_3 = 0.022^*$				
HbA1c %, Mean ± SD	5.03 ± 0.49	5.58 ± 0.57	6.02 ± 0.50	4.21 ± 0.25	
$P_{control}$	0.001*	<0.001*	<0.001*		<0.001*
P value between groups	$P_1 = 0.015^*$, $P_2 < 0.001^*$, $P_3 = 0.075$				
S . Cholesterol (mg/dL), Mean ± SD	98.93 ± 25.47	146.87 ± 59.05	208.27 ± 68.52	103.2 ± 24.08	
$P_{control}$	0.389	0.043*	<0.001*		K-W $\chi 2$, $P < 0.001^*$
P value between groups	$P_1 = 0.002^*$, $P_2 \leq 0.001^*$, $P_3 = 0.009^*$				
S. Triglycerides (mg/dL), Mean ± SD	131.67 ± 30.85	183.53 ± 60.90	273.07 ± 93.63	126.7 ± 23.53	
$P_{control}$	0.997	0.120	<0.001*		F, $P \leq 0.001^*$
P value between groups	$P_1 = 0.109$, $P_2 \leq 0.001^*$, $P_3 = 0.001^*$				
GFR (mL/min/1.73 m^2), Mean ± SD	105.91 ± 15.41	89.79 ± 8.42	73.07 ± 7.15	110.9 ± 15.44	
$P_{control}$	0.834	0.004*	<0.001*		F, $P < 0.001^*$
P value between groups	$P_1 = 0.018^*$, $P_2 \leq 0.001^*$, $P_3 = 0.013^*$				
Urinary podocin level (ng/mL), Mean ± SD	10.77 ± 5.30	18.27 ± 6.83	42.50 ± 9.04	3.50 ± 1.66	
$P_{control}$	<0.001*	<0.001*	<0.001*		K-W $\chi 2$, $P < 0.001^*$
P value between groups	$P_1 = 0.001^*$, $P_2 \leq 0.001^*$, $P_3 \leq 0.001^*$				

K-W χ^2: Chi-square for Kruskal Wallis test; *P* value between groups was done using Mann-Whitney test; F: F test (ANOVA), *P* value between groups was done using Tukey post hoc test; $P_{control}$: *P* value for comparing between control and each other groups; P_1: *P* value for comparing between normo and microalbuminuria; P_2: *P* value for comparing between normo and macroalbuminuria; P_3: *P* value for comparing between micro and macroalbuminuria.

less than 5 years, 16 patients suffered for 5-10 years and 4 were having the disease for more than 10 years. Comparing the three groups as regards the means of podocin levels showed a highly statistically significant difference ($P < 0.001$) respectively. Urinary podocin was significantly positively correlated with duration of DM months ($r = 0.655$, $P < 0.001$).

On comparing urinary podocin levels in patients with chronic kidney disease (CKD) stage 1 (GFR ≥ 90 mL/min/1.73m^2) (29 patients) versus those of stage 2 (GFR 89 – 60 mL/min/1.73m^2) (16 patients), a highly statistically significant difference was found ($Z=3.345$, $P=0.001$). Podocin was higher in stage 2 (34.50 ± 14.21 ng/mL) than stage 1 (17.97 ± 12.81 ng/mL).

Levels of urinary podocin in hypertensive patients (29.50 ± 12.61 ng/mL) were higher than those without hypertension (19.32 ± 16.17 ng/mL), and this difference was statistically significant ($Z = 2.788$, $P = 0.005$). Moreover, a significant positive correlation was found between urinary podocin level and diastolic blood

pressure (BP) (r = 0.300, $P = 0.045$)

Correlation between urinary podocin and the different studied parameters among patients' group is shown in Table 3. A significant positive correlation was found with body mass index (BMI), blood urea nitrogen (BUN), creatinine, FBG, HbA1c, cholesterol, triglycerides and ACR. Moreover, a statistically significant negative correlation was found between urinary podocin and each of the following; GFR, serum albumin and hemoglobin concentration.

Multiple linear regression analysis indicated that only the ACR levels were independent predictors of urinary podocin levels in type 2 DM patients (B = 0.077, $P < 0.001$).

4.1. Diagnostic efficacy of urinary podocin

Figure 1 illustrates receiver operating characteristic (ROC) curve analysis applied to the study results to examine the diagnostic performance of urinary podocin to predict diabetic nephropathy in normoalbuminuria cases at different cut-off values. At a cut-off level of 5.3 ng/mL the diagnostic performance of urinary podocin showed 93.3% sensitivity and 90% specificity, 93.3% positive predictive value and 90% negative predictive value with an AUC =0.977.

5. Discussion

In the present study, urinary podocin was detected in patients with type 2 DM in the pre-nephropathy stage. Urinary podocin was significantly higher in macroalbuminuric patients versus either microalbuminuric or normoalbuminuric patients in studied patients' group, with a highly significant positive correlation to ACR as a marker of severity of DKD. Podocytes' damage is essential for DKD development. Different pathological pathways can lead to podocyte injury including; neurohormonal changes, oxidative stress and decreased expression of adhesion molecules (12). The podocyte density in glomeruli seems to decrease with early stages of diabetic nephropathy and before establishing proteinuria (13).

These results are in accordance with Sahoo et al (13) who evaluated the podocyturia using indirect immune fluorescence by using antibodies against podocin. He reported that podocyturia appears to be an earlier marker of nephropathy in patients with DM Type 2. Moreover, they detected an increase in podocytes in urine with increasing duration of diabetes.

Moreover, Lioudaki et al (14) revealed that urinary podocyte markers (nephrin, podocin, and synaptopodin) were more elevated in normoalbuminuric DM patients in comparison to non-diabetic controls.

Urinary podocin was detected in diabetic patients with normal or increased GFR and was significantly and negatively correlated with GFR. However, a significant positive correlation was detected with serum creatinine and blood urea nitrogen which indicates podocytopathy in early stages of DKD. Podocyte density can predict the event of albuminuria in diabetic patients (15). Zheng et al (16) reported that urinary podocin mRNA level was significantly positively correlated with urinary albumin and serum creatinine levels. Unexpectedly, it did not

Table 3. Correlation between urinary podocin level (ng/mL) and different parameters in each group

	Podocin level (ng/mL)			
	Cases		Control	
	r_s	*P*	r_s	*P*
Age (y)	0.292	0.052	0.103	0.777
BMI (kg/m^2)	0.420*	0.004*	-0.555	0.881
Duration of DM (months)	0.655*	<0.001*	-	-
Systolic (mm Hg)	0.268	0.075	-0.333	0.347
Diastolic (mm Hg)	0.300*	0.045*	0.075	0.836
BUN (mg/dL)	0.459*	0.002*	0.428	0.217
Serum albumin (g/dL)	-0.755*	<0.001*	0.030	0.934
S. Creatinine (mg/dL)	0.442*	0.002*	0.018	0.960
Hemoglobin (g/dL)	-0.538*	<0.001*	0.018	0.960
FBS (mg/dL)	0.624*	<0.001*	-0.030	0.934
HbA1c %	0.508*	<0.001*	0.019	0.959
S. cholesterol (mg/dL)	0.643*	<0.001*	-0.018	0.960
S. triglycerides (mg/dL)	0.670*	<0.001*	0.018	0.960
ACR (mg/g)	0.874	<0.001*	-0.650*	0.042*
GFR (mL/min/1.73 m^2)	-0.584*	<0.001*	-0.018	0.960

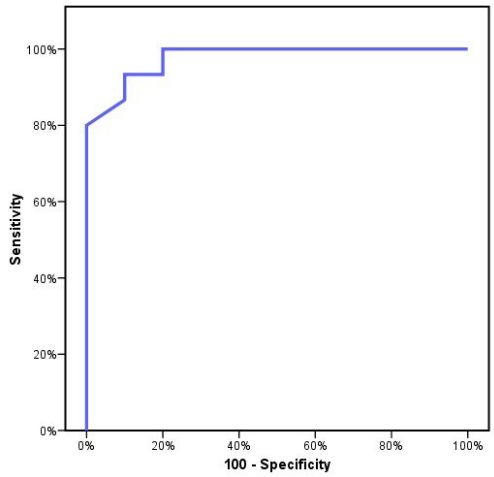

Figure 1. Receiver operating characteristics curve analysis showing the diagnostic performance of podocin for predicting diabetic nephropathy in normoalbuminuria cases at different cut-off values (area under the curve: 0.977).

correlate with GFR. Thus podocin can be used as a marker of progression of renal dysfunction caused by podocytes injury and loss.

The development of DKD is dependent on the duration of DM. In the present study urinary podocin was found to be related to DM duration. Moreover, it was detected in patients with DM of less than 5 years and was significantly higher in patients suffering from DM for more than 10 years.

A statistically significant positive correlation was found between urinary podocin and each of FBS and HbA1c in patients' group. This may be explained by glucotoxicity and hyperglycemia induces stimulation of inflammatory cell death pathways and oxidative stress with increased intracellular reactive oxygen species (ROS) contribute to podocyte injury (17). Thus, leading to apoptosis, detachment, increased albuminuria and exacerbates DKD (18).

Nephrin is a transmembrane protein of the podocytes. It is necessary for the proper functioning of the renal filtration barrier. Furthermore, nephrin is essential to maintain podocyte sensitivity to insulin. Nephrin's cytoplasmic domain enables insulin recognition and intracellular signaling (19). Doublier et al (20) found that albuminuria and histopathological features similar to DKD developed in podocyte-specific insulin-receptor knockout mice.

Podocin is one of slit diaphragm proteins that interacts with the cytosolic tail of nephrin. Hence, any disruption of slit diaphragm proteins may have a role in progression and severity of DKD and albuminuria.

Serum cholesterol and triglycerides were significantly positively correlated to urinary podocin levels in the patients' group. These findings are in agreement with other researchers, who found that cholesterol accumulation in podocytes may cause podocyte injury ending up in DKD (21). In our study diabetic hypertensive patients had a significantly higher urinary podocin than non-hypertensive diabetic patients. Additionally, diastolic blood pressure showed a significant positive correlation with urinary podocin. The renin-angiotensin-aldosterone system (RAAS) is up-regulated in DM leading to persistent elevation of intrarenal angiotensin II. The locally produced angiotensin II activates angiotensin receptor II type 1 directly, thus causing progressive injury of the podocytes and proteinuria (22). Moreover, it promotes podocyte injury through enhancement of independent hemodynamic changes (23).

A significant correlation between BMI and urinary podocin was also detected by Pereira et al (24) who demonstrated a correlation between evidence of podocyte injury and both obesity and hyperinsulinemia. Our results showed a significant negative correlation between urinary podocin and both serum albumin and hemoglobin concentration among the patients' group. These findings are in accordance with Han et al (25), who found that albuminuria was a significant risk factor for anemia in DKD patients independent of the estimated GFR.

The diagnostic performance of urinary podocin was assessed using ROC curve analysis. At a cut-off level of 5.3 ng/mL, podocin showed 93.3% sensitivity and 90% specificity to detect DKD in normoalbuminuric cases (versus control group). This makes urinary podocin a specific and sensitive early marker as a predictor of nephropathy in normoalbuminuric type 2 DM patients.

6. Conclusions

Urinary podocin is assumed to be a promising marker for early detection of DKD. It significantly correlated to the severity of kidney disease in type 2 DM patients. However, further studies are needed on a larger number of patients. And cohort studies to evaluate its applicability in monitoring treatment that preserves podocytes and decreases the progression of the disease.

Limitations of the study

Due to funding limitations, the study was conducted only on 45 diabetic patients, in addition to 10 healthy volunteers. Further studies are needed to evaluate the application of urinary podocin levels to follow up diabetic patients.

Authors' contribution

AE supervised, reviewed and validated the final manuscript. MAB was responsible for conceptualization, validation, writing and editing the manuscript. SAB contributed to setting the research methodology, while HAA validated the results; both of them contributed to the manuscript writing, review and editing. Finally, ES was responsible for original draft preparation and writing. All authors read, revised and approved the final manuscript.

Conflicts of interest

There were no points of conflicts to declare.

Ethical considerations

Ethical issues (including plagiarism, data fabrication, double publication) have been completely observed by the authors.

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