



## Original Article

## Urinary podocyte-associated mRNA profile in Egyptian patients with diabetic nephropathy

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## ABSTRACT

**Introduction:** Podocyte injury and subsequent excretion in urine play a crucial role in the pathogenesis and progression of diabetic nephropathy (DN). Quantification of messenger RNA expression in urinary sediment by real-time PCR is emerging as a noninvasive method of screening DN-associated biomarkers. We aimed to study the expression of podocyte-associated genes in urinary sediment and their relation to disease severity in type 2 diabetic Egyptian patients with diabetic nephropathy.

**Method:** Sixty patients with type 2 diabetes mellitus were recruited in addition to twenty non-diabetic healthy volunteers. Relative mRNA abundance of nephrin, podocalyxin, and podocin were quantified, and correlations between target mRNAs and clinical parameters were examined.

**Results:** The urinary mRNA levels of all genes studied were significantly higher in diabetics compared with controls ( $p < 0.001$ ), and mRNA levels increased with DN progression. Urinary mRNA levels of all target genes positively correlated with both UAE and HbA<sub>1c</sub>. The expression of nephrin, podocalyxin, and podocin mRNA correlated with serum creatinine ( $\{r = 0.397, p \text{ value} = 0.002\}$ ,  $\{r = 0.431, p \text{ value} = 0.001\}$ ,  $\{r = 0.433, p \text{ value} = 0.001\}$  respectively).

**Conclusion:** The urinary mRNA profiles of nephrin, podocalyxin, and podocin were found to increase with the progression of DN, which suggested that quantification of podocyte-associated molecules will be useful biomarkers of DN.

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## 1. Introduction

Diabetic nephropathy (DN) is a leading cause of end-stage renal disease. Diabetic nephropathy (DN), affects approximately one third of patients with either Type 1 or Type 2 diabetes mellitus [1]. Searching for the perfect biomarker of DN has become the holy grail of nephrology since the burden of this disease is untenable.

The only feasible way to tackle this health care crisis is by prevention of disease with early detection and treatment in a mechanistically rational approach. Therefore, the discovery of a specific, reliable diagnostic and prognostic biomarker for DN is imperative. Part of the difficulty in finding such a marker is the complex pathogenesis of DN; it is clearly multifactorial and involves multiple genes, proteins, metabolic pathways, and environmental

factors [2].

The current early biomarker is small amounts of albumin in the urine, or microalbuminuria. However, its association with progression to renal failure is unclear, as microalbuminuria does not always lead to progressive renal failure [3]. Furthermore, it is found in other disease states such as urinary tract infection [4], and hemodynamic stress (exercise, fever, congestive heart failure) [5].

Recent studies have shown that renal podocyte injury is pathogenetically and prognostically important in DN progression. The potential mechanisms of podocyte injury include foot process effacement, hypertrophy, detachment, apoptosis, and perhaps epithelial-to-mesenchymal transition (EMT), and these mechanisms are believed to be associated with the onset and progression of DN [6–9].

Several potential urine podocyte biomarkers have been described including podocytes themselves and fragments of podocytes, podocyte proteins and mRNAs, and exosomes [10].

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This potentially provides a noninvasive mechanism for monitoring podocyte well-being and raises the possibility that biomarkers of podocyte injury, stress, or loss could be used in combination with proteinuria to more reliably detect and monitor progression and response to treatment.

In the current study, we aimed to evaluate the expression of podocyte-associated genes in urinary sediment and their relation to disease severity in type 2 diabetic Egyptian patients with diabetic nephropathy.

## 2. Patient and methods

Kasr Al- Ainy Hospital is a major hospital and a tertiary referral center serving patients from Cairo and also patients referred from all other governorates of Egypt. Sixty patients with type 2 diabetes mellitus were recruited from the outpatient endocrinology clinic, Nephrology clinic and Internal Medicine Departments during June 2015 to December 2017. They were divided according to their urinary albumin excretion (UAE) into 3 groups: group 1 consisted of twenty diabetic patients with UAE less than 30 mg/g creatinine on random urine sample (normo-albuminuria group), group 2 consisted of twenty diabetic patients with UAE between 30 and 300 mg/g creatinine on random urine sample (micro-albuminuria group) and group 3 consisted of twenty diabetic patients with UAE above 300 mg/g creatinine on random urine sample (macro-albuminuria group) in addition to twenty age and sex matched non diabetic healthy individuals were enrolled in this study as controls.

Diagnosis of diabetes based upon the WHO classification. At least 5 years from the diagnosis of type 2 diabetes was required to be included in our study.

Exclusion criteria included Type 1 diabetes, Hypertensive patients, suspected non diabetic kidney disease, elevated kidney functions, Patients on either angiotensin converting enzyme “ACE” inhibitors or angiotensin II receptor blocker ARB’s, pregnant females, the Presence of infections and symptoms or signs of other systemic disease.

After discussing the procedure with each patient separately, a written consent was obtained. All our patients were subjected to full history, through clinical examination and fundus examination. Laboratory investigations included: routine laboratory investigations including; Complete blood count (CBC).

Fasting plasma glucose (FPG), glycosylated hemoglobin (HbA1c) and serum albumin, renal function: Creatinine and BUN, estimated glomerular filtration rate (eGFR) according to MDRD (Modification of Diet in Renal Disease) equation, random urine sample for testing for albumin/creatinine ratio (ACR) repeated twice 3 months apart if the first sample was positive for micro-albuminuria, patients had been instructed to refrain from heavy exercise 24 h before the test and urinary mRNA profile of podocyte-associated molecules, nephrin, podocalyxin, and podocin were quantified, and correlations between target mRNAs and clinical parameters were examined.

The current work was carried out as following: Collection of samples and storage; Extraction of mRNA of studied genes and mRNA of housekeeping gene. Detection and quantification of the amplified mRNA using real time PCR according to the following steps: it started with RNA extraction using quantitative real time PCR where total RNA was isolated using Qiagen tissue extraction kit (**Qiagen, USA**) according to instructions of manufacture. The following oligonucleotide primer sequences were used: NPHS1(Forward primer: 5'- AGAGCCCCATTCAAAGGCTC-3' - Reverse primer: 5'-ATTGGCATCGACAGTGCAGA-3'); NPHS2 (Forward primer: 5'- GATGATTGCTGCAGAAGCGG-3'- Reverse primer: 5'-ACTTTTC-TATGGCAGGCC-3'); PODX1(Forward primer: 5'-GACTCCGCA-CAAGGAGAACA-3'- Reverse primer: 5'- CTTCTGCAGCAATCATCCG-

3'); GAPDH (Forward primer: 5'-ACCACAGTCCATGCCATCAC-3'- Reverse primer: 5'-TCCACCACCCTGTGTCTGTA-3'). Lastly cDNA synthesis where the total RNA (0.5–2 µg) was used for cDNA conversion using high capacity cDNA reverse transcription kit Fermentas, USA).

Three µl of random primers were added to the 10 µl of RNA which were denatured for 5 min at 65 °C in the thermal cycler. The RNA primer mixture was cooled to 4 °C. The cDNA master mix was prepared according to the kit instructions Total volume of the master mix was 19 µl for each sample. This was added to the 31 µl RNA-primer mixture resulting in 50 µl of cDNA. The last mixture was incubated in the programmed thermal cycler 1 h at 37 °C followed by inactivation of enzymes at 95 °C for 10 min, and finally cooled at 4 °C. Then RNA was changed into cDNA. The converted cDNA was stored at –20 °C.

Real-time qPCR amplification and analysis were performed using an Applied Biosystem with software version 3.1 (StepOne™, USA). The qPCR assay with the primer sets were optimized at the annealing temperature. All cDNA were in duplicate and including previously prepared samples, and non-template control (water to confirm the absence of DNA contamination in the reaction mixture).

Two different methods of analyzing data from real-time quantitative PCR experiments exist: Absolute quantification and Relative quantification (Rq).

1. Absolute quantification: Determine the input copy number of the transcript of interest, usually by relating the PCR signal to a standard curve.
2. Relative quantification (Rq): It describes the change in the expression of the target gene relative to some reference group such as healthy control.

Absolute quantification should be performed in situations where it is necessary to determine the absolute transcript copy number. In some situations it may be unnecessary to determine the absolute transcript copy number and reporting the relative change in microRNA expression will suffice.

The  $2^{-\Delta\Delta CT}$  Method: Quantifying the relative change in mRNA expression using real-time PCR requires certain equations, assumptions, and the testing of these assumptions to properly analyze the data. The  $2^{-\Delta\Delta CT}$  Method was used to calculate relative changes in mRNA expression determined from real-time quantitative PCR experiments. The choice of calibrator for the  $2^{-\Delta\Delta CT}$  Method depends on the type of mRNA expression experiment that has been planned. The simplest design is to use the healthy controls as the calibrator.

The threshold cycle (CT), the cycle number at which the fluorescent signal of the reaction crosses the threshold, was detected and incorporated in quantifying the relative changes in mRNA expression (Rq).  $\Delta CT$  values are calculated by subtracting the CT value of the endogenous control for a given sample from the CT value of the target mRNA for the given sample.

The  $\Delta\Delta CT$  is calculated by subtracting  $\Delta CT$  of an experimental sample from a control. Fold change (FC) is calculated by raising 2 to the power of the negative  $\Delta\Delta CT$  value, since CT values are related to the amount of mRNA logarithmically.

$$\Delta CT = CT_{(mRNA \text{ of interest})} - CT_{(endogenous \text{ control})}$$

$$\Delta\Delta CT = \Delta CT_{(patient)} - \Delta CT_{(control)}$$

$$FC \text{ (or Rq)} = 2^{-\Delta\Delta CT}$$

Using the  $2^{-\Delta\Delta CT}$  method, the data is presented as the fold change in mRNA expression normalized to an endogenous control

and relative to the healthy controls. For the healthy control sample,  $\Delta\Delta CT$  equals zero and  $2^0$  equals one, so that the fold change in mRNA expression relative to the healthy control equals one. For the samples, evaluation of  $2^{-\Delta\Delta CT}$  indicates the fold change in mRNA expression relative to the healthy control.

### 3. Statistical analysis

Data were coded and entered using the statistical package SPSS (Statistical Package for the Social Sciences) version 25. Data was summarized using mean, standard deviation, median, minimum and maximum in quantitative data and using frequency (count) and relative frequency (percentage) for categorical data. Comparisons between quantitative variables were done using the non-parametric Kruskal-Wallis and Mann-Whitney tests. Correlations between quantitative variables were done using Spearman correlation coefficient. Receiver Operating Characteristics (ROC) curve analysis was performed to explore the ability of Nephtrin, Podocin and Podocalyxin to differentiate between different groups. P values less than 0.05 were considered statistically significant. Graphs were used to illustrate some information.

### 4. Results

In group 1 (normo-albuminuria group) 7 males and 13 females were included with average age  $53.3 \pm 2.8$  years; In group 2 (micro-albuminuria group) 7 males and 13 females were included with average age  $52.8 \pm 4.6$  years; In group 3 (macro-albuminuria group) 7 males and 13 females were included with average age  $54.3 \pm 3.9$  years where Control group: twenty healthy non-diabetic controls 10 males and 10 females were included with average age  $52.0 \pm 4.1$  years. Laboratory results of studied groups discussed in Table (1). Pairwise comparisons for comparison of each group versus each other group discussed in Table (2).

The urinary levels of the mRNA of podocyte-specific markers studied; nephtrin, podocin, and podocalyxin (expressed in number of folds of the control) were found to be significantly elevated in all diabetic patients participating in this study ( $p$  value  $< 0.001$ ). Urinary nephtrin, podocin and podocalyxin levels were higher in patients with microalbuminuria (group II) compared to normoalbuminuric diabetic patients (group I) ( $p$  value 0.045) ( $p$  value  $< 0.001$ ), ( $p$  value  $< 0.001$ ). Urinary podocyte markers are more prevalent in diabetic patients with macroalbuminuria (group III) compared to normo and microalbuminuria (group I & II) (urinary nephtrin  $p$  value  $< 0.001$ ) (urinary podocin  $p$  value  $< 0.001$ ), (urinary podocalyxin  $p$  value  $< 0.001$ ).

Correlation study among all diabetics found a statistically significant positive correlation between urinary nephtrin ( $r = 0.397$ ,  $p$  value = 0.002), podocin ( $r = 0.433$ ,  $p$  value = 0.001), and podocalyxin ( $r = 0.431$ ,  $p$  value = 0.001) against serum creatinine Table (3). Urinary podocyte markers were found to be significantly correlated

**Table 1**  
The laboratory characteristics of our patients. eGFR: estimated glomerular filtration rate; HbA<sub>1c</sub>: Glycated hemoglobin, A/C ratio: albumin to creatinine ratio.

Variables	Group I	Group II	Group III	Control Group
S.creatinine mg/d L	0.7 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.7 ± 0.1
eGFR ml/min/1.73m <sup>2</sup>	101.2 ± 18.8	99.6 ± 19.0	80.5 ± 11.7	115.2 ± 16.9
HbA <sub>1c</sub> %	7.5 ± 0.7	7.7 ± 0.6	8.4 ± 0.8	5.1 ± 0.4
A/C ratio mg/g	20.2 ± 4.7	110.0 ± 61.2	662.9 ± 190.5	
Nephtrin	2.73 ± 0.68	3.34 ± 0.93	11.68 ± 2.46	
Podocin	1.28 ± 0.23	2.26 ± 0.44	10.33 ± 2.81	
Podocalyxin	0.66 ± 0.19	1.30 ± 0.39	4.62 ± 1.22	

**Table 2**  
Pairwise comparisons for comparison of each group versus each other group. eGFR: estimated glomerular filtration rate; HbA<sub>1c</sub>: Glycated hemoglobin, A/C ratio: albumin to creatinine ratio.

Variables	Global P value	Group I vs Group II	Group I vs Group III	Group II vs Group III
S.creatinine (mg/d L)	<0.001	0.655	<0.001	0.005
e-GFR (ml/min/1.73m <sup>2</sup> )	<0.001	0.524	0.001	<0.001
A/C (mg/gm)	<0.001	<0.001	<0.001	<0.001
HbA <sub>1c</sub> %	<0.001	0.357	0.001	0.003
Nephtrin	<0.001	0.045	<0.001	<0.001
Podocin	<0.001	<0.001	<0.001	<0.001
Podocalyxin	<0.001	<0.001	<0.001	<0.001

( $p$  value  $< 0.001$ ) with glycated hemoglobin levels of all diabetics included in the study Table (3). A statistically significant **negative** correlation ( $p$  value  $< 0.001$ ) was found between urinary podocyte markers and e-GFR while studying correlations among all diabetics (urinary nephtrin  $r = -0.476$ ), (urinary podocin  $r = -0.514$ ), (urinary podocalyxin  $r = -0.486$ ) Table (3).

Urinary nephtrin ( $r = 0.919$ ,  $p < 0.001$ ), podocin ( $r = 0.983$ ,  $p < 0.001$ ) and podocalyxin ( $r = 0.965$ ,  $p < 0.001$ ) were significantly correlated with albuminuria while studying correlations among all diabetics Table (4). Statistically significant correlations ( $p$  value  $< 0.001$ ) were found between urinary podocyte markers and albuminuria among normo-, micro-, as well as macro-albuminuric patients Table (4).

#### 4.1. ROC curves for the diagnosis of increased albuminuria

The diagnostic performance and accuracy parameters of podocyte-associated proteins for increased albuminuria were determined using the ROC curve shown in Figs. 1–3. Greater areas under the curve and better accuracy were found for podocin and podocalyxin than for nephtrin. Overall, the sensitivity was greater than 85%, and the specificity ranged between 50% and 95%. Positive predictive values ranged between 63% and 95%. Cutoff points to define high or low expressions of gene mRNA in the urine were determined using the ROC curve. The highest sensitivity and specificity were found at 3.30 (nephtrin), 1.82 (podocin), 0.85 (podocalyxin).

### 5. Discussion

In our study, we firstly determined the expression of podocyte associated genes in the urine of patients with varying stages of DN. The results showed that urinary nephtrin, podocin, and podocalyxin mRNA were significantly increased in DN patients compared with healthy controls. Significantly higher mRNA of podocyte specific molecules -in the urine of subjects with diabetes compared with controls-is compatible with podocytopathy.

This result is consistent with the findings of Zheng et al. [11], Jim et al. [12], do Nascimento et al. [13], and Lioudaki et al. [14]. Those studies showed an increased urinary expression of markers of podocytes; nephtrin, podocin and/or podocalyxin reflecting shedding of these cells in the urine with the loss of podocyte slit diaphragms and cytoskeletal integrity, rendering them a reasonable urinary marker.

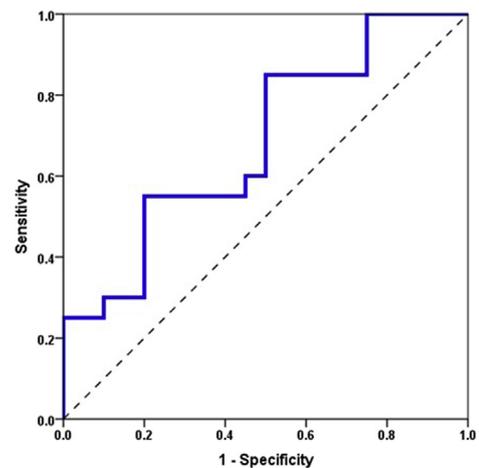
We investigated whether the expression of urinary podocyte mRNAs correlated with the progression of DN. Patients with DN were divided into three experimental groups based on their level of albuminuria. Interestingly, normoalbuminuric subjects with diabetes showed high mRNA levels of nephtrin, podocin, and podocalyxin (expressed in number of folds of the control) relative to controls, suggesting that podocyte damage may occur early in DN,

**Table 3**  
**Correlations between the mRNA levels of podocyte-associated proteins and different parameters in diabetic patients.** eGFR: estimated glomerular filtration rate; HbA<sub>1c</sub>: Glycated hemoglobin; Podxl: Podocalyxin; A/C ratio: albumin to creatinine ratio; r = Spearman correlation coefficient, P = P value.

			A/C (mg/gm)	Nephrin	podocin	Podxl	
Whole sample (n = 60)	Age	r	0.161	0.196	0.108	0.106	
		P	0.219	0.134	0.412	0.421	
	S.Creatinine	r	<b>0.415</b>	<b>0.397</b>	<b>0.433</b>	<b>0.431</b>	
		P	<b>0.001</b>	<b>0.002</b>	<b>0.001</b>	<b>0.001</b>	
	eGFR (ml/min/1.73m2)	r	<b>-0.479</b>	<b>-0.476</b>	<b>-0.514</b>	<b>-0.486</b>	
		P	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	
	HbA1c %	r	<b>0.561</b>	<b>0.657</b>	<b>0.535</b>	<b>0.542</b>	
		P	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	
	Normo-albuminuria	Age	r	0.126	0.083	-0.082	0.0
			P	0.596	0.727	0.730	1.0
		S.Creatinine	r	-0.122	-0.079	0.055	-0.254
			P	0.607	0.741	0.817	0.279
eGFR (ml/min/1.73m2)		r	0.001	-0.021	-0.318	0.180	
		P	0.997	0.930	0.171	0.447	
HbA1c %		r	<b>0.469</b>	<b>0.513</b>	0.265	<b>0.535</b>	
		P	<b>0.037</b>	<b>0.021</b>	0.259	<b>0.015</b>	
Micro-albuminuria		Age	r	0.095	0.091	-0.088	0.082
			P	0.691	0.702	0.711	0.733
		S.Creatinine	r	0.114	0.085	0.108	0.293
			P	0.633	0.721	0.652	0.209
	eGFR (ml/min/1.73m2)	r	-0.168	-0.166	-0.208	-0.279	
		P	0.478	0.484	0.378	0.233	
	HbA1c %	r	<b>0.673</b>	<b>0.712</b>	<b>0.656</b>	<b>0.585</b>	
		P	<b>0.001</b>	<b>&lt;0.001</b>	<b>0.002</b>	<b>0.007</b>	
	Macro-albuminuria	Age	r	<b>0.452</b>	<b>0.518</b>	0.367	-0.009
			P	<b>0.045</b>	<b>0.019</b>	0.111	0.971
		S.Creatinine	r	-0.089	-0.049	-0.111	-0.040
			P	0.710	0.837	0.642	0.866
eGFR (ml/min/1.73m2)		r	-0.290	-0.161	-0.303	-0.334	
		P	0.216	0.499	0.195	0.150	
HbA1c %		r	0.166	0.380	0.129	-0.225	
		P	0.485	0.098	0.587	0.340	

**Table 4**  
**Correlation of A/C (mg/gm) with Nephrin, Podocin and Podocalyxin within the whole group & within each group separately.** A/C ratio: albumin to creatinine ratio; r = Spearman correlation coefficient, P = P value.

		A/C (mg/gm)	
		r	P value
Whole sample (n = 60)	Nephrin	0.919	<0.001
	Podocin	0.983	<0.001
	Podocalyxin	0.965	<0.001
Normo-albuminuria (n = 20)	Nephrin	0.976	<0.001
	Podocin	0.698	0.001
	Podocalyxin	0.832	<0.001
Micro-albuminuria (n = 20)	Nephrin	0.994	<0.001
	Podocin	0.859	<0.001
	Podocalyxin	0.846	<0.001
Macro-albuminuria (n = 20)	Nephrin	0.880	<0.001
	Podocin	0.975	<0.001
	Podocalyxin	0.522	0.018

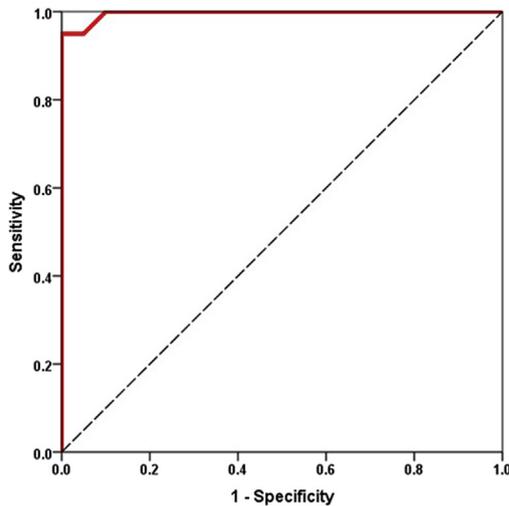


AUC	95% CI	Cut-off	Sensitivity	Specificity	PPV	NPV	Accuracy
0.685	0.520-0.850	≥ 3.30	85.0%	50.0%	63.0%	76.9%	67.5%

**Fig. 1.** ROC for Nephrin gene to differentiate group II from group I. AUC= area under the curve, CI= confidence interval, PPV= positive predictive value, NPV= negative predictive value.

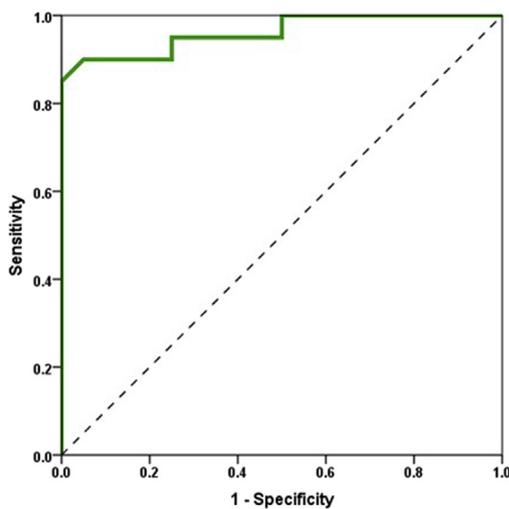
as has been reported by others [11,12,14,15].

A recent investigation by Jim et al. revealed that nephrinuria was present in 100% of type 2 diabetic patients with micro-albuminuria and macroalbuminuria [12]. In another study, nephrinuria was observed in 30% of normoalbuminuric type 1 diabetic patients, whereas none of the nondiabetic control subjects had



AUC	95% CI	Cut-off	Sensitivity	Specificity	PPV	NPV	Accuracy
0.996	0.986-1.0	≥ 1.82	95.0%	95.0%	95.0%	95.0%	95.0%

Fig. 2. ROC for Podocin gene to differentiate group II from group I. AUC= area under the curve, CI= confidence interval, PPV= positive predictive value, NPV= negative predictive value.



AUC	95% CI	Cut-off	Sensitivity	Specificity	PPV	NPV	Accuracy
0.961	0.904-1.0	≥ 0.85	90.0%	94.7%	90.5%	90.0%	92.5%

Fig. 3. ROC for Podocalyxin to differentiate group II from group I. AUC= area under the curve, CI= confidence interval, PPV= positive predictive value, NPV= negative predictive value.

nephriuria [16]. While further research is needed to confirm the above findings, urine nephrin levels could be a good biomarker of early diabetic kidney disease, as it appears to precede the development of microalbuminuria.

Elevated levels of urinary podocalyxin were observed in 53.8% of normoalbuminuric patients, indicating that urinary podocalyxin might be a useful biomarker for detecting early podocyte injury in diabetic patients [17]. Similar results were observed by Shoji et al. [15].

Pațari et al. proposed that before being shed, the injured podocyte suffers destabilization, altering the podocyte metabolism

and leading to the secretion of its molecular components, which can be detected earlier than albuminuria [16].

However, this result still seems controversial because of the damage to the filtration barrier and altered permselectivity may not be a uniform phenomenon. For example, Lemley et al. showed that only macroalbuminuric patients with DM type 2 had increased filtrations of high-molecular-weight dextrans through enlarged pores acting as molecular shunts. In patients with lower levels of albuminuria, the shunt size did not differ from that of normal controls [18].

To further analyze the correlation between urinary mRNA levels and renal functional parameters, we found statistically significant positive correlations between the three studied genes, serum creatinine and eGFR, on the other hand, showed an inverse relationship with the urinary expression of the three studied genes.

It is widely accepted that podocyte injury may trigger a sequence of events through epithelial-mesenchymal transition and apoptosis or detachment, to ultimately contribute to glomerulosclerosis and decline of renal function [19]. Our current study suggests that the detection of urinary podocyte-associated mRNAs may provide valuable information for evaluating the progression of diabetic nephropathy.

Overall, podocyturia was correlated with albuminuria and HbA1c, a finding that likely reflects the injury of uncontrolled hyperglycemia and associated mechanisms on the filtration barrier.

## 6. Study limitations

First, the study used a cross-sectional design and included a relatively small sample size. Second, molecular analyses were restricted to the urine sediment, because our patients had classical clinical presentations of DN and did not have a kidney biopsy. However, if we had included biopsies for atypical presentations, such as a faster decline of renal function and/or active urinary sediment, these could have led to skewed podocyte-protein expression of potential nondiabetic glomerulopathies.

## 7. Conclusion

Our study demonstrates that the urinary mRNA expression levels of nephrin, podocin and podocalyxin increase with DN progression. Quantification of urinary podocyte-associated molecules appears to reflect the severity of albuminuria and renal damage, suggesting that these podocyte specific genes may be used as biomarkers for DN progression.

## Ethical committee approval

The local ethical committee of the Internal Medicine department, School of Medicine, Cairo University, approved this work.

## Human and animal rights

All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

## Informed consent

“Informed consent was obtained from all individual participants included in the study”.

## Conflicts of interest

The authors have declared that no conflict of interest exists.

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