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ORIGINAL ARTICLE

Podocyte-associated messenger ribonucleic acids profile in urine of patients with idiopathic nephrotic syndrome and lupus nephritis.

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ABSTRACT

Background: Podocytes associated proteins are essential in the maintenance of a healthy glomerular filtration barrier, a spectrum of different glomerular diseases occurs due to podocyte abnormalities. Urinary podocytes mRNAs are a more accurate tool for monitoring the progression of different glomerular diseases than proteinuria.

Methods: Quantification of podocyte mRNAs in urinary sediment by real time polymerase chain reaction (PCR) of idiopathic nephrotic syndrome, lupus nephritis patients (LN) and the healthy controls, each group contains 15 individuals to be correlated with proteinuria level and eGFR (by MDRD equation).

Results: the urinary podocyte mRNAs in both idiopathic nephrotic syndrome and LN groups differed significantly in comparison to that of controls, there was a significant correlation when comparing renal function tests and estimated glomerular filtration rate (by MDRD equation) between nephrotic , LN groups and control group, the value of podocin showed significant correlation in active LN subgroup with its corresponding value in the non-active LN subgroup.

Conclusions: This study has revealed that urine pellet podocyte mRNAs can be used as a tool for monitoring the progression of idiopathic nephrotic syndrome and lupus nephritis patients and that urinary podocin can be used as a marker for lupus nephritis activity.

Keywords: Podocytes; nephrotic syndrome; Lupus nephritis; Proteinuria.

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INTRODUCTION

Podocytes have an essential role in the maintenance of the glomerular filtration barrier integrity by the expression of markers such as podocalyxin, synaptopodin, podocin and nephrin [1].Currently several experimental and clinical studies support the responsibility of podocyte injury in the occurrence of kidney disease progression and glomerulosclerosis [2].The clinical use of the urinary podocyte mRNAs in evaluation of the progression of various glomerular diseases may be more valuable than the current in use parameters, such as proteinuria levels and glomerular filtration rate, as revealed by preceding experimental studies and clinical trials [3].

Although the progression of glomerular diseases is associated with increase in the amount of proteinuria, some cases of membranous nephropathy and most patients with minimal change disease have marvelous prognosis with heavy proteinuria [4].Therefore, urinary podocyte mRNAs might be a better indicator of glomerular injury than proteinuria in different glomerulopathies [5].Different mechanisms of podocyte dysfunction in lupus nephritis patients were revealed such as podocyte structural damage due to immune complex deposition in classes III and IV, some cases of podocyte dysfunction is in the form of extensive effacement of foot process without evidence of inflammation, as in non-proliferative types of lupus nephritis **[6].**The levels of proteinuria are insignificantly correlated with immune complexes deposition and the activity of LN, but it correlates with the extent of histological pattern, degree of effacement of podocyte foot processes and the urinary podocyte mRNAs

[7]. Detection of podocyte mRNAs in urine has been proven to have an essential role in the glomerular diseases progression, So screening of podocyte mRNAs might be a novel technique in monitoring the glomerulopathies progression [8],[9].**The aim of this study** is to evaluate the value of detection urinary Podocyte mRNAs as a prognostic novel strategy in monitoring of idiopathic nephrotic syndrome and lupus nephritis patients

METHODS

This study is analytical case control study including 45 individuals was divided into three groups:Group (A) fifteen patients have idiopathic nephrotic syndrome.

- Group (B) fifteen patients have lupus nephritis.
- Group (C) fifteen healthy controls

All patients in this study were patients of the nephrology department at Theodor Bilharz Research Institute, (A) and (B) groups contained newly diagnosed, under treatment and clinically stable in remission patients.Inclusion criteria: patients according to clinical history and investigations suggesting the etiology of proteinuria, they were divided to two groups: idiopathic nephrotic syndrome patients and lupus nephritis patients.Exclusion criteria for patients**:** any Infection, symptoms and signs of other systemic diseases.All patients and controls were subjected to full clinical examination including age, weight, and clinical examination, routine laboratory tests (serum creatinine & blood urea, tests for albuminuria with a dipstick, 24-hour urinary proteins and estimated Glomerular filtration rate (eGFR) using the abbreviated modification of diet in renal disease (MDRD) equation).Serum creatinine and blood urea were measured by colorimetric assay, 24-hour urinary proteins were measured by Beckman Coulter AU480 analyzer (Beckman Coulter, Inc., Brea, California).Specific laboratory tests **{**quantification of podocyte mRNAs (synaptopodin, podocalyxin, α actin-4 and podocin) in urinary sediment by real time PCR}.

Methods of realtime PCR:

- **Collection of urine samples and total RNA extraction:**

Shortly after collection, the urine was centrifuged at 3000 rpm for 30 minutes at 4°C. The supernatant was discarded, and the remaining cell pellet was stored at -80 °C until use.

Total RNA was extracted according to the manufacturer's protocol (RNeasy Mini Kit, Qiagen, Germany)

The RNA concentration and purity were confirmed using the relative absorbance ratio at 260/280 on a nanodrop 2000 (Thermo, Wilmington, USA). RNA samples with a ratio higher than 1.8 were used for RT PCR.

- **Reverse transcription**

Reverse transcription according to the manufacturer's protocol RevertAid First Strand cDNA Synthesis kit (ThermoFisher Scientific, USA) 2 µL total RNA was mixed with 1 µL Oligo (dT)18 primer, 4 µL (5X) Reaction Buffer, 1 µL RiboLock RNase Inhibitor (20 U/µL), 2 µL (10 mM) dNTP Mix and 1 µL RevertAid M-MuLV RT (200 U/µL)

the solution and was completed to a volume of 20 µL with nuclease-free water.

Reverse transcription was performed at 42°C for 60 min, followed by an inactivation reaction at 70°C for 5 min. The resulting cDNA was stored at -20 °C until use.

- **Real-time PCR**

In the present study, relative abundance of synaptopodin, podocalyxin and α-actin4, podocin mRNA were quantified using the **StepOne Real-Time PCR System** (Applied Biosystems, California, USA). Human β-actin was used as a reference housekeeping gene. The following oligonucleotide primer sequences were used:

synaptopodin: forward 5'-CTTACGGCGGTGACATCTC, reverse 5'-GGTCCTGAGCCTCGATCC;

podocalyxin: forward 5' CTTGAGACACAGACACAGAG, reverse 5'-CCGTATGCCGCACTTATC;

α-actin4: 5'- GATGGTCTTGCCTTCAATG, reverse 5'- TGTTCACGATGTCCTCTG;

podocin: forward 5' TGGCTGTGGAGGCTGAAG, reverse 5'-TGAAGGGTGTGGAGGTATCG;

β-actin: forward 5'-TGGCACCCAGCACAATGAA, reverse 5'-CTAAGTCATAGTCCGCCTAGAAGCA

real-time PCR was proceeded as follow: 2 µL cDNA, 10 µL SYBR Green/ROX qPCR Master Mix (2X Maxima SYBR Green/ROX qPCR Master Mix (2X), 0.4 ml forward primer (10 mM), 0.4 µL reverse primer (10 mM), 0.4 µL ROX Reference dye and 6.8 µL nuclease free water were mixed to make a 20 µL reaction volume. All samples were run in duplicate.

The PCR technique was performed using a two-step process: 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60°C for 30 s. Then, dissociation curves (DC) and melting temperatures (Tm) were recorded.

The equation of target gene abundance/ housekeeping gene abundance was used to evaluate the level of expression of each gene. Controls consisting of ddH2O were negative in all runs.Nephrotic syndrome group consisted of eleven patients had membranous nephropathy

(MN) and four patients with focal segmental glomerulosclerosis (FSGS) according to renal biopsy, two patients from the patients presented with MN had a document for positive antiphospholipase A2 receptor antibody, but the others were diagnosed as idiopathic MN after exclusion of secondary causes.All patients in lupus nephritis (LN) group were diagnosed to be SLE patients according to American college of rheumatology diagnostic criteria; they were also diagnosed to have LN by clinical examination, proteinuria, active urinary sediments, elevation of renal function tests and kidney biopsy during their previous follow up.LN group is divided into 2 subgroups according to LN activity at the time of the study, active LN patients (9 patients) and Non-active LN patients (6 patients), the activity of SLE and LN is determined by clinical manifestations, renal function, ESR, C3 &C4 level, anti-double strand deoxyribonucleic acid (Anti ds DNA) titer, albumin, CBC, 24 hours urine collection for proteins and urine analysis for hematuria. CRP was done to all LN group with negative result (below 3 mg/l) **(table 1).**

ETHICAL CONSIDERATIONS

The work has been carried out in accordance with the code of ethics of the world medical association (Declaration of Helsinki) for studies involving humans.Written informed consents were obtained from all patients. Approval by ethical research committee in Theodor Bilharz Research Institute (TBRI) and IRB research committee of Zagazig Faculty of Medicine were included.

STATISTICAL ANALYSIS

Results are expressed as mean ± SD or numbers. Comparison between categorical data was performed using Chi square test or Fisher exact test instead if cell count was less than 5. Test of normality, Kolmogorov-Smirnov test, was used to measure the distribution of data. Accordingly, data were not normally distributed, so comparison between variables in the two groups was performed using Mann Whitney test while comparison between the three groups was performed using Kruskal Wallis ANOVA test followed by Mann Whitney test if significant results were recorded. Correlation between different variables in each group was performed using Spearman's Rank correlation coefficient, The Statistical Package for Social Sciences (SPSS) computer program (IBM Corp. Released 2013.IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp) was used for data analysis. P value ≤ 0.05 was considered significant**.**

RESULTS

In our study, we found a statistical significant increase in the value of alpha actin 4 in nephrotic group when compared with its corresponding value in control group (p= 0.001).There was a significant decrease in the value of synaptopodin and podoclyxin when compared with its corresponding value in control group (p= 0.001) **(table3).**

On the other hand in patients with LN group, we found that the increase in the value of alpha actin 4, synaptopodin and podocin differed significantly with its corresponding value in control group (p= 0.001) **(table3).**There was also a statistically significant correlation when comparing renal function tests and estimated glomerular filtration rate (by MDRD equation) between nephrotic, LN groups and control group **(table 2).**There was a significant difference between the active &non active LN subgroups and control group as regard the 4 markers, the value of podocin showed significant correlation in active LN subgroup with its corresponding value non-active LN subgroup (p= 0.007), but the other three markers (Alpha actin 4, synaptopodin and podocalyxin) there were no statistical significant difference between non-active and active LN subgroups **(table 4).**

Table 1: Comparison between the parameters of SLE and LN activity between non active and active subgroups.

	Non active (n= 6)	Active (n= 9)	p value
S. creatinine (mg/dl)	1.22 ± 0.44	1.75 ± 1.09	0.157
ESR (mm/1st hour)	11.17 ± 2.71	82.78 ± 16.41	0.001*
C3 (mg/dl)	138.50 ± 26.89	28.78 ± 10.78	0.001*
C4 (mg/dl)	36.33 ± 7.17	9.00 ± 1.94	0.001*
Antids.DNA (IU/ml)	32.17 ± 5.27	89.22 ± 10.28	0.001*
Albumin (mg/dl)	4.10 ± 0.31	2.99 ± 0.29	0.001*
Hb (g/dl)	11.30 ± 0.57	9.31 ± 0.78	0.002*
WBCs (10^9/L)	8.08 ± 1.45	4.28 ± 0.55	0.001*

	Non active (n= 6)	Active (n= 9)	p value
PLT(10^9/L)	320.67 ± 62.66	142.89 ± 17.58	0.001*
Proteinuria (gm/24 h)	1.04 ± 0.82	2.19 ± 0.75	0.013*
Hematuria			
<5/HPF	6 (100%)	0 (0.0%)	0.001*
>5/HPF	0 (0.0%)	9 (100%)	

ESR: Erythrocyte sedimentation rate, Antids.DNA: anti-double stranded deoxyribonucleic acid, HB:hemoglobin, Wbcs: white blood cells, PLT: platelets.
Data are expressed as mean ± SD or numbers, #= Chi square test or Fisher exact test,
$= Mann-Whitney test,*p< 0.05= significant,p> 0.05= Not significant.

Table 2: Comparison between the values of age, gender, weight, renal functions, proteinuria, eGFR by MDRD equation in the three studied groups.

	Control (n= 15)	Nephrotic (n= 15)	LN (n= 15)	p value	
				Control vs nephrotic	Control vs LN
Age (years)	30.87 ± 8.50	43.40 ± 13.92	31.27 ± 11.68	p= 0.010*	p= 0.755
Gender (F/M)	9/6	4/11	12/3	p= 0.139	p= 0.427
Weight (kg.)	74.67± 11.39	77.10 ± 11.30	7 3.40 ± 11.59	p= 0.406	p= 0.884
S. creatinine (mg/dl)	0.77 ± 0.10	1.19 ± 0.60	1.43 ± 0.78	p= 0.001*	p=0.001*
S. urea (mg/dl)	24.73 ± 3.26	42.74 ± 25.39	54.17 ± 37.96	p= 0.034*	P=0.001*
Proteinuria (gm/day)	0.30 ± 0.00	4.25 ± 2.67	1.73 ± 0.95	----	----
eGFR (MDRD) (ml/min/1.73m2)	106.95± 6.97	80.02 ± 29.04	59.15 ± 26.30	p= 0.008*	p=0.001*

LN: lupus nephritis, n: Number, kg: kilogram, F/M: female/male, eGFR: estimated glomerular filtration rate, MDRD: Modification of diet in renal disease.
Data are expressed as mean ± SD or numbers, *p< 0.05= significant, p> 0.05= not significant.

Table3: Comparison between values of alpha actin 4, podocin, synaptopodin and podocalyxin in the three studied groups.

	Control (n= 15)	Nephrotic (n= 15)	LN (n= 15)	p value	
				Control vs nephrotic$	Control vs LN$
Alpha actin 4	1.01 ± 0.14	3.21 ± 1.34	2.42 ± 0.82	0.001*	0.001*
Podocin	1.02 ± 0.25	1.81 ± 1.71	3.52 ± 2.96	0.494	0.001*
Synaptopodin	1.03 ± 0.24	0.49 ± 0.65	1.85 ± 0.35	0.001*	0.001*
Podoclyxin	1.03 ± 0.25	0.42 ± 0.45	0.87 ± 0.96	0.001*	0.021*

LN: lupus nephritis, n: Number.
Data are expressed as mean ± SD,*p< 0.05= Significant, p> 0.05= not significant.

Table4: Comparison between values of alpha actin 4, podocin, synaptopodin and podocalyxin in both non active and active LN subgroups and control group.

				Between groups p value			
	Control (n= 15)	**Non-active LN (n= 6)**	**Active LN (n= 9)**	**Control vs Non-active**	**Control vs active**	**Non-active vs active**	**Overall p value**
Alpha actin 4	1.01± 0.14	1.91 ± 0.75	2.76± 0.72	p= 0.001*	p= 0.001*	p= 0.126	p= 0.001
Podocin	1.02± 0.25	1.62 ± 0.82	4.79 ± 3.22	p= 0.016*	p= 0.001*	p= 0.007*	p= 0.001
Synaptopodin	1.03± 0.24	1.71 ± 0.27	1.94 ± 0.38	p= 0.001*	p= 0.001*	p= 0.126	p= 0.001
Podoclayxin	1.03± 0.25	0.64 ± 0.91	1.03 ± 1.01	p= 0.019*	p= 0.019*	p= 0.157	p= 0.035

LN: lupus nephritis, n: Number.
Data are expressed as mean ± SD,*p< 0.05= Significant, p> 0.05= not significant

DISCUSSION

Urinary podocytes loss accelerates glomerulosclerosis in the presence of a glomerular injury; this is mostly due to the inability of podocytes division in vivo. Normally daily podocyte loss does not lead to proteinuria as the podocytes pool exceeds the requirement of a glomerulus throughout a lifespan, and the exposed glomerular basement membrane (GBM) is shielded by podocytes. When urinary podocytes loss outnumbers its normal level, urinary protein loss appears, so proteinuria is considered a late event. So the search for early non-invasive urinary markers is essential to prevent the progression of glomerular injury and for follow-up after the treatment prescription **[10].**The detection of urinary mRNAs of podocytes by real time PCR can measure low abundance genes from even one single cell, and provides information for the progression of associated diseases **[11].Wickman et al** studied a large number of patients with different glomerulopathies detecting that, in those with biopsy-proven glomerular disease the urine podocyte mRNAs increased 79-fold in relation to controls, and these patients progressed to end-stage renal disease, thus supporting the hypothesis of podocyte depletion. After management, urinary podocyte mRNAs became at baseline values on disease remission. Subsequently, monitoring urine podocyte mRNAs may affect the management and outcome of patients with various glomerulopathies **[3].**The results of this study documented that there is low correlation between proteinuria and urinary m RNA of podocytes especially in patients with membranous nephropathy (most of the patients of the nephrotic group).

Hara et al and Fukuda et al both documented that renal function measures the effect of the accumulation of loss and damage of podocytes over time, which includes periods of increase and decrease podocyte loss that may be as a result of success of therapy. That cumulative podocyturia over time reflects the progression of glomerular disease. So, these result support that urinary mRNAs of podocytes provide disparate and supplementary information, which can be complementary to proteinuria **[12], [13].**

Yu et al stated that identification of podocyte mRNAs in urine is a more specific measure of disease activity than proteinuria**[14],** and this concept is supported by the data of **Troyanov et al and Heeringa et al** as they proved that various glomerular diseases exhibited different relations between urinary protein loss and podocyte depletion rate. in membranous nephropathy no correlation between podocyte detachment rate and proteinuria levels were recognized, This result is homogeneous with the clinical experience that disease progression in membranous nephropathy is not closely linked to the extent of proteinuria **[15],[16].Wang and his colleagues** documented that urinary mRNAs of podocytes were identified with lupus nephritis (LN) and diabetic nephropathy patients, and they have postulated these results to the disease progression **[17].Bollain et al** were proven that decrease of podocytes significantly correlated with the progressive excretion of podocytes in urine and proteinuria in LN patients also **Sabino et al** documented that urinary mRNAs of podocytes correlated with the albumin/creatinine ratio and both are associated with a significant correlation with the degree of LN

activity **[18],[19].**Also **Wang et al** showed that the levels of urine podocyte mRNAs were higher in active lupus nephritis patients compared with those with inactive disease; they also correlated with urinary protein levels and decline kidney function **[20].**As regarding, the observation in this study that Podocin is significantly correlated to LN activity, it was also supported by the result of **ElShaarawy et al** as they proved that urinary podocin was sensitive and specific in relation to LN activity and may be used clinically as a prognostic marker in LN patients **[21].**This concept was supported by the data of **Sabino AR et al** as they studied The podocyturia by indirect immunofluorescence technique by utlilizing primary antibodies to mRNAs of podocytes (anti podocin,synaptopodin,nephrin), they found that podocin positive cells were significantly associated with the LN severity so they concluded that anti-podocin antibody was the most appropriate biomarker in comparison to anti-synaptopodin and anti-nephrin in monitoring the LN activity**[19].**

In contrast to our results **Abo Ghanima et al** revealed that levels of urinary podocalyxin were increased in patients with various glomerularpathies and diabetic patients **[22].**

Sir Elkhatim et al documented that the levels of urine podocyte mRNAs have been clinically used with different degrees of success to evaluate various glomerular diseases and the detection of urine podocyte mRNAs may become a significant noninvasive method in different glomerular disease evaluation **[23].**So as consequence from the previous mentioned studies and data, these all hypothesize that urinary mRNAs of podocytes detection could be used as markers for the different glomerular disease prognosis and follow up**[13].**The limitation of our study includes the small sample size in each group, the inability to do serial follow up of m RNA of podocytes expression by real time PCR for each patient to correlate the results with serial renal function tests, and estimated GFR and no renal biopsy was done to any patient in the study so no correlation of the results of the urinary mRNAs of podocytes with classes of LN.This study is considered as a step in the way, and we are in need of further studies in the field of urinary podocyte mRNAs to support the clinical use of this novel technique in monitoring the glomerular disease progression.

CONCLUSION

This study has revealed that urine pellet podocyte mRNAs can be used as a tool for monitoring the progression of idiopathic nephrotic syndrome and lupus nephritis patients and that urinary podocin can be used as a marker for lupus nephritis activity.

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