ORIGINAL ARTICLE

EXPRESSION OF NEPHRIN IN EARLY DIAGNOSIS OF DIABETIC NEPHROPATHY

Moazzam A. Shahid[1], Saeeda Baig[2], Adnan M. Zubairi[3], Farha Ahmed
[1] Department of Biochemistry, Ziauddin University, Karachi
Department of Community Health Sciences, Ziauddin University, Karachi.

ABSTRACT

Background and Objectives: Diabetic nephropathy occurs as a result of proximal tubule dysfunction with podocyte damage due to increased advanced glycation end-products' insult in diabetes. Nephrin, one of the three proteins that make up the podocyte architecture, is excreted foremost following renal damage. The aim of this study was to evaluate the efficiency and reliability of nephrin for an early biomarker of kidney damage in diabetic patients.

Methods: Urine samples (78) were collected from diabetic center. Protein and glucose were determined by Dipstick. The patients were grouped on the basis of Albumin/creatinine ratio (UACR) as normoalbuminuric, Microalbuminuric and Macroalbuminuric with UACR less than 30 mg/g, from 30-300mg/g, and above 300mg/g respectively. ELISA KIT (Exocell USA) was used for Nephrin estimation. Statistical evaluation was done on SPSS version 20.

Results: Nephrinuria was present in 70(89.7%) out of 78 diabetic patients including 35(81.4%) of 43(55.1%) normoalbuminurics, 5(6.4%) of 30(38.5%) microalbuminurics and all 5 of macroalbuminurics (p=0.027). Nephrinuria was found associated with duration of diabetes, 21(91.3%)/23 were positive with less than three years of disease, 24(92.3%)/26 with three to seven years and 25(86.2%)/29 in more than seven years (p=0.039). Nephrin levels were found increasing from normo- (0.86ug/ml) to Micro- (11.6µg/ml) to Macroalbuminuria group (47.6µg/ml), compared to 0.15 µg/ml in comparison group.

Conclusion: The increase in Nephrin levels from 0.86µg/ml in patients with normal albuminuria to 47.6µg/ml in patients with macroalbuminuria suggests that Nephrin precedes albumin in urine predicting early signs of kidney damage. Nephrin biomarker can be used as predictor for early diagnosis of Nephropathy in diabetics.

KEYWORDS: Diabetic Nephropathy; Albuminuria; Podocytes

Corresponding Author
Moazzam Shahid
Research Assistant
Department of Biochemistry, Ziauddin University. Karachi.
Email: Moazzamshahid126@gmail.com

INTRODUCTION

Diabetic Nephropathy, the most frequent microvascular complication of diabetes mellitus worldwide, is prevalent in 20.2% population of Pakistan[1, 2]. Microalbuminuria appears as the first clinical sign of diabetic nephropathy which progresses to macroalbuminuria leading to complete renal failure and finally dialysis and renal replacement therapy[3]. Albumin is not part of the filtration barrier but leaks from the blood when the filtration barrier starts to break down. Microalbuminuria, therefore, cannot predict kidney damage and does not always lead to progressive renal failure in diabetes mellitus.[4] Yet, for complete renal failure albuminuria is considered as a good marker[5,6] and has been reported as a diagnostic biomarker for diabetic kidney disease as well as for other pathological conditions such as cardiovascular morbidity and cardiovascular mortality[7-9]. Thus a novel biomarker is required that would be part of the structural components of kidney. In the recent years many renal biomarkers have been researched for early prediction of renal damage. Among these biomarkers, Nephrin, a transmembrane protein, has been identified as the right candidate for not only prediction of diabetic

nephropathy but also the severity of the damage to podocytes by evaluating its levels in the blood.[10-12]

Nephrin is an important component of the slit diaphragm located between the foot processes of the podocytes and being a part of renal filtration diaphragm it is likely to be excreted first in case of damage to the filtration barrier[14]. Variations in nephrin can lead to the limitation of the size-selectivity of the slit diaphragm.[13] The aim of this study was to evaluate the efficiency and reliability of nephrin as an early biomarker of kidney damage.

METHODS

a) Study design and participants

Random urine samples from 78 patients were collected from a private diabetic clinic after a written consent from the patient and the permission from the primary diabetologist. The study was approved by Ziauddin Ethics Review Committee. Hypertensive patients and patients with urinary tract infection were excluded.

b) Procedures and variables assessment

A Urine dipstick test was performed for analysis of urinary glucose and protein by combur 10 Urine test strip (Roche), Germany. Albumin levels were determined by measuring albumin/creatinine ratio, using Roche kit by immunoturbimetic assay method on automated analyzer. We used Roche modular P-800i as an analyzer for albumin measurements.

Urinary nephrin levels were analyzed by Enzyme Linked Immunosorbent Assay (ELISA), Exocell Inc., Philadelphia,USA.

According to instructions by the manufacturer, Urine samples were diluted after checking the degree of turbidity and made into three dilutions 1:10, 1:20 and 1:40 in the given buffer. We added 50 µL of diluted sample in each well which were coated with rat nephrin then added 50-µL of rabbit anti-nephrin antibody. It was incubated for 60 minutes at room temperature. After washing with 100-µL second anti-rabbit HRP antibody to conjugate was added to each well and again incubated for 60 minutes at room temperature. After washing color developer was added, absorbance was read at 450 nm. Elevated levels of urinary nephrin or nephrinuria were compared with urinary nephrin 0.15 µg/ml. This value was based on eight healthy subjects who had constant urinary nephrin levels of 0.15 µg /ml.

c) Statistical analysis

Difference in Urinary nephrin between groups was determined by Kruskal-Wallis, Chi-square, Mann Whitney was used to assess associations of categorical variables, and Categorical variables were presented as percentages, while continuous variables were presented as median or mean ± standard deviation (SD). Difference in urinary nephrin between groups was determined by Kruskal-Wallis. Pearson's correlations were calculated. Analysis were done using SPSS version 20 and results were considered statistically significant at $P<0.05$.

RESULTS

The demographic details of all patients are given in **Table 1**. The 78 diabetic patients finalized included 37(47.4%) males and 41 (52.6%) females. Mostly diabetic patients were from Urdu speaking group 37(47.4%), belonging to low socioeconomic 55(69%) class with majority earning less than Rs. 20,000/month and having education up to Middle (class 8), 32(41.0%).

Table 1: Demographic details of population studied.

Variables	Sub type	Frequency n=78	Percent
Gender	Females	37	47.4%
	Males	41	52.6%
Ethnicity	Pathan	2	2.6%
	Punjabi	33	42.3%
	Sindhi	6	7.7%
	Urdu Speaking	37	47.4%
Income	<20,000	55	69.4%
	20000 to 30000	20	28.5%
	>30000	4	5.2%
Education	Bachelor	4	5.1%
	Intermediate	11	14.1%
	Master	4	5.1%
	Matric	24	30.8%
	Middle	32	41.0%
	No formal education	3	3.8%

The correlation of Nephrinuria with albuminuria (normoalbuminuria, microalbuminuria, and macroalbuminuria) was assessed **(Graph 1)**. Seventy patients out of 78 were positive for nephrinuria including 35(81.4%) of 43(55.1%) normo-albuminurics, 5(6.4%) of 30(38.5%) micro-albuminurics and all 5 of macro-albuminurics ($p=0.027$). Nephrinuria was found associated with duration of diabetes, 21(91.3%)/23 were positive with less than three years of disease, 24(92.3%)/26 with three to seven years and 25(86.2%)/29 in more than seven years ($p=0.039$).

Nephrin levels showed a steady increase with the progression of the disease, from normo-albuminuria group (0.86 µg/ml) to Micro-albuminuria (11.6 µg/ml) to Macro-albuminuria group (47.6 µg/ml), compared to 0.15 µg/ml in the comparison group **(Table 2)**.

Log graph shows correlation of log natural of micro-albuminuria and log natural of nephrinuria. Nephrinuria is directly proportional to subgroups of albuminuria include normal albuminoria, micro albuminoria & macro albuminoria. **(Figure 1)**

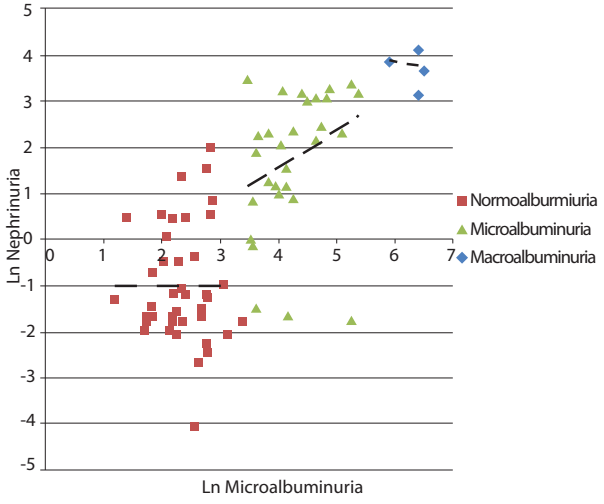

Figure 1: Correlation of Log graph of nephrinuria with albuminuria

Table 2: Association of Nephrinuria with Albuminuria

Groups according to albumin levels	Alnuminuria	Nephrinuria					
		Positive		Negative		P Value	Nephrin levels (mean ug/ml)
	Diabetics n=78(%)	n=70	%	N=08	%		
Normoalbuminuria	43 (55.1%)	35 (50%)	81.4%	8 (100%)	18.6%		0.86
Microalbuminiuria	30 (38.5%)	30 (42.9%)	100%	0 (0%)	0%	0.027	11.6
Macroalbuminuria	05 (6.4%)	05 (7.1%)	100%	0 (0%)	0%		47.6

Nephrinuria also correlated with duration of diabetes mellitus **Table 3** and with normo-, micro- and macroalbuminuria with the first group (n=23) having18 (78.3%), 05 (21.7 %), and none diabetic patients respectively. The second group (n=26) having 14 (53.84%), 10 (38.5%), and 02 (7.7%) patient respectively. The third group (n=29) having 12(14.4%), 14 (48.3%), and 03(10.4%) patient respectively.

Table 3: Association of Nephrinuria with duration of Diabetes mellitus

Duration of Diabetes	Nephrinuria n=78		P - value
	Low (Negative) N=8	High (Positive) N=70	
<3 years (n=23)	2(8.7%)	21(91.3%)	0.039
3 to 7 years (n=26)	2(7.7%)	24(92.3%)	
>7 years (n=29)	4(13.8%)	25(86.2%)	

DISCUSSION

Despite having normal albumin levels 90% of diabetic patients were positive for nephrin. Nephrin levels were found increasing from Normoalbuminurics (0.86µg/ml) to Microalbuminurics (11.6µg/ml) to Macroalbuminurics (47.6µg/ml), compared to 0.15 µg/ml in the comparison group. When patients were evaluated for presence of nephrin according to normo-, micro-, and macroalbuminuria, nephrinuria was found to be present in 100% of diabetic patients with micro- and macroalbuminuria, as well as 81.4% of patients with normoalbuminuria. Similar findings have been reported by studies in United States where nephrinuria also correlated significantly with albuminuria (rho = 0.89, p<0.001) detecting nephrinuria in 54% of normoalbuminuric Type 2 DM patients suggesting its potential role as an early biomarker of diabetic nephropathy (DN).[16]

Other studies on nephrin done on urine samples of patients with Type 1 Diabetes also substantiated our study. They detected nephrin in only 28% of those who developed albuminuria recently, whereas, 30% of normo-, 17% micro-, and 28% macroalbuminurics had nephrin present. They concluded that presence of nephrin in normal patients with normal albumin could be used as a prediction for developing Diabetes.[17] This makes Nephrin a podocyte-specific protein, presence of which can be linked with the course of diabetes and destruction of podocytes.[13] A comparison group (non diabetic), tested for urine nephrin, showed minute quantities of 0.15µg/ml which can be explained as the normal wearing and regeneration of glomerular apparatus, whereas, other studies on controls reported absence of nephrin.[17]

Nephrinuria and albuminuria, both were found associated with duration of diabetes. Ninety one

percent of diabetics who had onset history of less than three years were nephrin positive. This is in contrast to findings by other researchers who did not observe any statistical association of albuminuria with the 5 years of duration of the diabetes.[18] Albuminuria or microalbuminuria appears after five or more years of diabetes mellitus when almost or complete damage of podocytes has occurred. This proves that it cannot be a reliable early marker. The relationship between Microalbuminuria and renal impairments in type-2 diabetes has been reported by several studies that prove that clinical proteinuria appears after 9 years of DM on the average and demand a need to explore a new biomarker for assessing diabetic kidney disease in early or pre-clinical stage.[19,20] Whereas, our study also confirms that nephrin starts appearing in urine in less than 3 years of onset of diabetes.

In 43 patients UACR was less than 30 mg/g, with normal albuminuria, whereas, nephrin (0.86µg/ml) was present in 35(81.4%) of them. An independent correlation between nephrinuria and UACR eGFR exist which shows that nephrinuria is associated with decreased levels of eGFR, even in normo-albuminuric patients. This shows that nephrinuria may be potentially involved in the development of renal insufficiency even at the stage of normo-albuminuria, whereas, generally such patients are considered at low risk of developing Chronic Kidney Disease.[21] Thus a number of studies on Type 1 and Type 2 DM patients with normoalbuminuria have shown the importance of nephrin as an early biomarker of diabetes proving that Nephrinuria may precede albuminuria.[16, 17, 21]

Nephrin, since is a component of the slit diaphragm located between the foot processes of the podocytes, any variation in podocyte may lead to slit diaphragm size-selectivity restriction allowing leaking of certain elements. Nephrin may also leak out from podocytes in certain abnormal circumstances. This is currently uncertain whether these are shed as byproducts of an active vesicular transport process from podocyte foot or are released during apoptotic process after the podocyte is detached and leaks into urine.[22, 23] To further establish this fact a follow up study with bigger sample size should be done.

The steady increase in Nephrin levels from 0.86µg/ml in patients with normal albuminuria to 47.6µg/ml in patients with macroalbuminuria suggests that Nephrinuria predicts kidney damage and it precedes albuminuria. Nephrin estimation may be used as a pre-Clinical diagnostic biomarker of Nephropathy in diabetes mellitus.

Acknowledgement: This study was supported by Ziauddin University Postgraduate Program.

Competing Interest: Authors declare that they have no competing interests.

Authors' contributions: This work was carried out in collaboration between all authors. Authors MAS, SB designed the study. Author MAS collected the samples, did the bench work, wrote the protocol and wrote the first draft of the manuscript. Author SB facilitated in bench work, literature search and finalization of manuscript. Author AMZ helps in research protocol and FA assisted in analyses of the data. All authors read and approved the final manuscript.

REFERENCES

1. Abougalambou SSI, Mohamed AH, Syed Azhar SS. et al. Prevalence of Vascular Complications among Type 2 Diabetes Mellitus Outpatients at Teaching Hospital in Malaysia. J Diabet Metabol.2011; 2:115.
2. Shera AS, Jawad F, Maqsood A.et al.Prevalence of chronic complications and associated factors in type 2 diabetes.J Pak Med Assoc. 2004; 54:54-9.
3. Remuzzi G, Schieppati A. and Ruggenenti P., "Nephropathy in patients with type 2 diabetes," NEJM. 2002; 346:1145–51.
4. Karalliedde J, Viberti G. Proteinuria in diabetes: bystander or pathway to cardiorenal disease?J Am Soc Nephrol. 2010; 21:2020–27.
5. Matheson A., Willcox M. D. P., Flanagan J., and Walsh B. J., Urinary biomarkers involved in type 2 diabetes: a review,"Diabetes Metabolism Research and Reviews.2010; 26:150–71,.
6. De Zeeuw D., Ramjit,D. Zhang, Z. et al., Renal risk and renoprotection among ethnic groups with type 2 diabetic nephropathy: a post hoc analysis of RENAAL, Kidney International.2006; 69:1675–82.
7. Ruggenenti P. and Remuzzi, G. Time to abandon-microalbuminuria?Kidney International. 2006; 70: 1214–22.
8. Ninomiya T, Perkovic V, de Galan BE, et al., Albuminuria and kidney function independently predict cardiovascular and renal outcomes in diabetes.-JASN.2009; 20: 1813–21.
9. Klaus K, Knut BJ, Bo FR, et al., Very low levels of microalbuminuria are associated with increased risk of coronary heart disease and death independently of renal function, hypertension, and diabetes. Circulation. 2004; 110: 32–35.
10. Langham RG, Kelly DJ, Cox AJet al, Proteinuria and the expression of the podocyte slit diaphragm protein, nephrin, in diabetic nephropathy: Nephrol Dial Transplant .2004 19: 262–64.

11. Proletov I, Galkina O, Bogdanova Eet al, Clinical significance of podocyte injury markers evaluation in patients with primary glomerulopathies. Nephrol Dial Transplant .2014; 29:193.
12. Tchebotareva N, Bobkova I, Kozlovskaya Let al, Assessment of podocyte dysfunction and urinary podocyte loss in chronic glomerulonephritis (CGN): Significance for estimation of clomerular damage and glomerulosclerosis risk. Nephrol Dial Transplant. 2012; 27:195.
13. Ligia P, Adrian V,Gheorghe G, et al. Proximal Tubule Dysfunction Is Associated with Podocyte Damage Biomarkers Nephrin and Vascular Endothelial Growth Factor in Type 2 Diabetes Mellitus Patients: A Cross-Sectional Study. 2014; 9, plose one.
14. Kawachi H, Koike H, Kurihara H, et al. Cloning of rat nephrin: Expression in developing glomeruli and in proteinuric states. Kidney Int .2000; 57:1949-61.
15. Proletov I, Galkina O, Bogdanova E, et al: Clinical significance of podocyte injury markers evaluation in patients with primary glomerulopathies. Nephrol Dial Transplant. 2014; 29:3193.
16. Jim B, Ghanta M, Qipo A, et al. Dysregulated nephrin in diabetic nephropathy of type 2 diabetes: a cross sectional study. PLoS One. 2012; 7: 3604.
17. Patari A, Forsblom C, Havana M,et al, Study Group Nephrinuria in diabetic nephropathy of type 1 diabetes. Diabetes. 2003; 52:2969–74.
18. Kandasamy Y, Smith R, Lumbers ER, et al ; Nephrin - a biomarker of early glomerular injury. 2014; 23:2:21.
19. Mogensen CE: Microalbuminuria predicts clinical proteinuria and early mortality in maturity-onset diabetes. N EnglJ Med. 1984;310:356–60,
20. Seema D, Pierre F, Flbert M, et al,Microalbuminuria in type 2 diabetes and hypertension diabetes care.2008;31: 194 -20.
21. Ng DPK, Tai BC, Tan E, et al : Nephrinuria associates with multiple renal traits in type 2 diabetes. Nephrol Dial Transplant. 2011; 26:2508–14.
22. Petermann AT, Pippin J, Krofft R, et al. Viable podocytes detach in experimental diabetic nephropathy: potential mechanism underlying glomerulosclerosis. Nephron Exp Nephrol. 2004; 98:114-23.
23. Russo LM, Sandoval RM, Campos SB, et al. Impaired tubular uptake explains albuminuria in early diabetic nepropathy. J Am Soc Nephrol. 2009; 20: 489–94.