RESEARCH ARTICLE

Determination and comparison of urinary albumin creatinine ratio, neutrophil gelatinase associated Lipocalin and Cyclophilin A in relation to type 2 diabetes duration

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Abstract

Objective: To determine and compare the levels of Urinary Albumin Creatinine Ratio, Neutrophil Gelatinase Associated Lipocalin and Cyclophilin A among groups based on duration of Type 2 diabetes.

Methodology: This is a cross-sectional comparative study. The study was conducted in Chemical Pathology Department of University of Health Sciences, Lahore, Pakistan from December, 2021 to July, 2022. Convenient sampling technique was used. All together 84 patients were included from outpatient department in this study and were classified into 3 groups according to the duration of Type 2 Diabetes Mellitus. Group A= ≤ 1 year (Control group), Group B= > 1 year and < 5 years, Group C= ≥ 5 years and < 10 years. Data was analyzed using SPSS 24. ANOVA was used to compare mean levels of urinary albumin creatinine ratio (ACR), Neutrophil Gelatinase Associated Lipocalin (NGAL) and Cyclophilin A (Cyp A) among the groups. A p-value ≤ 0.05 was considered as significant.

Results: The mean age of the patients in Group A, B and C was 49.1±12.8, 51.1±13.5 and 50.0±12.3 years respectively. The results of the comparison showed that the patients having diabetes for more than five years had the highest levels of urinary Cyp A (26.9±1.8 ng/mL), NGAL (119.9±6.6 ng/mL) and ACR (1284.8±222.1 mg/g). The comparison of urinary ACR with Cyp A and NGAL in the three groups showed that the urinary Cyp A and NGAL started appearing in the urine before the ACR in diabetics even when the ACR was still in normal range (< 30 mg/g).

Conclusion: The urinary Cyp A, NGAL and ACR showed significant rise with the duration of the disease. Additionally, urinary NGAL and CypA could be used as a marker for early detection of diabetic nephropathy.

Key Words: Lipocalin-2, NGAL protein, Cyclophilin A, Diabetes Mellitus, Type 2, Albumin to Creatinine ratio, Diabetic Nephropathy, Albuminuria.

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Introduction

Type 2 Diabetes Mellitus (T2DM) is a chronic metabolic disease which has many serious effects on human health.[1] International Diabetes Federation (IDF) has estimated that this disease will affect 693 million adults worldwide till year 2045.[2]

Chronic hyperglycaemia caused by T2DM can result in both macro- and microvascular diabetic problems. Patients with T2DM frequently experience microvascular complications including retinopathy, nephropathy and peripheral neuropathy.[3,4] The most commonly occurring complication is diabetic nephropathy (DN) and this is a significant cause of patient's morbidity and mortality.[5] Approximately 10-20% of T2DM patients develop end-stage renal disease (ESRD).[6]

When DN is in silent phase, there is no excretion of albumin in urine (normoalbuminuria); microalbuminuria is present in incipient nephropathy and macroalbuminuria is a characteristic feature of overt nephropathy. Overt diabetic nephropathy occurs 10-20 years after onset of diabetes and 5-10 years after beginning of microalbuminuria. Albumin excretion in urine is commonly used as a non-invasive biomarker for DN. Albuminuria determines the severity of DN. It has usually been studied to primarily reveal glomerular damage and increased glomerular permeability to macromolecules.[7] However, factors such as urinary tract infections, exercise, heart failure, and acute illness can influence microalbuminuria levels.[8]

Diabetes is a systemic disease with manifestations that affect the kidneys, and the primary target for pathological impacts is renal tubular cells. It has also been proven that damage to the renal tubules may occur before microalbuminuria and without glomerular proteinuria.[9] It

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can take more than 5 years after the diagnosis of diabetes to develop microalbuminuria.[1]

Another marker for renal tubular injury is urinary Neutrophil Gelatinase-Associated Lipocalin (NGAL), a 25-kDa protein from the lipocalin family. It is produced by neutrophils and is significantly elevated in damaged renal tubular epithelial cells.[10]

Cyclophilin A (CypA) has been identified as a valuable biomarker for diagnosing DN. It is an 18-kDa protein classified as an immunophilin with ubiquitous characteristics. It is rapidly and directly secreted by mesangial cells and proximal tubular epithelial cells in the kidneys in response to hyperglycaemia. Its levels consistently rise as DN progresses.[11] According to Tsai et al., novel urinary marker i.e. CypA levels rise in stage 2 DN after five years of diabetes, even when the albumin-to-creatinine ratio (ACR) remains in the normoalbuminuric range.[12] Additionally, a study by Amer et al. found positive correlation of urinary CypA levels with duration of T2DM. CypA levels could serve as an early marker for detecting DN as elevated levels were observed in diabetic patients before albuminuria appeared in stage 2 DN.[13]

The purpose of this study was to find out and compare urinary ACR, NGAL and CypA levels in the patients of T2DM with less than 10 years of diabetes and to find out which urinary marker appears early and can help in the detection of kidney damage in the silent phase of diabetic nephropathy before the appearance of albuminuria.

Materials and Methods

A prospective cross-sectional comparative study was carried out in Chemical Pathology Department of University of Health Sciences, Lahore and the patients were selected from outpatient department. This study was approved by the Ethical review committee of UHS, Lahore vide letter number UHS/REG-21/ERC/1479 and conducted between December, 2021 to July, 2022. Convenient sampling technique was used. Study subjects who matched the inclusion criteria were selected and written informed consent was obtained from each of them. The study included patients aged 40 to 70 years with a proven history of type 2 diabetes mellitus (T2DM) documented in hospital health records by a medical practitioner, as well as individuals receiving oral hypoglycaemic agents.

Patients with history of non-diabetic renal disease, T2DM of duration more than 10 years and on insulin treatment were excluded from the study.

The sample was calculated using the following formula:[11]

$Z\ 1\text{-}\alpha/2$ = Level of significance = 5% = 1.96

$Z\ 1\text{-}\beta$ = Power of study = 80% = 0.8

$\mu 1$ = Mean CypA in Group 1 = 0.74

$\mu 2$ = Anticipated level of CypA in Group 2 = 1.87

$\sigma 1$ = Anticipated standard deviation of CypA in Group 1 = 0.45

$\sigma 2$ = Anticipated standard deviation of CypA in Group 2 = 2.01

n = Calculated sample size in each group = 27

Total 84 patients were included in this study after sample size calculation and were divided into 3 groups based on the duration of T2DM.

Group A= ≤ 1 year (Control group)

Group B= > 1 year and < 5 years

Group C= ≥ 5 years and < 10 years

Demographic data was taken and written in a pre-designed proforma. From the study subjects, spot midstream random urine sample was collected in a sterile container at the time of outpatient department visit. Urine samples were centrifuged to obtain clear supernatant; aliquoted in different eppendorf according to the three parameters needed to be performed, tagged and stored at -80°C until analysis.

Laboratory parameters that were performed were Urinary creatinine, Urinary albumin, Urinary NGAL and Urinary CypA.

Determination of urinary albumin and creatinine in urine sample was done by immunoturbidimetric method and Jaffe method[14] respectively on spectrophotometer. Calibration was carried out prior to sample analysis using standard calibrators supplied by the reagent manufacturers. Albumin to creatinine ratio (ACR) was calculated by the following formula:

$$\text{ACR (mg/g)} = \frac{\text{Urinary albumin mg/dl}}{\text{Urine creatinine g/dl}}$$

ACR Ranges: Normal value < 30 mg/g, Microalbuminuria 30-299 mg/g, Proteinuria ≥ 300mg/g.[15]

Determination of urinary NGAL and CypA was done by ELISA kits where each step of the procedure was done according to the manufacturer's guidelines. The kits included specific calibrators, and with these, the calibration curves were constructed. These calibrators'

serial dilutions were processed alongside the samples and their absorbance recorded in a microplate reader.

Statistical Analysis: Data was analyzed using SPSS 24. Data was given as mean ± SD. One-way ANOVA was used to compare mean levels of urinary ACR, NGAL and CypA among the groups (Group A, Group B and Group C). p¬-value ≤ 0.05 was considered as significant. Shapiro wilk test was used to assess the normality of data.

Results

A total of 84 T2DM patients were enrolled in the study. 42 patients were males and 42 patients were females. The demographic data of the patients in the three groups is given in the table 1.

The gender and age distribution across the three groups was comparable, with no significant association observed between these variables and the duration of the disease.

The results of the comparison showed that the Group C including the patients having diabetes for more than five years had the highest levels of Urinary CypA, NGAL and ACR as shown in the Table 2.

The Urinary CypA, NGAL and ACR showed significant rise with the duration of the disease. Multiple comparisons of all the three parameters using ANOVA and independent sample t-test shows statistically significant difference with p-value of ≤0.05 among various groups as shown in table 3. The analysis of the parameters in the three groups showed that the Urinary CypA and NGAL appeared in the urine before the ACR. This means Urinary CypA and NGAL can be used as promising early markers for the detection

Table 1: Age and Gender distribution in the three groups.

Variables		Group A(n=29)	Group B(n=27)	Group C(n=28)	p-value
Age in years	Mean±SD	49.1±12.8	51.1±13.5	50.0±12.3	0.902 [b]
Gender	M (%)	14(48.3)	14(51.9)	14(50.0)	0.965[a]
	F (%)	15(51.7)	13(48.1)	14(50.0)	

[a] denotes association between the gender among the three groups (Chi-square test)
[b] denotes the comparison of the mean of ages in the three group (One-way ANOVA)

Table-2: Mean levels of Urinary CypA, NGAL and ACR in the three groups of diabetic patients

	(Mean ± SEM[a])			p-value
	Group A	Group B	Group C	
Cyclophilin A ng/mL	9.6±0.4	21.5±1.5	26.9±1.8	<0.0001*
NGAL ng/mL	60.7±3.6	101.7±4.7	119.9±6.6	0.0082*
ACR mg/g	18.6±0.7	182.5±19.6	1284.8±222.1	<0.0001*

a Standard error of means (SEM), * Statistically significant p-value ≤0.05

Table 3: Multiple pairwise comparisons

Variables	Groups	Mean ± SEM	p-value [a]	p-value
Cyclophilin A ng/mL	A	9.6±0.4	<0.0001*	<0.0001*[b]
	B	21.5±1.5		0.0229*[c]
	C	26.9±1.8		<0.0001*[d]
NGAL ng/ml	A	60.7±3.6	0.0082*	<0.0001*[b]
	B	101.7±4.7		0.0297*[c]
	C	119.9±6.6		<0.0001*[d]
ACR mg/g	A	18.6±0.7	<0.0001*	<0.0001*[b]
	B	182.5±19.6		<0.0001*[c]
	C	1284.8±222.1		<0.0001*[d]

ACR=Albumin creatinine ratio
* Denotes statistically significant p-value ≤0.05
a Denotes the comparison among the three groups (ANOVA)
b Denotes the comparison of group A and group B (t-test)
c Denotes the comparison of group B and group C (t-test)
d Denotes the comparison of group A and group C (t-test)

and diagnosis of DN.

The results show that levels of Urinary ACR, CypA and NGAL are strongly associated with duration of diabetes which means all three parameters are increasing as the disease is progressing with increasing duration of diabetes.

Discussion

One of the most prevalent microvascular complications of diabetes is diabetic nephropathy (DN), which is considered a leading cause of end-stage renal disease (ESRD). Early detection and treatment can help prevent the irreversible kidney damage caused by diabetic nephropathy.

The most common indicator used to anticipate the emergence and progression of DN is albuminuria. Unfortunately, classic markers of DN are not sensitive or specific enough to catch the disease at its earliest stages. For this reason, it is crucial to find more accurate DN markers that can enable timely intervention before irreparable damage occurs due to the disease.[11]

For this purpose, a total of 84 Diabetic patients were enrolled and divided into three groups (Group A, B, C) on the basis of the duration of their disease. The mean ages of the patients in the three groups (A, B, C) were 49.1±12.8, 51.1±13.5 and 50.0±12.3 years respectively. Saif and his colleagues in Egypt recruited patients of stage 1 to 5 of DN and the mean age of the patients was 53.5 years which is comparable to our patients' age i.e. 50.0 years.[15] Another group of scientists in our neighbouring country i.e., India, found that the group of patients having DM for less than 10 years were of mean age 54.5 years which is comparable with the present study too.[16]

There were 42 male and 42 female patients in our study. There were no significant differences in gender distribution according to a study by Tsai et al.[11] According to a study done in Egypt, gender has no significant association with serum cyclophilin A which is in line with our study.[17]

Multiple studies have been conducted worldwide in which urinary NGAL and CypA have been estimated. Vijay et al. determined the levels of urinary NGAL for detection of early DN and microalbuminuria. The levels of urinary NGAL were measured and found that urinary NGAL was 146.12 ng/ml in group with the duration of disease less than five years with no albuminuria compared to our study in which the value of urinary NGAL was 101.67 ng/ml. In Vijay's study the patients with microalbuminuria and disease duration for more than five years had urinary NGAL levels 228.18 ng/ml as compared to 119.85 ng/ml in our study.[18] These findings are consistent with the current study and lend support to our findings.

Sueud and his fellows found significantly higher urinary NGAL levels in microalbuminuria as well as macroalbuminuria groups in comparison to healthy individuals. The urinary ACR and NGAL levels in the group of patients with duration of disease for five years were 19.7 and 58.7 ng/ml respectively and as the disease progressed with duration for more than five years, the levels were 74.4 and 54 ng/ml respectively. This increment of ACR with the increase in the duration of disease is consistent with the findings of the present study but decrease in urinary NGAL is not present in our study. The researchers concluded that urinary ACR measures DN more accurately than urinary NGAL.[19]

In the present study, when the urinary CypA levels were determined, it was found that urinary CypA levels to be 9.58 ng/ml, 21.53 ng/ml and 26.90ng/ml in the group A, B and C respectively showing the increase in mean levels of urinary CypA with the disease progression. In 2020, Saif and his colleagues estimated urinary ACR and CypA levels in various stages of Diabetic patients. They found that patients having Diabetes for less than five years had urinary ACR levels to be 18.4±3.7 mg/g and CypA levels to be 0.63 ng/ml which is raised when ACR is still in normal range and as the disease progressed, the patients with Diabetes for more than five years had levels of ACR to be 18.1± 5.7 mg/g and CypA to be 2.85 ng/ml and keep on increasing with the increased in the duration of diabetes. These results of CypA are consistent with the present study.[15] Another in vivo study was done by El-Ebidi and his colleague in 2020 in which adult rats were used and grouped as a control group and a DM induced group. The control group had urinary ACR levels to be 1.5 mg/g and DM induced group had urinary ACR levels 6.4 mg/g. Whereas urinary CypA levels were 1.4 ng/ml in the control group and in the DM induced group, levels were 2.1 ng/ml demonstrating urinary CypA as a more sensitive and rapid biomarker for detecting renal disease in diabetic patients. They showed concordant results to the present study that the levels of urinary CypA were significantly increasing soon after the DM.[20]

An Egyptian study found high levels of urinary CypA in diabetic patients with renal involvement at any level, the levels being higher in diabetics with renal involvement even without albuminuria. They found that healthy individuals have urinary ACR levels as 9.29 mg/g and CypA levels to be 0.55 ng/ml. Whereas, when the disease progressed and the duration of disease increased to more than five years, the urinary ACR and CypA levels were 20.96 mg/g and 1.69 ng/ml i.e., they were increased with

the disease progression. Urinary ACR was still in normoalbuminuric range, but urinary CypA was increased. These findings are in correlation to the present study and shows urinary CypA can be used as an early marker.[13] Another recent study done in Egypt assessed urinary CypA as a biomarker for diagnosis of DN found positive co-relation of urinary CypA and urinary ACR. They identified that urinary CypA starts to rise before urinary ACR and progresses with the stages of DN.[20]

A study from Spain in 2021 found urinary CypA a potential biomarker of kidney injury, which is independent from decreased kidney function.[21] An elaborative study done in Thailand in 2024 shows that urinary CypA could be used as a biomarker for distinguishing early-stage CKD as well as T2DM complications.[22]

Although albuminuria is the most common marker for diagnosis and follow-up of the progression of DN, the search for more sensitive markers for its early detection is increasingly important. It is recommended that all patients with T2DM be screened for DN at diagnosis and should be reviewed annually.[6, 23] According to our study, the above-mentioned markers (urinary CypA and NGAL) can be used as early markers which is consistent with recent studies done in different regions of the world.

Conclusion

The urinary CypA, NGAL and ACR showed significant rise with the duration of the disease. Our finding showed that urinary NGAL and CypA could be used as a biomarker for early detection of diabetic nephropathy before the appearance of albuminuria and detects kidney damage in silent phase of diabetic nephropathy. Patients may benefit from being aware of their health state and altering their attitude towards type 2 diabetes in order to slow the progression of kidney disease.

Limitations of the study: Our study focussed on evaluating the predictive role of urinary NGAL and Cyclophilin A (CypA) in relation to the duration of T2DM. The findings suggest that these biomarkers may be useful in the early detection of kidney involvement in diabetic patients. However, to establish their role in predicting disease progression, further studies are needed in patients with established CKD associated with T2DM. Additionally, future research should consider excluding patients with poor glycaemic control, as uncontrolled blood glucose levels may influence biomarker levels and affect the accuracy of the results.

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AUTHORS' CONTRIBUTIONS:

ZR: Concept, design, data interpretation, drafting and agreement to be accountable for all aspects of the work.

SG: Concept, design, revision and agreement to be accountable for all aspects of the work.

NM: Data acquisition, analysis, revision, final approval and agreement to be accountable for all aspects of the work.

SJ, KB & SR: Concept, drafting, critical review, final approval and agreement to be accountable for all aspects of the work.