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# CLINICAL UTILITY OF ESTIMATION OF URINARY NEPHRIN LEVELS AS AN EARLY BIOMARKER IN DIABETIC NEPHROPATHY

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## ABSTRACT

### Background

Diabetes mellitus (DM) is major cause of chronic kidney disease (CKD). Proteinuria is the clinical significance of diabetic nephropathy (DN). In diabetes mellitus, a disruption of the glomerular capillary blood barrier with the development of proteinuria will leads to clinical diabetic nephropathy. Nephrin is an important protein component of the slit diaphragm that maintains the integrity of the filtration barrier. So estimation of nephrin levels is important to rule out as early biomarker for diabetic nephropathy.

### Objectives

This study investigates whether if nephrin, as opposed to albumin, can serve as a earlier sing of CKD in diabetic mellitus.

### Methods

This quantitative correlational study included 75 participants of age group between 45-60 years in the month June 2013, of which 15 did not have DM or CKD confirmed by testing blood sugar levels and blood urea-creatinine levels, 15 participants had DM but not CKD confirmed by testing blood sugar levels and blood urea-creatinine levels and 45 participants had both DM and CKD confirmed by testing blood sugar levels and blood urea-creatinine levels, further CKD divided into subgroups CKD -Stage 2,3 and 4 based on the creatinine levels in the blood, where all data was collected from the medical records in the month of June 2013 at JSS Hospital, Mysore. Urinary nephrin was measured using enzyme linked immunosorbent assay (ELISA) and correlated with other parameters using Statistical package for social sciences (SPSS) Software.

**Results**
The concentration of urinary nephrin increased progressively as the kidney function worsened. Nephrinuria had significant positive correlation with urine creatine ratio (r=0.237). Receiver operator curve (ROC) analysis showed that urinary nephrin had high sensitivity and but low specificity. At urinary nephrin level of 1.317 ng/µL, sensitivity and specificity were maximum at 90.3% and 75%, respectively and hence can be used as the cut off value.
**Conclusions**
Compared to albumin, urinary nephrin can serve as an early biomarker of diabetic nephropathy and can help in the staging of CKD.

**Keywords:** Biomarker, Diabetic Nephropathy, Urinary Nephrin.

## INTRODUCTION

Diabetes mellitus is a group of metabolic disorder resulting from defects in insulin secretion, insulin action or both, leads to elevated blood glucose levels (hyperglycemia).[1] Diabetes is a condition primarily defined by the increased glucose levels giving rise to risk of microvascular damage (retinopathy, nephropathy and neuropathy).[2] Diabetic kidney diseases is a complication that take place 20-40% of all diabetics.[3]

More than 40% of persons with diabetes have elevated urinary albumin excretion, and the prevalence is higher in those with diabetes of longer duration. In insulin-dependent diabetes mellitus (IDDM), the incidence of persistent proteinuria rises during the first 10 years of diabetes and begins to decline after ~15 years of diabetes.[4]

Hyperglycemia induces renal damage directly or through hemodynamic modifications. It induces activation of protein kinase C, increased production of advanced glycosylation end products, and diacylglycerol synthesis. In addition, it is responsible for hemodynamic alterations such as glomerular hyper filtration, shear stress, and microalbuminuria.[5]

Proteinuria is the clinical hallmark of diabetic nephropathy (DN). In diabetes mellitus a breakdown of the glomerular capillary blood barrier with the development of proteinuria (Albustix positive) is usually accepted as marking the onset of clinical diabetic nephropathy. This condition progresses to end-stage renal failure.[6]

Nephrin is crucial components of the Slit diaphragm. They are assumed to play key roles in cell-cell adhesion through their interactions, thus serving as a structural framework of the Slit diaphragm.[8] Podocytes (specialized visceral epithelial cells) are important for the maintenance of the dynamic functional barrier.[9] Nephrin, a protein found in these cells, is crucial for maintaining the integrity of the intact filtration barrier.

Thus, nephrin excretion could be an early finding of podocyte injury, even before the onset of albuminuria.[10] Treatment with blockers of the renin– angiotensin–aldosterone system might help protect nephrin expression, the nephrin expression is reduced in diabetic nephropathy. Hence the estimation of nephrin excretion in urine and proteinuria is important.[7]

ELISA is simple, rapid, highly sensitive and specific method for the estimation of human nephrin in urine with the advantage that they can handle large number of samples that may be analyzed rapidly. It is also easy to titre the nephrin molecule in the normal people, diabetic patients without kidney damage and CKD different stages patients.

## MATERIALS AND METHODS

Fifteen normal individuals who were non-diabetic and did not suffer from any kidney diseases had been selected randomly in the age group of 45 – 60 years (Group 1). Another 15 diabetic subjects without any features of diabetic nephropathy would be enrolled into the study (Group 2). Forty five diabetic patients who attended the Nephrology or Medicine OPD or patients admitted at JSS Medical College Hospital, Mysore and who had suspected features of diabetic nephropathy would be included in the study (Group 3).

In group 3 the subjects were further divided into 3 groups: 15 patients of CKD stage 2 (group 3a), 15 patients of CKD stage 3 (group 3b), 15 patients of CKD stage 4 (group 3c) and all parameters will be compared in all the groups. The study was approved by institutional ethical committee and informed consent was collected from all participants of the study.

## RESULTS

The study was conducted on 75 subjects who were divided equally into 5 groups viz. 15 Diabetes mellitus without CKD, 15 CKD stage 2, 15 CKD stage 3, 15 CKD stage 4 and 15 normal subjects. Based on the age the study groups were divided into 3 groups: - 40-50 yrs in first group, 51-60 yrs in second group and 61-75yrs in the third group (Fig 1). The figure 5.2 shows that more number of males suffer from CKD than the number of females.

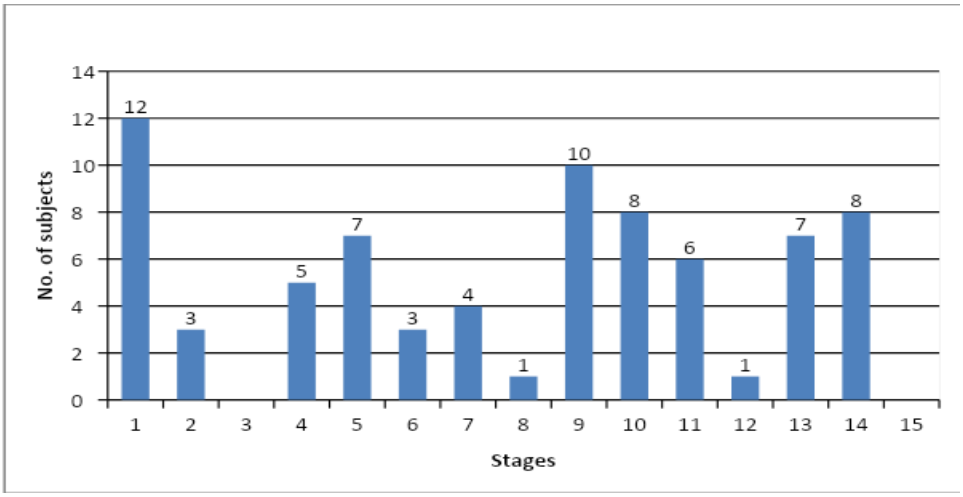


**Figure 5.1: - This graph shows that distribution of subjects based on their age across control subjects, diabetes mellitus subjects & different stages of CKD**

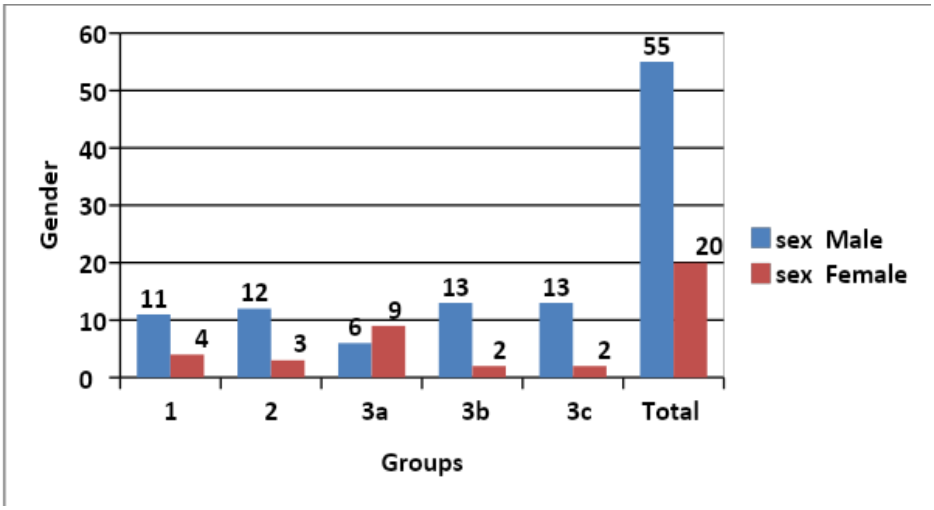


**Figure 5.2: - This graph shows that distribution of subjects based on their sex across control subjects, diabetes mellitus subjects & different stages of CKD**

| Groups | Mean ± Std. Deviation | | | | | | |
|---|---|---|---|---|---|---|---|
| | FBS in mg/dl | HbA1c in % | Blood urea in mg/dl | Serum creatinine in mg/dl | eGFR | Urine protein creatinine ratio | Urinary nephrin in ng/µl |
| 1 | 95.2 ± 4.77 | 5.1 ± 0.3 | 20.3 ± 1.2 | 0.9 ± 0.15 | 86.67 ± 19.47 | 0.24 ± 0.29 | 0.40 ± 0.40 |
| 2 | 183.4 ± | 8.9 ± | 37.3 ± 17.6 | 1.12 ± 0.33 | 70.13 ± | 0.42 ± 0.62 | 1.14 ± 0.80 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 60.18* | 1.7* | | | 21.81 | | |
| 3a | 130.8 ± 39.14 | 6.8 ± 1.7 | 51.7 ± 24.3 | 1.04 ± 0.10 | 59.07 ± 14.92 | 1.39 ± 1.72 | 1.31 ± 0.97 |
| 3b | 140.6 ± 46.75 | 8.1 ± 1.0 | 44.4 ± 14.6 | 1.95 ± 0.20 | 35.73 ± 4.67 | 4.04 ± 2.80* | 2.05 ± 0.66 |
| 3c | 137.3 ± 21.67 | 8.4 ± 1.4 | 79.5 ± 30.1* | 3.28 ± 0.51* | 19.80 ± 4.26 | 3.90 ± 2.53 | 2.06 ± 0.50 |

**Table 5.1: Comparison of all parameters across control subjects, diabetes mellitus subjects & different stages of CKD**

The mean and standard deviation of all the parameters is shown in table 1. The mean FBS levels were more in Diabetes mellitus without CKD group compared to controls and patients in different stages of CKD. The mean blood urea and serum creatinine levels in subjects with group 3c (CKD Stage 4) was statistically higher when compared to all other study groups. This shows that as CKD stages progresses the kidney damage increases and urea and creatinine excretion in urine decreases, as a compensatory mechanism blood urea and serum creatinine level increases. The mean eGFR values is more in controls compared diabetes mellitus without CKD and subjects at different stages of CKD. It states that as the progression to CKD stage increases the eGFR values will decrease. Mean urine protein creatinine ratio (UPCR) in CKD stage 3 was more compared to controls, diabetes mellitus without CKD and subjects at different stages of CKD. Though the urinary protein excretion increased steadily as the CKD stage advanced, the UPR in group 3c was slightly lower than that of group 3b. This could be due to altered creatinine excretion in the urine. The higher creatinine concentrations in the group 3c might lower the urine protein creatinine ratios. The mean Urinary Nephrin levels in group 3c (CKD Stage 4) was more compared to controls, Diabetes mellitus without CKD and subjects at different stages of CKD. This shows that as CKD progresses the urinary nephrin excretion levels increases.

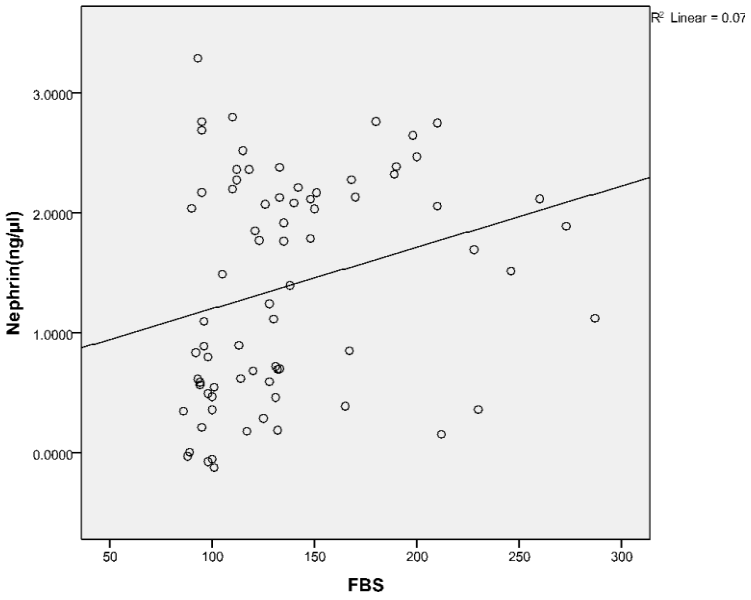


**Figure 5.3: - Correlation of Urinary Nephrin levels with FBS levels**

The figure 5.3 implies that there was a positive correlation between urinary nephrin and Fasting blood glucose levels. This correlation was found to be statistically significant.

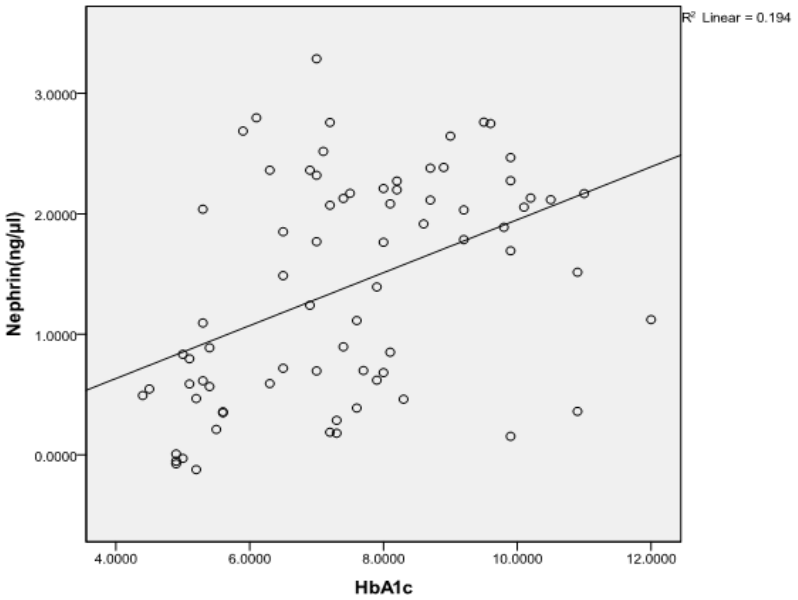


**Figure 5.4: - Correlation of Urinary Nephrin levels with HbA1c**

The **figure 5.4** implies that there is a positive correlation between urinary nephrin and HbA1c levels. This correlation was found to be statistically significant.

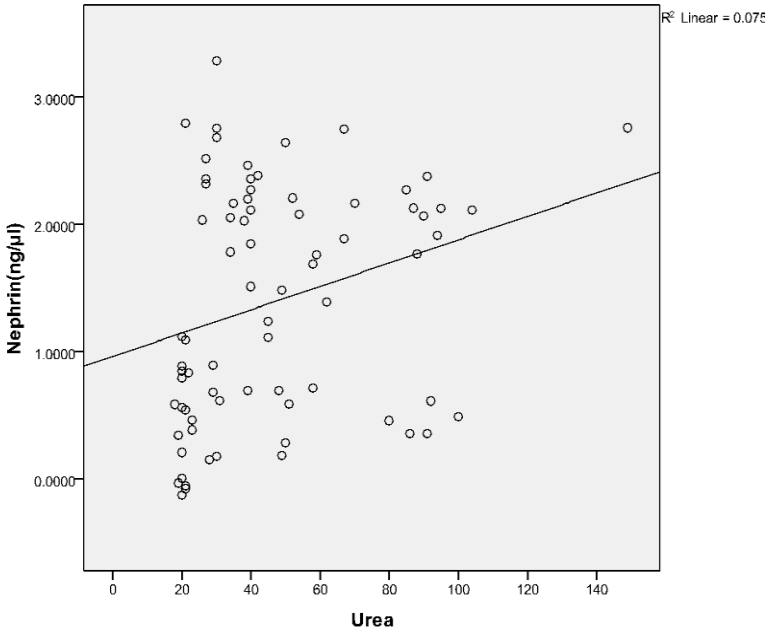


**Figure 5.5: - Correlation of Urinary Nephrin levels with urea levels**

The figure 5.5 implies that there is a positive correlation between urinary nephrin levels and serum urea levels. This correlation was found to be statistically significant.

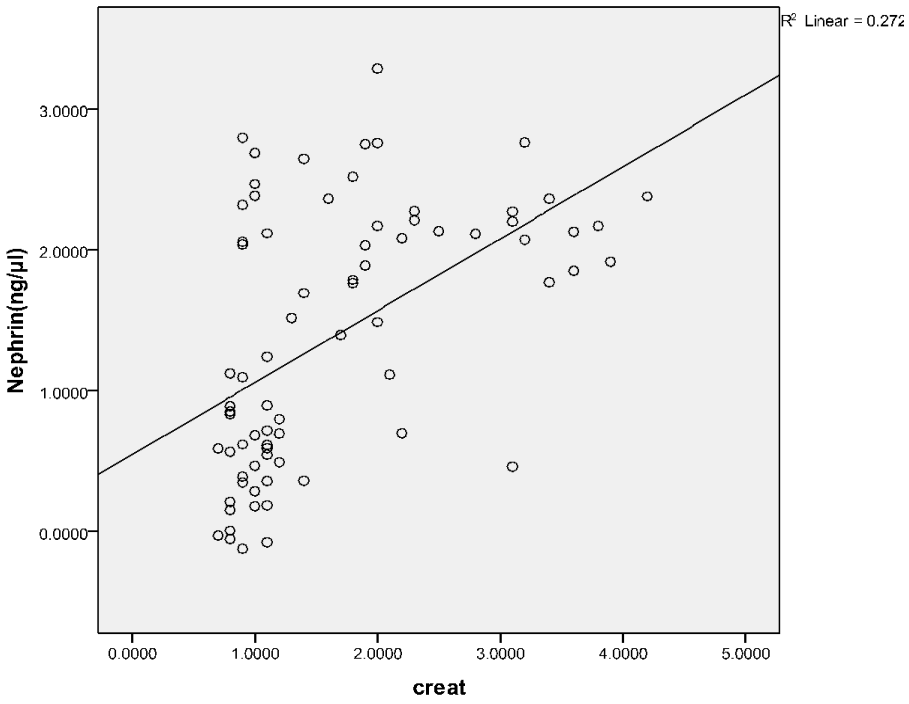


**Figure 5.6: - Correlation of Urinary Nephrin levels with creatinine levels**

Figure 5.6 implies that there was a positive correlation between urinary nephrin and creatinine levels. This correlation was found to be statistically significant.

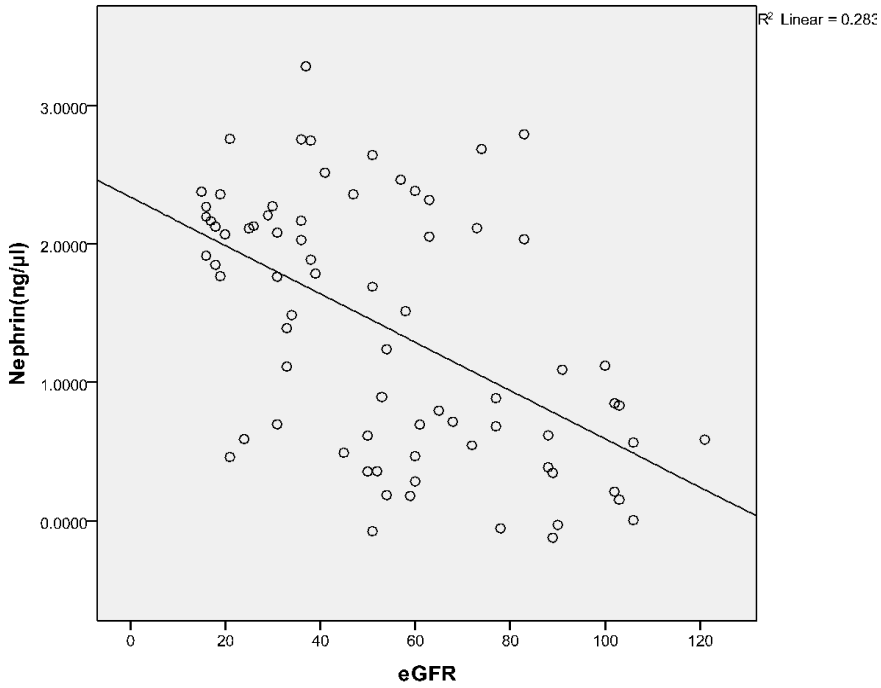


**Figure 5.7: - Correlation of Urinary Nephrin levels with eGFR levels**

The figure 5.7 implies that there was a negative correlation between urinary nephrin and eGFR values levels. This correlation was found to be statistically significant.

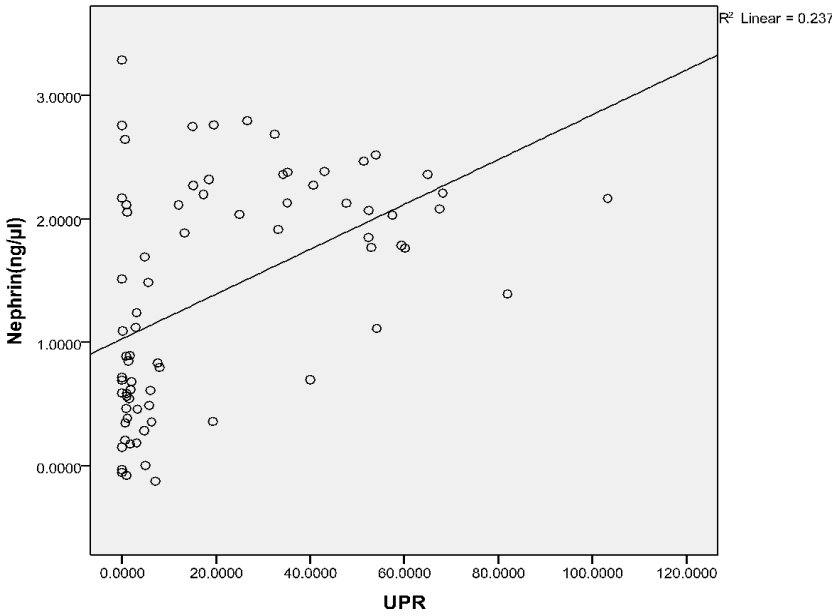


**Figure 5.8: - Correlation of Urinary Nephrin levels with urine protein creatinine ratio**

The figure 5.8 implies that there is a positive correlation (r = 0.237) between nephrin and urine protein creatinine ratio levels. This correlation was found to be statistically significant. The relation is stronger at higher urine protein creatinine ratios.

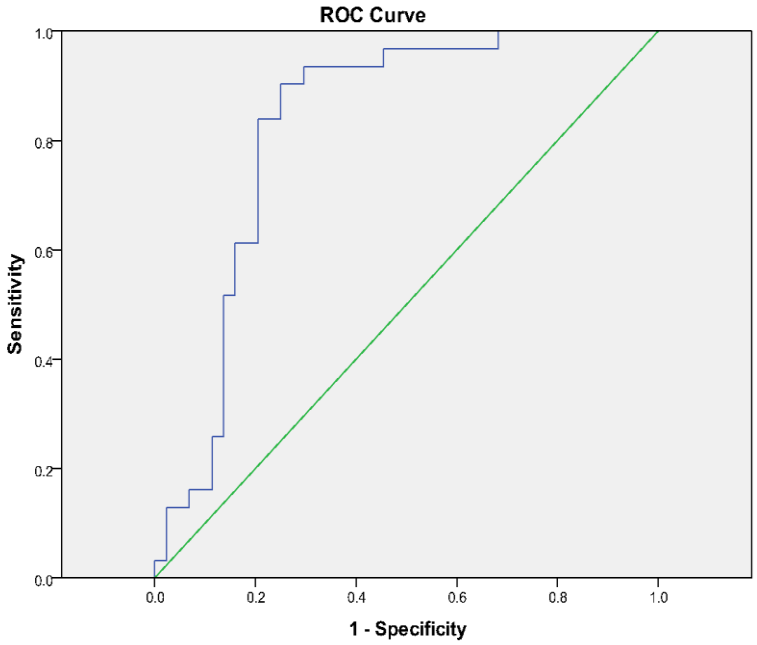


**Figure 5.9: Receiver Operator Characteristics (ROC) Curve for urinary nephrin levels**

When a ROC curve was plotted for urinary nephrin levels for the detection of chronic kidney diseases the sensitivity was very high indicating that urinary nephrin estimation is a sensitive biomarker to detect the early kidney damage in diabetic nephropathy but the marker could not be highly specific. At a urinary nephrin cut off level of 1.317ng/µL, there was highest sensitivity (90.3%) and specificity (75.0%). Thus serum nephrin level of 1.317 ng/dl can be considered as cut off for progression to chronic kidney disease.

## DISCUSSION

It is seen in our study that majority of the patients with CKD stage 3 and 4 were from 40-50 and 51-60 age groups. This indicates that if the disease progression from stage 2 to 3 is rapid in the early elderly age groups then the progression to stage 4 is also rapid. This is in contrary to the published literature which says patients with prolonged history of diabetes mellitus were more prone to diabetic nephropathy and further progression to the end stage renal disease.

There were a distinctly higher number of males in the study group than females. This could indicate that more number of males suffers from CKD or males are more prone for progression of the disease to end stage renal disease. Since this was a hospital based study a true picture of prevalence of CKD in males cannot be confirmed. Larger cohort community based studies or multi centric studies will be required to confirm the findings of this study.

The mean fasting blood sugar (FBS) levels in subjects with diabetes mellitus without CKD was more (183±60.17) compared to controls and subjects at different stages of CKD. This could be due to the reason that patients with CKD are more closely monitored and hence have a better control of blood glucose levels. This study re-emphasizes that good glycemic control is important before the kidney damage has occurred.

The correlation curve shows there was a positive correlation between urinary nephrin and serum glucose levels. Though many of them in the study groups did not have very high blood glucose levels, the small number of samples with higher blood glucose levels did show a linear relation when compared to urinary nephrin levels.

The mean Serum HbA1c levels in subjects with Diabetes mellitus without CKD was more (8.86±1.70) compared to controls and subjects at different stages of CKD. Since this group also had the highest levels of blood glucose levels, their HbA1c levels also were high as compared to all the other groups. Our results are in contrast to some studies which have seen higher levels of glucose and HbA1c levels in diabetic patients with impaired renal function on dialysis when compared to diabetic groups with normal renal function[11] but few studies have seen published that patients on renal dialysis have better glycemic controls compared to diabetics without kidney diseases.[12]

The correlation curve for HbA1c when plotted against urinary nephrin levels showed a positive correlation indicating that nephrin levels are higher in uncontrolled diabetic patients. Uncontrolled glucose levels might add on to the severity of the disease and hence more leakage of urinary nephrin from the podocytes in to the renal tubules leading to an increased excretion of nephrin in the urine.

The mean serum urea levels in subjects with CKD stage 4 was more (79.47 ± 30.111) compared to controls, diabetes mellitus without CKD and subjects at different stages of CKD. The mean serum creatinine levels in subjects with CKD stage 4 was more (3.28±0.51) compared to controls, Diabetes mellitus without CKD and subjects at different stages of CKD. It is a well-known fact that urea and creatinine levels increase in chronic kidney disease. In our study also we have seen a steady increase in the levels of urea and creatinine as the stage of chronic kidney disease advances.

Though the use of creatinine in the management of chronic kidney disease is undoubted, its role in diagnosing the disease early is questioned and many studies are being undertaken to identify a marker which can identify kidney insults earlier than creatinine. As seen in our study the comparison of creatinine levels between group 2 patients and group 3a patients did not show difference in the serum creatinine levels but the eGFR levels were reduced in the group 3a patients classifying them as stage 2 CKD. Hence it is very obvious that creatinine cannot be used as a marker for early diagnosis of kidney disease subsequent to diabetes mellitus.

In an earlier study done by Levey et. Al in 1999, they have very clearly shown that though an inverse relation exists between serum creatinine and GFR levels, the relation is not linear and many a time the serum creatinine might be normal when the GFR has declined to significant low levels.[13]

The levels of serum urea and creatinine correlated well with the levels of urinary nephrin levels indicating that they have a positive relationship with nephrin. The relation was stronger in the case of creatinine than urea. This indicates that as levels of creatinine are used to find out the severity of the chronic kidney disease, urinary nephrin may also be used to predict the staging of chronic

kidney diseases. Thus urinary nephrin not only act as early biomarkers of kidney disease they also predict the staging of the kidney disease and may be useful for the management of CKD patients.

Proteinuria is an early and sensitive marker of kidney damage in many types of chronic kidney disease. Albumin is the most abundant urine protein in most types of chronic kidney disease. Low molecular weight (LMW) globulins are the most abundant urine proteins in some types of chronic kidney disease. In this and later guidelines, the term proteinuria includes albuminuria, increased urinary excretion of other specific proteins, and increased excretion of total urine protein. On the other hand, the term albuminuria has been used only when referring to increased urinary albumin excretion. Older laboratory methods, such as the urine dipstick or acid precipitation, detect most urine proteins. Microalbuminuria refers to excretion of small but abnormal amounts of albumin, which requires recently developed, more sensitive laboratory methods that are now widely available. The most widely used tests are estimation of urinary microalbumin, urinary albumin creatinine ratio, 24hrs urinary protein and protein creatinine ratio.

Measurement of albuminuria and total proteinuria are a central aspect of the management and prognosis of patients with CKD. The mean urine protein levels in subjects with CKD stage 4 (Group3C) was more (1.83±0.89) compared to controls, diabetes mellitus without CKD and subjects at different stages of CKD. The mean Urine protein creatinine ratio levels in subjects with CKD stage 3 was more (4.037±2.8) compared to controls, Diabetes mellitus without CKD and subjects at different stages of CKD.

Estimation of urinary proteins is central for the diagnosis and classification of diabetic nephropathy. Currently the earliest marker of detecting nephropathy is estimation of urinary microalbumin or albumin creatinine ratio (ACR). There are studies done recently which have shown that protein creatinine ratio correlates well with the ACR levels and hence can be considered instead of ACR. However the guidelines laid by KDIGO (Kidney Disease: Improving Global Outcomes) takes only ACR into consideration for classification and staging of CKD. There has been a lot of emphasis on interpretation of urinary protein levels along with eGFR levels.[14]

The mean eGFR values in Control subjects was more (86.6±19.4) compared to controls, Diabetes mellitus without CKD and subjects at different stages of CKD. There was a progressive decline the eGFR levels of patients as the stage of chronic kidney disease advanced. Even in the normal and diabetic group without CKD there was mild decline in the eGFR levels though it was not significant. However significant decrease in the levels of eGFR were seen between diabetic subjects without CKD and subjects with various stages of CKD and the levels decreasing with progression of staging in CKD.

There was also a strong negative correlation between urinary nephrin levels and the eGFR levels. As the eGFR levels declined the urinary nephrin levels were elevated. Hence urinary nephrin can be used in the staging of CKD along with eGFR. eGFR and urinary proteins form an important part of classification of staging for chronic CKD

Elevated urinary nephrin level is considered as one of the important risk for diabetic nephropathy. Many studies have shown urinary nephrin levels are increased in chronic kidney diseases patients. Urinary nephrin levels have also been shown as early markers of glomerular basement damage. The increase in urinary nephrin levels is more significantly seen in type 1 diabetes than type 2 diabetes mellitus where podocyte damage is more prominent. However this does not negate its use in early identification of nephropathy due to type 2 DM.

In our study we have seen a steady increase in the urinary nephrin levels as the stage of chronic kidney disease advances. Hence it is worthy to note that not only does urinary nephrin help in early diagnosis of diabetic nephropathy, it also can be used in staging of CKD. In present study, the mean urinary nephrin levels in 5 groups was 0.042, 1.135, 1.3074, 2.054 and 2.057 respectively showing an increasing trend as the stage advances. Our results are consistent with the results of Jim et al (2012) who have also seen a similar trend in diabetic nephropathy patients. CKD Stage 4 were (2.05±0.501) high compared to controls, Diabetes mellitus without CKD and subjects at different stages of CKD.[15]

That nephrinuria is seen in early disease (in 54% of patients with normoalbuminuria) and increases in overt disease (macroalbuminuria) supports a previous finding by Patari et al. who had described nephrinuria in one-third of diabetic patients with normoalbuminuria. In contrast to Patari et al., however, is our report of a significant correlation between nephrinuria and albuminuria. Patari et al. has described the presence of similar levels of nephrinuria in all stages of albuminuria, i.e. normo-, micro-, newly discovered micro-, and macroalbuminuria.[16] However, their method of detection for nephrinuria was via Western blotting, which showed either the presence or absence of nephrin fragments. They used an antibody that detected parts of the intra and extracellular domains of nephrin (amino acids 1031–1055 and 1096–1215). Since the level of nephrinuria was not quantified, it is not immediately obvious whether there would have been an association with albuminuria. We, on the other hand, employed the ELISA method which used an antibody that detected the extracellular domain of nephrin (amino acids 23–322) which allowed for quantification of nephrinuria. Thus it is difficult to compare our results from those of Patari et al. as methodologies of nephrin measurement were different.

Receiver operator characteristic (ROC) curve plotted for urinary nephrin levels showed that at a level of 1.317 ng/µl urinary nephrin has 90% sensitivity and 75 % specificity to detect chronic kidney disease. The ROC curve shows that urinary nephrins have a high sensitivity to detect early diabetic kidney disease but it might not be very specific to kidney damage alone.

## CONCLUSION

The purpose of this research was to identify urinary nephrin can be an early biomarker in diabetic nephropathy. Based on this correlation studies conclude that compared to albumin, urinary nephrin can serve as an early biomarker of diabetic nephropathy and can help in the staging of CKD

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