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DIAGNOSTIC SENSITIVITY OF BIOMARKERS IN ASSESSING THE OCCURRENCE AND PROGRESSION OF DIABETIC NEPHROPATHY

Author details

First author : Mr.Kondapi Kishore

Senior Lecturer, Department of Biochemistry, GSL Medical College, Rajahmundry, Andhra Pradesh, India

Second Author : Dr Santhi Silambanan

Professor, Department of Biochemistry, Sri Ramachandra Medical College,
Sri Ramachandra Institute of Higher Education and Research, Porur, Chennai, Tamilnadu , India

Third Author : Dr Swathy Moorthy

Associate Professor, Department of General Medicine,
Sri Ramachandra Medical College, Sri Ramachandra Institute of Higher Education and Research, Porur, Chennai, Tamilnadu, India

Corresponding Author : Dr Swathy Moorthy

Associate Professor, Department of General Medicine,
Sri Ramachandra Medical College, Sri Ramachandra Institute of Higher Education and Research, Porur, Chennai, Tamilnadu, India

Phone : 9444016401

Email : drswathymoorthy@sriramachandra.edu.in

ABSTRACT

Background

Diabetic nephropathy is a major end stage kidney disease and its timely diagnosis helps in combating the morbidity and mortality associated with it, thereby improving the quality of the patients suffering from the condition. Though identification of microalbuminuria has long been used to diagnose the nephropathy among diabetics, there has always a need for sensitive and specific biomarker that could predict the susceptibility of the diabetic patients to develop nephropathy much ahead of the onset of microalbuminuria.

Methods

A prospective study was conducted among 125 diabetic patients at various levels of nephropathy like the normoalbuminuric, microalbuminuric and macroalbuminuric patients and 45 healthy controls based on the Joint Committee of Diabetic Nephropathy Classification. A detailed history, clinical characteristics, duration of illness and other baseline investigations along with urinary podocin, urine nephrin, serum carboxy-methyl lysine levels, glycated albumin, glycaemic control, lipid profile were analyzed among the various nephropathy stages and compared with the controls.

Results

The sensitivity, specificity, positive and negative predictive value of the various studied biomarkers were compared between the controls and diabetic patients with

progressing levels of albuminuria. According to the AUC values the studied biomarkers were categorised into five groups as follows :

i. AUC 1.0-0.9 : excellent diagnostic accuracy : podocin, nephrin, CML, microalbumin

ii. AUC 0.9-0.8 : very good diagnostic accuracy : glycated albumin, HbA1c

iii. AUC 0.8-0.7 : good diagnostic accuracy : HOMA-IR

iv. AUC 0.7-0.6 : sufficient diagnostic accuracy : none among the studied biomarkers

v. AUC 0.6-0.5 : no diagnostic accuracy : serum albumin, bilirubin

Conclusion

Urinary Podocin, Nephrin, n-Carboxy methyl lysine, Glycated Albumin seem to have very good to excellent diagnostic value in accordance to microalbumin and HbA1c followed by HOMA-IR in the diagnosis of onset and progression of diabetic nephropathy.

INTRODUCTION

Diabetes Mellitus is the leading cause of end stage renal disease and the higher morbidity and mortality rates associated with it. Though Diabetic nephropathy is diagnosed by the presence of microalbuminuria, it has its own pitfalls. Structural damage of the glomeruli preceed the occurrence of proteinuria [1] and microalbuminuria is not diabetes specific but can occur in any progressive kidney disease and its presence need not be associated with the progression of diabetic kidney disease (DKD). Hence there is always a need for sensitive and specific biomarkers that could predict the susceptibility of patients to develop diabetic kidney disease.

Though traditionally DKD is considered a non-immune disease, there is strong evidence that inflammation progresses for years before clinically overt disease occurs in diabetic nephropathy [2]. The pathophysiology of glomerular and tubulointerstitial damage and the occurrence of albuminuria are complex. Proximal renal tubular damage can lead to albuminuria and at the same time increased protein reabsorption from the tubular lumen induces a proinflammatory and profibrotic cascade.

Numerous studies have shown multiple urinary and serum biomarkers with varying diagnostic accuracy in predicting kidney damage. Many earlier studies focussed on one diabetic nephropathy stage or studied a single biomarker in assessing their diagnostic value during the course of diabetic kidney disease [3]. The current study investigated the diagnostic sensitivity of biomarkers like nephrin, podocin, carboxy-methyl lysine and glycated albumin HOMA-IR, HbA1c, Serum Bilirubin, Serum Albumin along with the standard microalbumin in predicting the occurrence and progression of diabetic nephropathy.

MATERIALS AND METHODS

The prospective study was conducted in South India over a period of 24 months in a medical college hospital. We studied 125 diabetic patients along with 45 age and sex matched healthy controls. Patients at various stages of diabetic nephropathy were studied, involving 45 with normoalbuminuria, 40 with microalbuminuria and 40 with macroalbuminuria.

All the patients with Type 2 Diabetes Mellites (T2DM) over the age of 18 years as per the WHO criteria baring the controls were included in the study after getting written informed consent and approval from the Institutional Ethics Committee. Patients with systemic hypertension on medications which interfere with proteinuria like angiotensin converting

enzyme inhibitor or angiotensin receptor blockers, cardiovascular diseases, malignancies, urinary tract infections and any acute febrile illness were excluded as these conditions could interfere with the proteinuria assessments in these patients.

A detailed history, clinical characteristics, duration of illness and other baseline investigations along with urinary podocin, urine nephrin, serum carboxy-methyl lysine levels, glycated albumin, glycaemic control, lipid profile were documented and analyzed among the various nephropathy stages. The diabetic patients were grouped into normoalbuminuria, microalbuminuria and macroalbuminuria groups based on the Joint Committee of Diabetic Nephropathy Classification [4] with their urinary albumin- creatinine ratio as less than 30 mg/g, 30-299 mg/g and more than and equal to 300 mg/g respectively and a healthy control group. The various biomarkers levels were compared among the various study groups and with other parameters among the cases and controls.

STATISTICAL ANALYSIS

The data was analysed using SPSS software version 21.0. Mean and standard deviation were used to represent the quantitative data. Student t test was used for comparison between groups and Anova (F test) followed by post hoc analysis were used for comparison between groups. The diagnostic performances of various biomarkers were assessed by their sensitivity and specificity while the overall performance was assessed by area under curve in Receiver operating Characteristic curve.

RESULTS

A total of 170 study participants with 125 patients with 45 normoalbuminuria, 40 microalbuminuria and 40 macroalbuminuria and 45 healthy controls were included with 65.88% males and 34.12% females with their mean age (SD) of 51.82 (11.94) years. Table-1 shows the baseline characteristics of the study population according to their stage of diabetic nephropathy. In the index study patients with progressing stages of albuminuria were significantly older with longer the duration of diabetes compared to the non diabetic and normoalbuminuric diabetic patients. The diabetic patients had significantly higher Body mass index (BMI), Waist hip ratio (WHR) and both systolic and diastolic blood pressures. There was an increase in the triglyceride levels with advancing stage of nephropathy. However, the LDL did not show a significant difference between the cases and controls, but patients with macroalbuminuria had significantly higher levels than the controls.

The levels of each biomarker in various stages of diabetic nephropathy were represented by box plots as shown in figure-1 (a to i). the median value of these biomarkers namely the n-CML, podocin, nephrin, glycated albumin, HbA1c, HOMA-IR had significantly increased in patients with nephropathy and progressed as the stage advanced. Table 2 and Table 3 show the correlation between the biomarkers and patients clinical and laboratory parameters respectively. Podocin, CML, Serum Albumin, and microalbumin showed a significant correlation with age of the study participants. However, all the biomarkers demonstrated significant positive correlation with BMI, WHR except Serum bilirubin and a significant negative correlation with increasing value of HOMA-IR, HbA1c, FBS and PPBS except for serum bilirubin.

Similarly, they showed significantly increased values with higher levels of serum cholesterol, triglyceride and LDL except Serum Bilirubin. HDL levels seemed to increase with increasing levels of the biomarkers. All the biomarkers positively correlated with systolic and diastolic blood pressure but only podocin, microalbumin, HOMA-IR and serum creatinine correlated with the presence of prior Systemic Hypertension.

The receiver operating characteristics (ROC) curve analysis of the study results is illustrated in the Figure-2, which demonstrates the diagnostic performance of the biomarkers in study. The cutoff level of HbA1c to identify cases of diabetic nephropathy with cardiac complications was 6.55% with sensitivity of 88% and specificity of 67%. Table-4 shows the area under curve, sensitivity, specificity, positive predictive value and the negative predictive value of the various studied biomarkers.

The sensitivity and specificity and area under curve (AUC) of the biomarkers were calculated in order to assess the significance of the diagnostic value of each biomarker in predicting various proteinuric stages of diabetic nephropathy which had sensitivity, specificity and AUC of almost 1 in all the groups when compared to the controls. According to the AUC values the studied biomarkers were arranged in descending order and categorised into five groups as follows :

i. AUC 1.0-0.9 : excellent diagnostic accuracy : podocin, nephrin, CML, microalbumin
ii. AUC 0.9-0.8 : very good diagnostic accuracy : glycated albumin, HbA1c
iii. AUC 0.8-0.7 : good diagnostic accuracy : HOMA-IR
iv. AUC 0.7-0.6 : sufficient diagnostic accuracy : none among the studied biomarkers
v. AUC 0.6-0.5 : no diagnostic accuracy : serum albumin, bilirubin

DISCUSSION

Over the last three decades the gold standard diagnostic and prognostic biomarker for the onset and progression of diabetic nephropathy has been albuminuria or the albumin creatinine ratio [5]. But, albuminuria lacks both sensitivity and specificity in the diagnosis of diabetic nephropathy. This is so, because diabetic nephropathy can progress quite often without increased albumin excretion and difficulty in diagnosing the progression of diabetic nephropathy when the urinary albumin excretion is less than 300mg in 24 hours respectively. Hence it does not serve as a surrogate endpoint diagnostic test for diabetic nephropathy.

An attempt was made in the current study to study a few biomarkers in urine and serum aiding in the diagnosis of the onset and progression of diabetic kidney disease and grade their diagnostic accuracy from excellent to very good to good according to the sensitivity, specificity and area under curve.

Advancing age was strongly positively associated with DKD progression. Age and the estimated GFR (eGFR) bear an inverse relationship with each other while using the CKD-EPI formula for calculating the GFR [6]. Female patients did not have significant DKD as was observed in previous studies [7]. Females have lower total GFR than males because their kidneys have few nephrons than the males causing lower total GFR among them, but no demonstrable difference in the single nephron GFR was noted between the females and males [8].

Albuminuria is a continuum across the progression of Diabetes [9] and is associated with endothelial dysfunction. Microalbuminuria starts within 10–15 years of onset of DM

among 20-40% of the diabetic population and progress to macroalbuminuria over the next 5 years. [9] Diabetic patients with microalbuminuria have fair chance of spontaneously reverting back to normal with adequate control of the risk factors. However, in patients with macroalbuminuria, complete remission is highly unlikely but with adequate control of the risk factors the progression of DN can be significantly delayed.[10]

The filtered albumin in the glomerulus escaping from the glomerular filtrate is reabsorbed by the cubilin–megalin protein complex in the proximal tubular cells. Reabsorbed albumin through activation of oxidative stress and inflammation causes further renal damage. It leads to tubular cell apoptosis, monocyte infiltration, complement activation, accumulation of extracellular matrix in the interstitium resulting in renal fibrosis and eventually renal function loss. [11] Due to biological variability in urinary albumin excretion, two of three specimens collected within a 3- to 6-month period should be abnormal before establishing albuminuria. [12] Albuminuria levels vary based on collection of urine on different days, exercise, hyperglycemia, urinary tract infections, inflammation, water consumption, menstruation, obesity, exercise, diet, smoking and posture. There are few limitations which discourages albuminuria from being a potential marker of DN such as lack of standardized methodology for estimation, lack of uniformity in reporting, lack of age and sex specific reference intervals. [13]

As GFR decreases, the urinary albumin creatinine ratio (uACR) and microalbuminuria progressively increase with the progression of DN. Poor glycemia is a strong risk factor for the diabetic nephropathy. The albuminuric effect of high sugars has its impact on the glomerulus and proximal tubules altering the glomerular endothelium and podocytes in glomerulus and impacts the albumin absorption in proximal tubules. Duration of diabetes affected both albuminuria and GFR in the index study which was in slight contradiction to the previous studies [14].

Urine nephrin was found to be steadily progressing across the groups. The significant difference between controls and normalbuminuric group shows that urine nephrin started increasing even before the onset of proteinuria. This showed that nephrin is a better biomarker of early diabetic nephropathy in T2DM patients; probably earlier than urinary albumin. Phosphorylation of tyrosine residues of nephrin mediates recovery of injured glomerulus and thus preserves glomerular function. Decrease in nephrin expression as found in diabetes, impairs the ability of podocytes to recover following injury making them susceptible to detachment.[15] Nephrin is a better indicator of early kidney injury in DN even before the onset of albuminuria. In T2DM, around 35–57% of patients with chronic kidney disease (CKD) do not present with albuminuria. It has been found that nonalbuminuric CKD has been associated with advanced glomerular lesions compared with patients with albuminuric CKD.

A strong relationship exists between albuminuria and obesity mediated by insulin resistance which increased oxidative stress, proinflammatory and fibrogenic cytokines and adipokines [16]. We observed a strong corelation between body mass index (BMI) and all biomarkers except bilirubin which could probably indicate the risk for metabolic syndrome. Uribarri J et al [17] observed correlation between serum AGE levels and BMI.

The metabolism of excess glucose leads to glycolytic intermediates which increase the reactive aldehydes and eventually advanced glycation end products (AGEs) in diabetes. AGEs accumulation which is independent of hyperglycemia is also well established [18]. AGE formation is further contributed by the oxidative stress among diabetic nephropathy patients. Glycotoxins such as N-carboxymethyl lysine, methyl glyoxal derivatives and pentosidine have been shown to be significantly associated with the chronic kidney disease progression, regardless of the sources.

Serum AGE levels were found to be elevated in the normoalbuminuric patients and progressively increased from normoalbuminuric to microalbuminuric and further to the macroalbuminuric groups in the present study. Wagner Z et al, found a positive correlation between AGE level and urinary ACR, creatinine and GFR among diabetic patients with impaired renal function [19], but in the present study the serum AGE levels significantly positively correlated with urinary ACR, creatinine and GFR which suggest AGE elevation in early diabetic nephropathy and mark progression of DKD.

Fructosamine (FA) refers to all glycated serum proteins with ketoamine linkages, which consists of glycation of albumin (around 90%), apolipoprotein B100 present on LDL, IgA, collagen and laminin. GA is considered to be a glucose status indicator for 1 to 3 weeks since the half-life of albumin is 20 days. Hence GA gives an idea about the glycaemic variability over a short period, haemolytic anemia, diabetic retinopathy, T2DM with comorbidities or who is on HD. GA was associated with intima media thickness (IMT) of carotid arteries, dementia and cancer. GA values are also correlated with postprandial plasma glucose levels and oscillations in plasma glucose concentrations. Albumin is highly sensitive to glycation; it is 4.5 times faster than HbA1c, probably due to two reasons: albumin is present extracellularly and has more glycation sites. GA is present at a concentration of 1% to 10% in normal individuals. It increases to two to three fold in T2DM.[20]

In the present study, HbA1c was 5.15±0.34, 6.98±0.70, 7.02±0.65 and 8.45±0.76 in the controls and progressing proteinuric stages respectively. GA was normal in the control group, but had increased further from normoalbuminuric to macroalbuminuric groups corresponding to the deterioration in kidney function.(Table I). There was significant difference between the groups in HbA1c and GA; but this significant difference did not exist between normoalbuminuric and the microalbuminuric groups. This was probably due to the fact that the extent of damage was almost the same in both stages of normoalbuminuria and microalbuminuria.

The cutoff level of HbA1c to identify cases of diabetic nephropathy with cardiac complications was 6.55% with sensitivity of 88% and specificity of 67%. The cutoff level of GA was 176.50pmol/mL with sensitivity of 90% and specificity of 76%. The area under the curve for HbA1c and GA were 0.858 and 0.882, showing that GA is a better prognostic marker of CVD in DN with better sensitivity and specificity than HbA1c.

There has been emerging data on the development of metabolic memory in diabetic complications and AGEs could be a key factor for the same as its level is directly proportional to the duration of diabetes and degree of glycaemic control [21]. This is well highlighted by the strong positive correlation between the serum AGE levels and each of glycated haemoglobin, fasting and post prandial sugars in the different patient groups.

However, serum AGE levels did not increase with increasing duration of diabetes in our study.

One of the key essentials in the development of DN is Podocyte damage, which can happen due to oxidative stress, neurohumoral changes or decreased adhesion molecules expression. This causes decrease in the podocyte density within the glomeruli even before the onset of proteinuria in DKD [22]. Sahoo et al observed podocyturia as an early marker of nephropathy among T2DM patients, which increased with prolonged duration of diabetes as noted in our study [22].

Urinary podocin was detected even in normoalbuminuric diabetic patients. The urinary podocin progressively increased from normoalbuminuric to microalbuminuric and further to macroalbuminuric groups, which significantly positively correlated with the urinary ACR and creatinine suggesting podocyte damage in the early stages of diabetic nephropathy and mark the diabetic nephropathy progression as noted by Zheng et at [23], however it did not correlate with GFR as observed by them.

Bilirubin is an endogenous antioxidant, Okada et al [24] found low levels of serum bilirubin to be a risk factor for diabetic kidney damage. In the current study the serum bilirubin levels in the macroalbuminuric group differed from the normoalbuminuric and the microalbuminuric groups though not statistically significant. Therefore, it may not be considered as potential candidate for the early detection of diabetic kidney disease, however it may be useful in assessing the renal damage severity.

The HOMA IR values also progressively increased with worsening proteinuria stages and negatively correlated with GFR similar to the observations of Viswanath et al. Several mechanisms including circulating hormones, neuro endocrine pathways and chronic inflammation could contribute to the worsening of insulin resistance at different stages of diabetic nephropathy as reported by Svensson et al. Both IR and diabetic nephropathy could be predisposed by genetic and environmental factors. Being a cross sectional design our study could not conclude whether IR was a cause of the decline in the renal function. A Japanese study suggested that insulin resistance and hyperinsulinemia contribute to renal function in the general population along with other risk factors by causing glomerular hypertension and hyperfiltration which may predispose to progressive glomerulosclerosis, leading to renal dysfunction. [25] Insulin resistance also indirectly damages the kidney through systemic atherosclerosis process forming a link between the hormone insulin and kidney function.

Higher incidence of atherosclerosis has been observed among diabetic patients probably the human LDL-C oxidation playing a role in atherosclerosis initiation. In our study, all biomarker levels strongly positively correlated with total cholesterol and triglycerides except bilirubin. LDL was significantly correlated with all except Bilirubin and serum albumin. This favours them as a potential marker for atherosclerosis and atherosclerotic vasculopathy among the diabetic patients and their correlation gradually increased with progressive nephropathy stage.[26]

ROC curve analysis was used to assess the diagnostic performance of the biomarkers. Podocin showed 98.75% sensitivity and 98.89% specificity to detect the nephropathy in the normoalbuminuric diabetic patients at a cut off level of 18.5 ng/ml. thus, it makes urinary podocin not just an early but a sensitive and specific biomarker for predicting the onset of nephropathy among the normoalbuminuric diabetic patients. This is in agreement with the

observations of El Shaaraway et al favouring urinary podocin as a highly sensitive and specific marker to predict diabetic nephropathy [27].

N-carboxymethyl lysine showed 97.5% sensitivity and 97.8% specificity to detect the nephropathy in the normoalbuminuric diabetic patients at a cut off level of 1455 ng/l. Thus, it makes N-carboxymethyl lysine not just an early but a sensitive and specific biomarker for predicting the onset of nephropathy among the normoalbuminuric diabetic patients. This is in agreement with the observations of Hirata and Kubo favouring N-carboxymethyl lysine as a highly sensitive and specific marker to predict diabetic microangiopathy both nephropathy as well as retinopathy [28] but in contradiction to the observations made by Busch et al where they noted that N-carboxymethyl lysine did not predict renal outcomes in diabetic population [29].

Nephrin excretion was found to be significant in diabetic patients with normoalbuminuria whereas the control group who were nondiabetics, were not excreting nephrin in urine. Thus, establishing nephrinuria as a marker of preclinical diabetic nephropathy.[30] The cut-off level of urine nephrin was 97.5ng/ml with the sensitivity of 92.5% and specificity of 76.7%; the cut-off level of microalbuminuria was 31mg/g of creatinine with the sensitivity of 97.5% and specificity of 100% and the cut-off level of serum creatinine was 0.95mg/dL with the sensitivity of 88.8% and specificity of 74.4%. Area under the Curve (AUC) for urine nephrin, albuminuria and serum creatinine were found to be 0.943, 0.998 and 0.912 respectively. Even though AUC for urine albuminuria was as high as 0.998, it could not satisfy the criteria of early diagnosis of DN since it is not elevated in normoalbuminuria. The threshold of albuminuria varies greatly between individuals; DN can progress without significant albuminuria.[31] Nephrin expression and nephrin tyrosine phosphorylation are significantly reduced by diabetes earlier than the onset of microalbuminuria. Further research on podocyte metabolism might confirm nephrinuria to be a biomarker of pre-clinical DN.[28]

Since multiple pathophysiological processes are involved in diabetic kidney disease, it may be difficult for a single biomarker to predict the disease. It is better to develop a panel of biomarkers that capture several pathophysiological processes which might improve prediction of DN progression.[32]

LIMITATIONS

The present study being a cross-sectional study design provides basis for association rather than causality. Also, adjustment for Hypertension and dyslipidaemia as confounding factors could not be done because such adjustments would be difficult since predisposition to both hypertension and dyslipidaemia occur with diabetic kidney disease.

CONCLUSIONS

Urinary Podocin, Nephrin, n-Carboxy methyl lysine, Glycated Albumin seem to have very good to excellent diagnostic value in accordance to microalbumin and HbA1c followed by HOMA-IR. However Serum Albumin though well known as a negative acute phase reactant and serum Bilirubin – the endogenous antioxidant did not stand the test of time in predicting the onset and prognosis of Diabetic Nephropathy.

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Table 1: Baseline Characteristics of the study cohort.

Variables	Study cohort				Diabetic Subgroups					
	All	No DM	DM	P value	Normo albuminuria	P value	Micro albuminuria	P value	Macro albuminuria	P value
Age (y)	51.68±6.2	49.5±6.0	52.43±6	0.031	50.95±5.5	.284	52.8±6.6	0.015	53.7±6.0	.002
Sex M	N=112	N=30	N=82	------	N=29	-------	N=28	-------	N=25	-----
F	N=58	N=15	N=43		N=16		N=12	--	N=15	
Height (ft)	5.52±0.2	5.6±0.2	5.5±0.21	.769	5.5±0.22	.483	5.58±0.21	.710	5.57±0.21	.510
Weight (kg)	79.4±10.8	71.4±6.8	82.3±10	<0.01	78.2±10.2	.002	79.6±10.5	<0.001	89.6±6.6	<0.01
BMI(kg/m2)	27.66±3.2	24.7±1.5	28.7±3.0	<0.01	27.2±2.5	<0.01	27.73±2.7	<0.01	31.3±2.0	<0.01
Waist (cm)	92.49±7.6	89.7±5.9	93.5±7.9	0.014	89.4±5.1	.842	91.5±6.0	.179	100.5±8.0	<0.01
Hip (cm)	95.05±5.9	98.1±5.5	93.9±5.6	<0.01	92.2±5.8	<0.01	94.6±5.6	.005	95.1±5.0	.013
W/H ratio	0.97±0.06	0.91±.03	0.99±.05	<0.01	0.97±.04	<0.01	0.96±.03	<0.01	1.05±.05	<0.01
Systolic BP	143.3±8.9	136±6.0	145±8.3	<0.01	139±4.5	.118	148±5.4	<0.01	151±9.0	<0.01
Diastolic BP	88.22±7.3	82.1±3.1	90.4±7.1	<0.01	84±4.6	.113	91.5±5.6	<0.01	95±5.9	<0.01
Diab. Duration(y)	6.31±4.3	---------	8.58±2.3	<0.01	8.13±2.17	<0.01	8.85±3.11	<0.01	8.8±1.68	<0.01
FBG (mg/dl)	121.56±37	75.7±9.0	138±28	<0.01	133±31	<0.01	123±21	<0.01	157±21	<0.01
PPBS (mg/dl)	176.87±52	113.6±13	199.6±42	<0.01	195±46	<0.01	181±30	<0.01	222±37	<0.01
HbA1c (%)	6.85±1.3	5.15±0.3	7.46±0.9	<0.01	6.9±0.7	<0.01	7.02±0.6	<0.01	8.4±0.7	<0.01
Glycated Albumin (pmol/ml)	216±129	97.3±21	259±125	<0.01	194±60	<0.01	211±76	<0.01	379±133	<0.01
BUN (mg/dl)	16.78±4.4	15.5±3.2	17.2±4.7	0.105	14.33±5.1	.128	20.5±4.2	<0.01	17.12±1.8	.065

Urea (mg/dl)	36.04±9.5	37.5±4.4	35.5±10.8	0.487	33.4±6.8	0.049	28.0±7.4	<0.01	45.3±10.9	<0.01
S.Creat (mg/dl	1.21±0.58	0.78±0.1	1.36±0.6	<0.01	0.91±0.14	0.012	1.04±0.14	<0.01	2.18±0.34	<0.01
eGFR (ml/min/1.73m2)	76.56±31	107.5±15	65.4±28	<0.01	86.6±20.8	<0.01	75.6±12.6	<0.01	31.3±7.12	<0.01
ACR (mg/G)	116±135	25.08±4.06	148.7±145	<0.01	25.46±3.2	.947	84.9±43	<0.01	351±35	<0.01
Microalbumin(mg)	102.8±113	25.07±3.7	130.7±120	<0.01	24.8±3.2	.973	84.7±35.7	<0.01	296±41.9	<0.01
Total.chol (mg/dl)	183.9±32	175.9±24	186±34	<0.01	183.9±34.4	0.223	175±35.2	.899	202±28.4	<0.01
LDL(mg/dl)	110.3±28	107±23	111±30.4	.701	109.8±29.5	.659	99.7±31	.212	125±25.2	.003
HDL(mg/dl)	39.76±3.9	41.7±4.13	39±3.6	<0.01	40.73±3.5	0.170	38.8±3.02	<0.01	37.3±3.4	<0.01
TGL(mg/dl)	169.6±53	134±19	182±56.5	<0.01	170.15±56	.004	180±63	<0.01	197.6±46.3	<0.01
U.Nephrin (ng/ml)	126.5±74	66.1±8.2	148±76	<0.01	97.5±10.9	.001	116±19.7	<0.01	237±74	<0.01
U.Podocin (ng/ml)	20.1±16.7	4.5±0.6	25±16	<0.01	11.11±3.2	<0.01	21.2±2.0	<0.01	46.5±10.2	<0.01
Serum CML (ng/l)	1512±388	1138±82	1647±366	<0.01	1238±124	0.003	1713±145	<0.01	20141±176	<0.01
HOMAIR	2.54±1.32	0.9±0.19	3.12±1.04	<0.01	3.0±1.06	<0.01	2.8±0.85	<0.01	3.6±1.03	<0.01
S.Bilirubin (mg/dl)	0.6±0.2	0.58±0.13	0.62±0.15	<0.01	0.69±0.13	<0.01	0.66±0.10	0.050	0.51±0.14	0.055

Table.2 Correlation coefficient (r) between biomarkers and Patients clinical parameters.

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Biomarkers	Clinical parameters										Comorbid conditions				Diabetic complications					
	Age		Sex (Male)		SBP		DBP		BMI		SHT		Dyslipidemia		retinopathy		LV dysfunction		Neuropathy	
	r	P	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	P
Nephrin (ng/ml)	.149	.053	-.021	.393	.516	<0.01	.650	<0.01	.610	<0.01	-.175	.120	-.044	.155	.073	.315	-.007	.484	.073	.315
Podocin (ng/ml)	.213	0.005	-.068	.189	.618	<0.01	.683	<0.01	.651	<0.01	.094	<0.01	.051	.051	-.017	.686	-.100	.001	-.017	.686
Gly.Albumin (pmol/ml)	.134	.082	-.103	.091	.515	<0.01	.564	<0.01	.624	<0.01	.086	.310	-.001	.076	-.389	<0.01	-.085	.145	-.389	<0.01
N-CML (ng/L)	.222	.004	-.059	.221	.644	<0.01	.732	<0.01	.606	<0.01	-.522	.652	-.609	.851	-.766	<0.01	-.656	.044	-.766	<0.01
S.albumin (gm/dl)	-.268	<0.01	.029	.352	-.516	<0.01	-.534	<0.01	-.492	<0.01	-.699	.317	-.794	.075	.774	.003	-.699	.317	.774	.003
T.Bilirubin (mg/dl)	.062	.418	-.014	.427	-.223	.004	-.206	.007	-.104	.177	-.375	.752	-.304	.149	-.379	.183	-.338	.299	-.379	.183
Microalbumin (mg)	.189	.013	-.019	.401	.573	<0.01	.638	<0.01	.658	<0.01	.094	.004	.059	.052	.206	.134	.167	.002	.206	.134
HOMAI	.134	.081	-	.05	.67	<0.	.46	<0.	.45	<0.	.54	<0.0	.49	<0.	.34	<0.	.45	<0.	.34	<0.0

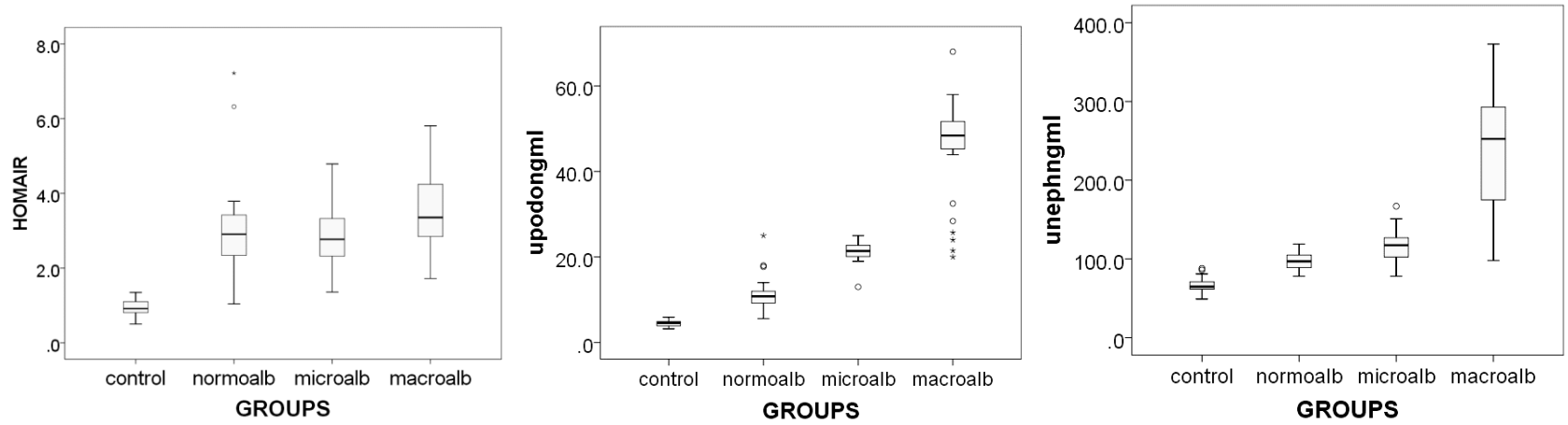
R			.145	8	7	01	5	01	7	01	2	1	4	01	9	01	9	01	9	1
Serum Creatinine	.195	.011	.014	.859	.581	<0.01	.610	<0.01	.661	<0.01	.496	<0.01	.372	<0.01	.342	<0.01	.362	<0.01	.342	<0.01

Table 3. Correlation coefficient (r) between biomarkers and study group laboratory parameters.

Biomarkers	Glycemic markers										Kidney function markers								Lipid Profile					
	Homa-IR		**HbA1c**		**FBS**		**PPBS**		**DM duration**		**BUN**		**Creat.**		**u.ACR**		**eGFR**		**T.chol**		**TGL**		**LDL**	
	r	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**
Nephrin ng/ml	**.521**	**<0.01**	**.727**	**<0.01**	**.600**	**<0.01**	**.582**	**<0.01**	**.474**	**<0.01**	**.112**	**0.145**	**.793**	**<0.01**	**0.832**	**<0.01**	**-.739**	**<0.01**	**.294**	**<0.01**	**0.351**	**<0.01**	**.252**	**<0 01**
Podocin ng/ml	**.541**	**<0.01**	**.734**	**<0.01**	**.601**	**<0.01**	**.527**	**<0.01**	**.523**	**<0.01**	**0.173**	**.024**	**0.875**	**<0.01**	**.903**	**<0.01**	**-.837**	**<0.01**	**.256**	**<0.01**	**.349**	**<0.01**	**.207**	**.007**
Glycated Albumin pmol/ml	**.633**	**<0.01**	**.906**	**<0.01**	**.758**	**<0.01**	**.630**	**<0.01**	**.480**	**<0.01**	**.095**	**0.219**	**.731**	**<0.01**	**.710**	**<0.01**	**-.718**	**<0.01**	**.239**	**.002**	**.349**	**<0.01**	**.200**	**.009**
N-CML ng/l	**.527**	**<0.01**	**.735**	**<0.01**	**.580**	**<0.01**	**.563**	**<0.01**	**.544**	**<0.01**	**.271**	**<0.01**	**.755**	**<0.01**	**.820**	**<0.01**	**-.780**	**<0.01**	**.143**	**0.062**	**.305**	**<0.01**	**.096**	**.215**
S.Albumin gm/dl	**-.348**	**<0.01**	**-.503**	**<0.01**	**-.372**	**<0.01**	**-.345**	**<0.01**	**-.444**	**<0.01**	**-.313**	**<0.01**	**-.542**	**<0.01**	**-.597**	**<0.01**	**.529**	**<0.01**	**-.158**	**.04**	**-.303**	**<0.01**	**-.109**	**.156**
T.Bilirubi	**.04**	**.56**	**-**	**.18**	**-**	**.40**	**.01**	**.83**	**.03**	**.64**	**-**	**.20**	**-**	**<0.**	**-**	**<0.**	**.26**	**<0.**	**-**	**.20**	**-**	**.18**	**-**	**.29**

n mg/dl	**5**	**1**	**.103**	**2**	**.064**	**5**	**6**	**1**	**6**	**6**	**.099**	**0**	**.367**	**01**	**.357**	**01**	**6**	**01**	**.018**	**1**	**.103**	**3**	**.081**	**7**
Microalbumin mg	**.488**	**<0.01**	**.685**	**<0.01**	**.543**	**<0.01**	**.491**	**<0.01**	**.396**	**<0.01**	**.136**	**0.078**	**.903**	**<0.01**	**.989**	**<0.01**	**-.819**	**<0.01**	**.270**	**<0.01**	**.300**	**<0.01**	**.237**	**0.002**

Fig- 1 : Biomarker levels in various stages of diabetic nephropathy



(a) (b) (c)

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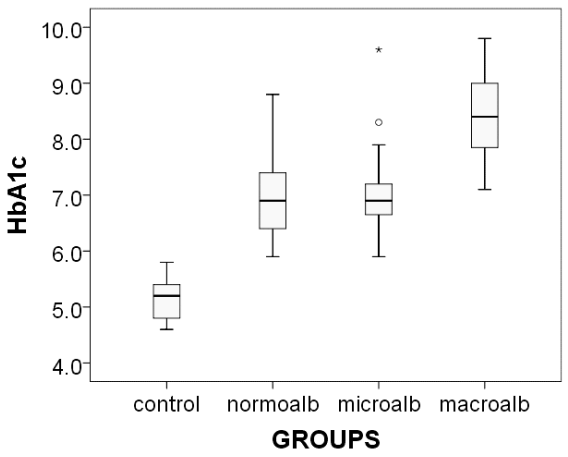

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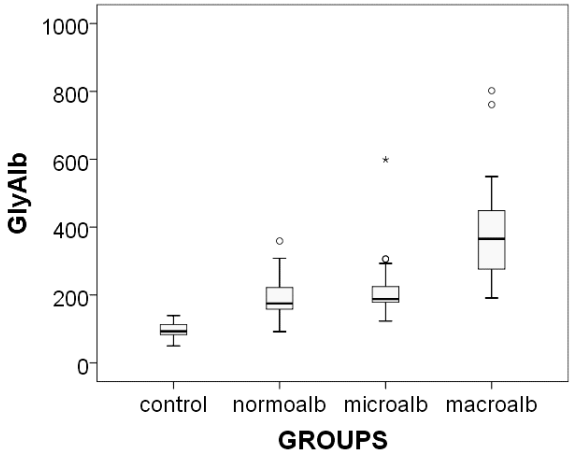

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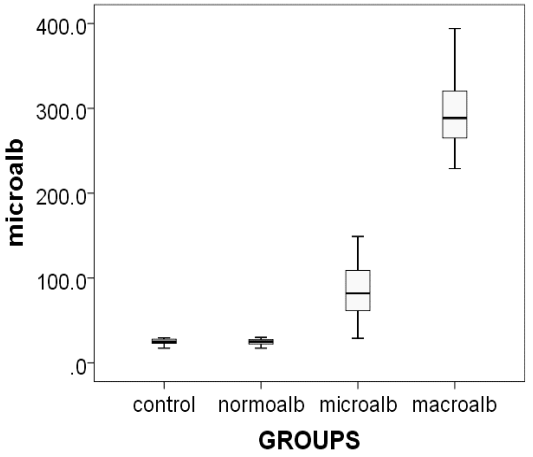

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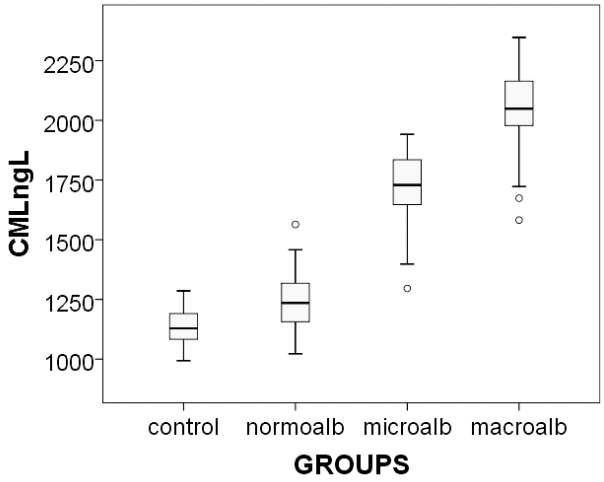

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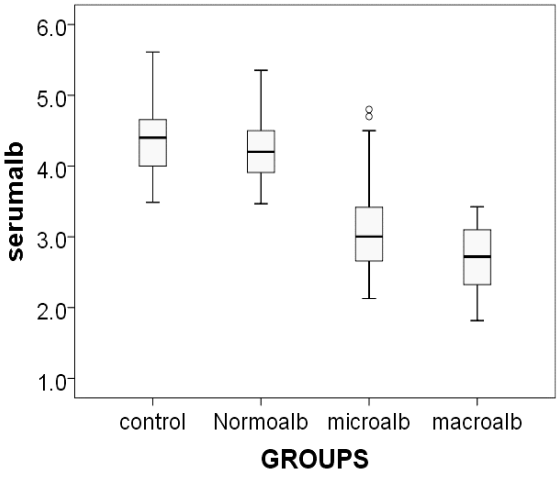

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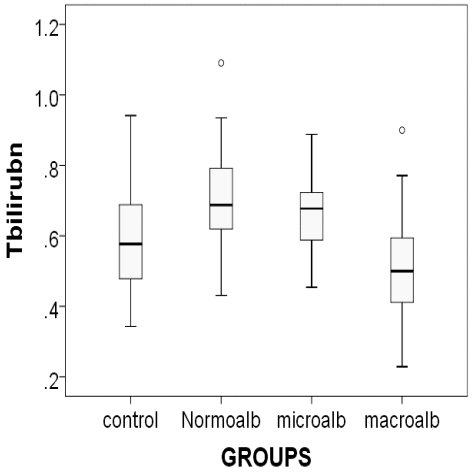

(i)

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Table.4 : AUC ,Sensitivity ,Specificity ,PPV, NPV of Biomarkers.

Parameter	Area under curve	Cut off	Sensitivity	specificity	PPV	NPV
HoMA IR	.792	2.45	81.3%	64.4%	67%	79.5%
Serum albumin	.043	3.98	93.8%	71%	74.3%	92.8%
Total bilirubin	.082	0.6	80%	90%	87.7%	83.5%
HbA1c	.858	6.8	83.8%	68.9%	70.5%	82.7%
Glycated albumin	.882	179	87.5%	77.8%	77.8%	87.8%
Urinary nephrin	.943	102.5	86.3%	82.2%	81.2%	87.1%
Urinary podocin	.993	18.5	98.8%	98.9%	98.8%	98.9%
Serum AGE(CML)	.997	1455	97.5%	97.8%	97.5%	97.8%

Fig-2 : Receiver Operating Characteristic Curves of the biomarkers