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"Performance of Urinary Clusterin as a Biomarker for Diagnosis of Early Diabetic Nephropathy in type 2 Diabetes"

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ABSTRACT:

Aims Diabetic kidney disease (DKD) is considered one of the rising issues nowadays due to the growing incidence of diabetes especially type 2. The use of variable novel biomarkers for early detection and correlation with DKD is a general direction in most of the current literature, so we aim to evaluate the diagnostic significance of urinary clusterin as an early marker of DKD and correlate its level with the traditional markers of DKD.

Methods An observational study included 90 subjects, 45 patients with type 2 diabetes (T2DM) and another 45 subjects without diabetes who were age, sex, and BMI matched. The group of diabetes was classified according to the level of albuminuria into 3 subgroups; normoalbuminuric, microalbumineric, and macroalbuminuric.

Results urinary clusterin was higher in the group with diabetes even in the absence of albuminuria compared to the control group with significantly higher levels of clusterin with advancing of the albuminuric stage.

Conclusion Urinary clusterin is considered an early sensitive marker in predicting DKD in T2DM at a cutoff point of 16.9 ng/ml (Sensitivity 97.8%, specificity 97.8%), and its level showed a significant progression with the advancement of the stage of nephropathy.

Keywords Clusterin; diabetic nephropathy; CKD marker; T2DM; albuminuria

1. Introduction

Diabetic kidney disease (DKD), which is defined as chronic kidney disease caused by diabetes, is a very common health burden worldwide. Diabetic nephropathy is usually used to describe the typical histological pattern of glomerular changes in diabetes on renal biopsy which is less commonly used in clinical practice. [1]

The actual percentage of chronic kidney disease (CKD) in diabetes is not assessed, most probably owing to heterogeneous confounders in such patients and the paucity of diagnostic precise biopsies. [2]

Although the presence of albuminuria is considered an early sensitive marker of DKD and a marker of greatly increased cardiovascular morbidity and mortality for patients with either type 1 or type 2 diabetes, when albuminuria presents a significant degree of glomerular dysfunction is always present. Furthermore, several limitations have been described when considering albuminuria as a marker of DKD; first albuminuria doesn't occur in all cases of DKD as shown by many studies on people with either type 1 or type 2 who developed advanced-stage CKD without albuminuria (nonalbuminuric diabetic kidney disease" NADKD"). [3,4] Second, albuminuria is considered an excellent marker for glomerulopathy which occurs more commonly in type 1, while in type 2 diabetes glomerulopathy is not the sole pathology, where tubulointerstitial and sometimes macroangiopathy of renal vessels is the cause of CKD. [5] Finally, albuminuria is not a specific marker of DKD which can occur due to other causes than diabetes.[6] Consequently, an evolving trend for using new markers with a sensitivity for minor renal dysfunction either at the glomerular or tubular level which is usually underestimated due to lacking of sensitive tools in clinical practice.

Clusterin is a glycoprotein (named apolipoprotein J) that is expressed in various tissues and body fluids with a very high concentration in endocrinal and central nervous system tissues. It was first isolated in 1979 from rats' testicles and subsequently named due to its ability to produce clustering and aggregations with blood cells in vitro. No known significance was detected for this glycoprotein at such time [7 - 9]

Clusterin (76–80 kDa protein) was found to be present in very high concentrations in dedifferentiated tubular cells, a point that considers urinary clusterin as a precise marker of acute tubular injury and suggested to exert an antiapoptotic role allowing for cell aggregations and attachment on exposure to injury with subsequent cell protection effect. [10] Also, in animal model studies, urinary clusterin is upregulated in glomerular injury in DN and on exposure to prerenal insult. This upregulation also has been demonstrated to have a protective antioxidant effect.[11] On the other hand, circulating Clusterin is strongly exhibited as a component of cardiometabolic syndrome, where it is overexpressed in obese rather than lean adipocytes and seems to exert a deleterious metabolic effect. [12]

So, in the current study, we investigate the role of urinary clusterin as an early marker of DKD in T2DM, and its correlation with the studied population variables, and other markers of CKD such as eGFR, creatinine, and albuminuria.

2. Participant and Method:

2.1. Study design and participant

In this cross-sectional study, we included a total of 90 subjects: 45 with Type 2 diabetes (T2DM) and 45 healthy controls. Patients with T2DM were diagnosed based on the American Diabetes Association criteria and divided into three groups according to albuminuria level: normoalbuminuric group (albumin/creatinine ratio, ACR < 30 mg/g, n = 15), microalbuminuria group (ACR 30-300 mg/g; n = 15), and the macroalbuminuria group (ACR > 300 mg/g; n = 15). [13] Subjects were excluded from the study if they had renal disease other than diabetic nephropathy, cardiovascular diseases, active infection, or malignancy.

Routine investigations were performed on all included subjects such as; complete blood count (CBC), liver function tests, serum creatinine, lipid profile, blood urea nitrogen (BUN), albumin creatinine ratio (ACR), glycosylated hemoglobin (HbA1C) and estimated GFR was calculated for all cases using a modification of diet in renal disease (MDRD) formula.

Urinary clusterin was measured in the first-morning urinary sample. Then the serum was stored at −80∘ C till analysis. Urinary clusterin levels were evaluated using an ELISA kit developed by Sun Red biotechnology co. Sensitivity: 0.905ng/ml and assay range: 1ng/ml to 300ng/ml with Intra-Assay coefficients of variations (CVs) <8% and Inter-Assay CV<10%.

2.2. Statistical Analysis

The sample size was determined through power analysis using preliminary data obtained in our laboratory with the following assumptions: α of 0.05 (two-tailed) and power of 90%. A minimum of 11 subjects in the three T2DM subgroups and control group allowed the detection of differences in urinary clusterin concentrations. Data were expressed as means ± standard deviation or median (interquartile range). The differences in characteristics between three groups of T2DM patients and control subjects were compared using Chi-square tests, one-way ANOVA, or the Kruskal-Wallis test. Logistic regression analysis was used to determine the risk factors for developing DN. The correlations between urinary clusterin and other parameters were analyzed by Pearson correlation analysis. Multiple linear regression analysis was used to determine the contribution of various factors to urinary clusterin. *P* values less than 0.05 were considered statistically significant.

3. Results:

The demographic characteristics of the studied populations were matched regarding age (53.56±5.68, and 55.18±3.56), BMI (27.61±1.59, and 28±1.75), and sex (62.2% & 37.8 % versus 60% & 40% females, and males), in control and diabetes group respectively. **Table 1**

Statistical analysis of laboratory data revealed a significant difference between the control and diabetes group regarding hemoglobin level (gm/dL) (mean± SD; 11.91±1.83 &10.30±1.15 with P value <0.001), Total Protein (gm/dL) (mean± SD; 7.29±0.50 & 5.23±0.93 with P value <0.001), Albumin (gm/dL) (mean± SD; 4.31±0.38 & 3.12±0.44 with P value <0.001), blood urea nitrogen (BUN) (mg/dl) (mean± SD; 14.31±4.73 & 36.89±12.31with P value <0.001), Creatinine (mg/dL) (mean± SD; 0.89±0.14 & 1.38±0.41 with P value <0.001), ACR (mg/gm) (mean± SD; 17.22±8.57 & 267.84±144.18 with P value <0.001) HbA1C % (mean± SD; 4.91±1.77 & 8.64±0.42 with P value <0.001), Random blood sugar (mg/dL) (mean± SD; 171.36±83.77 & 303.11±72.32 with P value <0.001), cholesterol (mg/dL) (mean± SD; 157.22±33.28 & 243.84±17.42 with P value <0.001), high-density lipoprotein (HDL) (mg/dL) (mean± SD; 62.09±11.62 & 37.64±7.08 with P value <0.001), low density lipoprotein (LDL) (mg/dL) (mean± SD; 75.69±29.48 & 161.20±19.05 with P value <0.001), and Triglyceride (mg/dL) (mean± SD; 98.16±95.43 & 338.87±114.18 with P value <0.001) as shown in **Table 1.**

Table 1. Basic characteristics and laboratory investigation of the studied groups:

*¥ SD, # Student t-test, £ Chi-square test, ^ interquartile range, * Statistically significant at p ≤ 0.05*

[a] blood urea nitrogen, [b] albumin creatinine ratio, [c] estimated glomerular filtration rate,

Variable		Control Group (n=45)		Diabetes Group (n=45)		Tests	
						***T*#**	***P* value**
Age (years) Mean ± SD¥		53.56±5.68		55.18±3.56		-1.624	0.108
BMI Mean ± SD		27.61±1.59		28±1.75		-1.099	0.275
Variable		**No**	**(%)**	**No**	**(%)**	**X^{2} £**	**P value**
Sex	**Female**	28	62.2	27	60	0.047	0.829
	Male	17	37.8	18	40		
Variable		**Control Group (n=45)**		**Diabetes Group (n=45)**		***Test* t/z**	***P value***
Complete blood count Mean ± SD	**Leucocytes (/mm)**	7.79±2.12		7.31±1.57		1.222	0.225
	Hemoglobin (gm/dl)	11.91±1.83		10.30±1.15		5.005	**<0.001***
	Platelets	303.22±83.77		324.82±86.14		-1.206	0.231
Blood protein levels (g/dl) Mean ± SD	**Total Protein**	7.29±0.50		5.23±0.93		13.043	**<0.001***
	Albumin	4.31±0.38		3.12±0.44		13.819	**<0.001***
Renal function Mean ± SD	**BUN [a] (mg/dl)**	14.31±4.73		36.89±12.31		-11.483	**<0.001***
	Creatinine (mg/dl)	0.89±0.14		1.38±0.41		-7.510	**<0.001***
	ACR [b] (mg/g)	17.22±8.57		267.84±144.18		-11.640	**<0.001***
Glycemic levels Mean ± SD	**HbA1c (%)**	4.91±1.77		8.64±0.42		-6.346	**<0.001***
	Random blood sugar (RBS) (mg/dL)	171.36±83.77		303.11±72.32		-7.986	**<0.001***
Lipid profile (mg/dl)	**cholesterol**	157.22±33.28		243.84±17.42		-15.470	**<0.001***
	HDL	62.09±11.62		37.64±7.08		12.052	**<0.001***
	LDL	75.69±29.48		161.20±19.05		-16.340	**<0.001***
	TG Median (IQR^)	98.16±95.43 70 (56.5-111)		338.87±114.18 300 (251-400)		-7.808	**<0.001***
eGFR[c] (Mean ± SD) (ml/min/m^2)		100.42±12.38		110.53±17.64		-3.148	**0.002***
Clusterin (ng/ml) Mean ± SD Median (IQR)		1.60±3.21 1.5 (0.02-2.5)		114.91±80.13 100.25 (33.5-169)		-8.163	**<0.001***

A comparison of the studied group regarding estimated GFR revealed a statistical difference with a relatively higher GFR in the group with diabetes (110.53±17.64) than the control (100.42±12.38) with a p-value of 0.002. Also, urinary clusterin showed significantly higher values in group with diabetes (114.91±80.13,) than in the control group (1.60±3.21,) with a P-value <0.001**. (Table 1)**

Comparison of urinary clusterin levels in the diabetes subgroups revealed a significant difference with higher clusterin values in advancing the stage of albuminuria (28.50±2.12, 105.89±79.02, 185.99±42.26 in normoalbuminuric, microalbuminuric, macroalbuminuric group respectively) with no significant difference between the control group (1.6 ± 3.2) and normoalbuminuric subgroup **Table 2**.

Table 2. Relation between clusterin level as regard level of albuminuria

	N	Mean ±SD	f	P value	LSD
Control group	45	1.60±3.21	42.6	<0.001*	Ref
Normoalbuminuria	15	28.50±2.12			0.473
Microalbuminuria	15	105.89±79.02			<0.001*
Macroalbuminuria	15	185.99±42.26			<0.001*

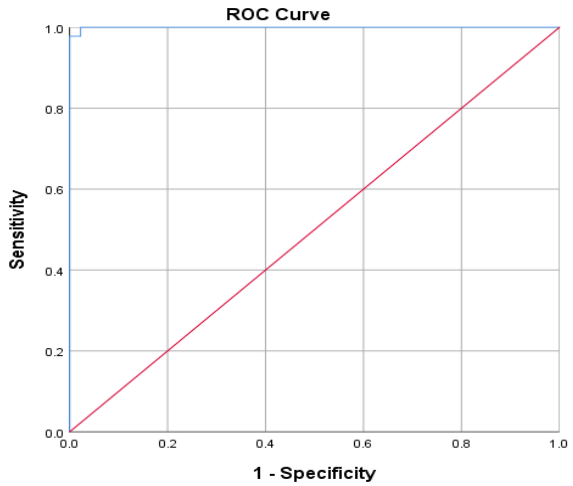
The receiver operator characteristic curve (ROC) revealed a significant predictive value for diabetic nephropathy at a cutoff point of 16.9 ng/dl at a sensitivity of 97.8%, specificity of 97.8%, positive predictive value of 97.8%, and negative predictive value of 97.8%. (With AUC 1.000, 95%CI (0.998-1.0) (**Table 3, figure 1**)

Table 3. Validity of Clusterin at cut off= 16.9 ng/dl in predicting diabetic nephropathy:

Variables	AUC[a]	95%CI[b]	Cutoff	Sensitivity	Specificity	PPV[c]	NPV[d]	Accuracy
DT index	1.000	0.998-1.0	16.9	97.8%	97.8%	97.8%	97.8%	97.8%

[a] area under the curve, [b] confidence interval, [c] positive predictive value,[d] negative predictive value

Fig.1 ROC curve illustrating the validity of Clusterin at cut-off= 16.9 in predicting diabetic nephropathy



The correlation of urinary clusterin with the studied parameters in the group with diabetes revealed a significant positive correlation with creatinine (r = .783, p= 0.00), BUN (r = .509, p= 0.00), ACR (r = .695, p= 0.00), blood glucose (r = .463, p= 0.001), cholesterol(r = .740, p= 0.000), LDL(r =.838, p= 0.000), TG (r = .713, p= 0.000), eGFR (r = .695, p= 0.00), while a significant negative correlation with albumin(r = -.680, p= 0.00), total protein (r = -.795, p= 0.00), platelet (r = -.617, p= 0.00), and HDL (r = -.817, p= 0.000). No significant correlation was found regarding age, BMI, hemoglobin, WBCs, and HbA1c. **(Table 4)**

The correlation of urinary clusterin with the studied parameters in the control group revealed a significant positive correlation with BMI (r = .414, p= 0.005), HbA1c (r = .785, p= 0.00), ACR (r = .296, p= 0.049), blood glucose (r = .764, p= 0.00), eGFR (r = .363, p= 0.014), while no significant correlation was found regarding other parameters. **(Table 4).**

Table 4. Correlation between clusterin and different parameters within the group with diabetes and control group

Variables		Clusterin in the group with diabetes (n=45)	Clusterin in the control group (n=45)
Age	r	0.139	-0.108
	p	0.364	0.48
BMI	r	-0.043	**.414****
	p	0.78	**0.005**
Leucocytes	r	-0.111	0.185
	p	0.47	0.224
Haemoglobin (g/dl)	r	0.057	-0.115
	p	0.71	0.453
PLT	r	**-.617****	-0.158
	p	**0.00**	0.301
Creatinine (mg/dl)	r	**.783****	-0.06
	p	**0.000**	0.696
BUN (mg/dl)	r	**.509****	-0.12
	p	**0.00**	0.434
Albumin (gm/dl)	r	**-.680****	-0.148
	p	**0.00**	0.333
Total Protein(gm/dl)	r	**-.795****	-0.024
	p	**0.00**	0.877
HbA1c (%)	r	-0.018	**.785****
	p	0.907	**0.00**
RBS (mg/dl)	r	**.463****	**.764****
	p	**0.001**	**0.00**
ACR (mg/g)	r	**.695****	**.296***
	p	**0.00**	**0.049**
Cholesterol (mg/dl)	r	**.740****	0.22
	p	**0.000**	0.147
HDL (mg/dl)	r	**-.817****	-0.027
	p	**0.000**	0.858
LDL (mg/dl)	r	**.838****	0.2
	p	**0.000**	0.187
TG (mg/dl)	r	**.713****	-0.095
	p	**0.000**	0.533
eGFR (ml/min/m^2)	r	**.695****	**.363***
	p	**0.00**	**0.014**

4. Discussion:

It is well-documented that early detection of CKD with a prompt application of suitable management protocol can improve the outcomes. In diabetes, the paucity of application of renal biopsy for precise diagnosis renders the diagnosis based on certain clinical criteria and traditional markers such as ACR. Thus, the current evolving trend is directed toward the assessment of different urinary proteomes for early detection of CKD as well as differentiating the cause of CKD in certain situations. Clusterin is an example of many proteomes present in urine that seems to have a specifically higher increase in the urine of patients with CKD due to diabetes and hypertension compared to other causes of CKD.[14]

The current study found a statistically significant difference between the overall group of diabetes and the control regarding urinary clusterin with relatively higher levels in type 2 diabetes. This finding agrees with a study by Kim et al investigating the predictive role of clusterin for the development of DKD as an early marker. The previous study has documented a significant difference in baseline urinary clusterin levels between diabetic and nondiabetics.[15]

The eminent link between insulin resistance and the pathogenesis of T2DM could explain the previous finding. Many previous studies on humans have demonstrated the crucial relation between plasma clusterin (Apolipoprotein J), and the magnitude of insulin resistance with a decline in its level with an improvement of insulin resistance.[16] Also, the isolation and purification of adipocyte-derived clusterin revealed a significant effect on insulin resistance mediated mainly through hepatic action.[17]

In the current study, the Receiver operator characteristic curve revealed a validity of Clusterin at cut-off= 16.9 ng/dl in predicting diabetic nephropathy with sensitivity, specificity, PPV, and NPV of 97.8%. Also, the comparison of urinary clusterin levels in the 3 diabetes subgroups revealed a significant difference with a significantly higher value of clusterin with the progression of albuminuria stage (normoalbuminuric group, mean ± SD; 28.50±2.12, microalbuminuric group, mean ± SD; 105.89 ± 79.02, and the macroalbuminuric group, mean ± SD; 185.99±42.26), while no significant difference was found between the control (mean ± SD; 1.59±3.21) and the normoalbuminuric group.

This significant progression of urinary clusterin level at different chronological stages of albuminuria could suggest the possible predictive role of this biomarker in the early detection of DKD as previously observed in a study by Zeng et. al. [18]

Although urinary clusterin can be produced as a result of podocyte injury in the glomeruli in DKD, it is considered a marker of tubular injury rather than glomerular injury as demonstrated in multiple animal models. [19, 20] DKD includes multiple inflammatory pathological mechanisms involving different structures in the kidney most of which are the glomeruli and tubules. Hyperglycemia, as an incentive event, with its sequel including advanced glycation end-products, carbonyl intermediates, and ultra-filtered growth factors can induce glomerular injury with disruption of the glomerular basement membrane allowing passage of albumin through renal tubules which in turn starts an inflammatory cascade in tubulointerstitial tissues enhanced by the effect of hyperglycemia itself with resultant activation of production of tubular specific biomarkers such as urinary clusterin. [21]

A comparison of eGFR in diabetes and control groups revealed a significantly higher level in the former (mean± SD 110.53±17.64), which can reflect the early stage of glomerular hyperfiltration that develops in some diabetics before the appearance of proteinuria and without clinical manifestations. Great variability is documented regarding the staging of DKD with a lack of correlation with albuminuria. Many T1DM and most T2DM, don't follow the classic course of DKD.[2]

In the current study, urinary clusterin was correlated with parameters of renal injury, with a positive correlation with ACR, and eGFR in both studied groups, as well as a correlation with serum creatinine and BUN in the group of diabetes only. Serum creatinine and BUN are incomplete markers of kidney function as the rise means the loss of approximately 75% of kidney function and they reflect only one aspect of renal insults involving decreasing GFR. [22]

Microalbuminuria is considered an independent risk factor for cardiovascular events and acts as a marker of endothelial dysfunction in multiple well-established pieces of literature. [23, 24] However, a lack of sensitivity and specificity of ACR for progressive decline in eGFR is reported with only about one-third of those with microalbuminuria developing a progressive decline in GFR, also about half of those developing a decline in renal function were albuminuric. [25]

A correlation of urine clusterin with lipid profile in the group of diabetes revealed a significant positive correlation with total cholesterol, LDL, and triglyceride and a negative correlation with HDL. To our knowledge, this is the only study that correlated urinary clusterin with metabolic parameters after the finding of Kim et al that revealed a positive correlation of urinary clusterin with LDL.[15]

Serum clusterin is considered a modulator in lipid transport and metabolism. It is present in plasma as a soluble protein or as a component of HDL and seems to exert a protective antiatherogenic effect. In tissue culture, clusterin acts by transferring cholesterol from macrophage-derived foam cells allowing the mobilization of cholesterol from damaged tissue and delivering it to HDL particles with reverse cholesterol transport.[26]

In many reviews, serum clusterin was shown to be correlated with LDL, HDL, or even total cholesterol with many controversial effects on cardiovascular and metabolic parameters. Some studies demonstrated this correlation as a part of a potential protective role in the atherogenic process as Clusterin is considered a protein component of dense HDL cholesterol that aids in the transfer of HDL cholesterol from peripheral tissues to the liver, reducing the risk of atherosclerosis progression, also it acts by binding enzymatically to LDL and reducing fatty acid-mediated cytotoxicity. [27, 28]

By contrast, many observations suggest that serum clusterin is strongly correlated with insulin resistance, atherogenesis, and cardiovascular risk. Clusterin can induce a deleterious effect on paraoxonase-1 (PON1), an antioxidant, with subsequent enhancement of the atherogenic process. [29] Further, clusterin is strongly associated with the proinflammatory factor C-reactive protein. [30] A study by Hoofnagle et al showed that lower clusterin levels within HDL molecules lead to loss of the protective effect of HDL and are associated with insulin resistance and obesity and unfavorable lipid profile .[31]

A positive correlation was found between urinary clusterin and blood glucose levels in both groups also with glycosylated hemoglobin and BMI in the control group. Clusterin has shown a strong association with the cardiometabolic axis especially Clusterin derived from adipocytes. Clusterin exerts an effect on appetite and obesity mediated through the affection of leptin transport across the blood-brain barrier. In addition, clusterin mediates the activation of pro-inflammatory mediators of insulin resistance. Thus, higher clusterin levels are found to be correlated with obesity, insulin resistance, and overall cardiovascular risks and mortality. [12] However, in one study lower serum clusterin was associated with higher mortality in chronic heart failure patients creating some debates regarding its effect on cardiovascular disease. [32]

Conclusion and recommendation:

Urinary clusterin is considered an early sensitive marker in predicting CKD in T2DM with a cutoff point of 16.9 ng/ml (Sensitivity 97.8%, specificity 97.8%). It is significantly correlated with serum urea, creatinine, ACR, lipid profile, and blood glucose. A higher level of clusterin was observed with the progression of the stage of CKD in subjects with diabetes.

Limitations:

Our study had several limitations. First, the sample size was small and was from a single center. Second, serum clusterin wasn't assessed for its correlation with metabolic parameters. Finally, a long follow-up period is needed for re-evaluation of urinary clusterin in the studied groups with disease advancing.

Statements and declarations:

Competing interests: no funding was received to assist with the preparation of this manuscript and the authors have no competing interests to declare that are relevant to the content of this article.

Ethical approval: the study was approved by an International Review Board (IRB) at Zagazig University (on July 2023/No ZU-IRB # 10944, and the study was performed following the declaration of Helsinki.

Consent to participate: Informed consent was obtained from all individual participants included in the study.

Author contributions: All authors contributed to the study's conception and design. All authors shared in material preparation, data collection, and analysis and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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