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The Role of Soluble Klotho in Diabetic Nephropathy Patients

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Abstract

Background: Diabetic nephropathy (DN) is serious microvascular complication of diabetes which may be complicated by renal failure, cardiovascular disease and premature mortality. **Objective:** to evaluate the role of soluble Klotho in diabetic nephropathy patients and its relation with albuminuria and determine its ability to slow down the progression of renal disease and reduce the risk of cardiovascular diseases. **Patients and methods:** this this case control study included seventy patients with type 2 diabetes, at the diabetic outpatient clinic of Zagazig University Hospitals, as well as 20 healthy controls. All laboratory investigations were done in clinical pathology department in Zagazig University Hospitals. **Results:** There was highly statistically significant difference between diabetic and control groups regarding presence of hypertension and between the 3 diabetic subgroups regarding duration of DM and type of the antidiabetic therapy. Macroalbuminuria group had the longest duration of DM and the highest proportion ofpatients on insulin therapy. However, no statistically significant difference was found regarding presence of hypertension between diabetic subgroups. We found an interesting decrease in hemoglobin concentration from 12.9 g/dl in normoalbuminuria group and 13.1 g/dl in microalbuminuria group to 11.2 g/dl in macroalbuminuria group (p value <0.001).There were highly statistically significant differences among diabetic and control groups regarding fasting blood sugar level, HbA1c, hematocrit and urinary creatinine. A highly statistically significant differences among the studied groups regarding urinary albumin/creatinine ratio. **Conclusion:** Serum klotho concentrations are negatively associated with renal function. Serum klotho could serve as a biomarker for predicting the risk of developing diabetic nephropathy. Klotho may be involved in the pathogenesis of kidney disease, not only DN. Future studies performed in patients with another kidney disease are needed to explain the precise role of klotho in kidney disease. Our findings that urine klotho correlates with nephropathy markers in type 2 diabetic subjects, suggest that elevation of urine klotho could be an independent predictive marker for the progression of diabetic nephropathy.

Keywords: Soluble Klotho ; Diabetic Nephropathy Patients ;

Introduction

Diabetes is the most common cause of end stage renal disease requiring renal replacement therapy. Diabetic nephropathy (DN) is defined by increased urinary albumin excretion in the absence of other renal diseases **(1).** Approximately one-third of all patients with diabetes are affected by DN, in addition, renal involvement is a major cause of morbidity and mortality in the diabetic population **(2).**

Microalbuminuria (MA) is one of the earliest manifestations of diabetic kidney disease (DKD) in diabetes mellitus. MA, a marker of endothelial dysfunction, is associated with a higher risk of cardiovascular disease (CVD) morbidity and mortality. Increased oxidative stress and endothelial dysfunction are key events contributing to the pathogenesis of DKD and CVD **(3).**

Klotho is a nephroprotective transmembrane protein predominantly expressed in the distal renal tubules, parathyroid glands, and choroid plexus, it implicated in regulating phosphate metabolism, together with fibroblast growth factor-23 (FGF-23) **(4).**

Klotho is an aging suppressor gene encoding a 130-kDa β-glucuronidase that catalyzes the hydrolysis of steroid β-glucuronides and found in two forms: an intermembrane form and a secreted form. The Klotho gene is mainly expressed in the kidneys, and its mutation induces many aging-associated diseases **(5).**

Soluble Klotho confers vascular renal protection in different experimental models of metabolic and kidney diseases by enhancing antioxidant, antisenescence, and antiapoptotic mechanisms. Angiotensin II (Ang-II), a proinflammatory and oxidant agent, is upregulated in a variety of kidney diseases, including diabetic kidney disease **(6).**

In vivo, soluble Klotho deficiency is accompanied by activation of the renin angiotensin system (RAS) and endothelial dysfunction. Individuals with type 2 diabetes and an eGFR >60 ml/min have reduced tissue levels of Klotho compared with individuals with IgA nephropathy. Previous studies summarized that Klotho levels have been associated with DKD **(7).** However, with regard to diabetic nephropathy, the role of s-Klotho in the pathogenesis of kidney injury has not been fully studied. Renal s-Klotho expression is markedly decreased in diabetic nephropathy in humans **(8).**

Therefore, this study aimed to evaluate the role of soluble Klotho in diabetic nephropathy patients and its relation with albuminuria and determine its ability to slow down the progression of renal disease and reduce the risk of cardiovascular diseases helping to decrease morbidity and mortality.

Patients and Methods

Seventy patients with type 2 diabetes, at the diabetic outpatient clinic of Zagazig University Hospitals between March 2022 and February 2023, as well as 20 healthy controls, were enrolled for this case control study. All laboratory investigations were done in clinical pathology department in Zagazig University Hospitals.

Inclusion criteria:

The age of patients.≥18years. Estimated GFR(eGFR) ≥ 60ml/min/1.73m² by MDRD6 variable equation, GFR=170× $(SCr)^{-0.999}$× $(Age)^{-0.176}$×0.762(if patients female) ×1.18(if patient is black) × $(BUN)^{-0.170}$× $(Alb)^{0.318}$ Where S.Cr is serum creatinine, BUN is blood urea nitrogen and Alb is serum albumin. Stable renal function without doubling of serum creatinine in last 5months. Diabetic patients on oral hypoglycemic medications or insulin therapy. Patients consents to participate in the study.

Exclusion criteria:

Patients known to have acute vasculitis, malignancy or sever liver dysfunction; age<18 years; patients denied participating; evidence of current inflammation or infection; recent or currant use of steroids; chronic kidney disease other than diabetic nephropathy; pregnancy; history of acute myocardial infarction, recent stroke or occlusive peripheral vascular disease within last 6 months; history of renal transplantation.

I. **Full history taking and thorough clinical examination** including: general and systemic examination.

II. **Laboratory investigations** including:

1- Fasting blood sugar (FBS), HbA1c.
2- Complete blood count.
3- Renal function tests: serum creatinine and BUN.
4- Liver function tests: serum albumin, ALT, AST, bilirubin (total and direct).
5- Lipid profile.

Assay of urinary micro albumin:

Immunometric Enzyme Immunoassay kits (ORGENTEC Diagnostika GmbH, Mainz, Germany) were used for the quantitative determination of micro albumin in urine. Spot morning urine samples were collected andstoredat-20ºCtillassay. Urinary micro albumin level was divided by urinary creatinine level to calculate urinary albumin/creatinine ratio in mg/gm.

Highly purified human albumin is bound to micro wells. Calibrators, controls and undiluted patient samples arepipetted together with anti-human-albumin-peroxidase conjugate in the wells. Microalbumin, if present in diluted urine,will compete with coated albumin for binding of the anti-albumin-conjugate. Washing of the micro wells removes unspecific components. In the presence of bound conjugate, an enzyme substrate hydrolyzes to form a blue color. When an acid is added, it stops the reaction forming a yellow end-product. The intensity of this yellow color is measured photometrically at 450 nm. The amount of color is inversely proportional to the concentration of albumin present in the original sample.

Assay procedure:

20μlofcalibrators,controls and undiluted patient samples were pipetted into the wells. 100µl of enzyme conjugate solution was added into each well. Incubationfor30 minutes at room temperature(20-28 °C). The contents of the micro wells were discarded and washed 3 times with 300 µl of wash solution. 100 µl of tetramethylbenzidine (TMB) substrate solution was dispensed into each well. Incubationfor15 minutes at room temperature. 100µl of stop solution was added to each well of the modules. The optical density at 450 nm was read and the results were calculated. The developed color is stable for at least 30 minutes. Optical densities were read during this time.

Assay of plasma soluble α-Klotho level:

Plasma levels of soluble α-klotho were analyzed by human solubleα-Klotho (S-KLα) ELISA Kits (Glory Science, catalog #:90282, Del Rio, Texas, USA) according to the manufacturer's protocol. Plasma samples were centrifuged at aspeed of 2000-3000 rpm for 20 minutes. Supernatants were removed and samples were kept in -20^{o}C to preserve till assay.

The kit is for the quantitative level of S-KLα in the sample, adopt purified Human S-KLα to coat microtiter plate, make solid-phase antibody, then add S-KLα to wells, Combine S-KLα antibody with labeled Horseradish Peroxidase (HRP) to form antibody-antigen- enzyme-antibody complex, after washing completely, add tetramethylbenzidine (TMB) substrate solution, TMB substrate becomes blue color at Horseradish Peroxidase (HRP) enzyme-catalyzed, reaction is terminated by the addition of a stop solution and the color change is measured at a wavelength of 450 nm. The concentration of S-KLα in the samples is then determined by comparing the optical density (OD) of the samples to the standard curve.

Assay procedure:

40 µl of sample dilution was pipetted to testing sample well, then 10µl of testing sample was added (sample final dilution is 5-fold), sample was pipetted to wells, and mixed gently.Incubation for 30 min at 37^{o}C.mWash solution was diluted 30- fold(or 20-fold)with distilled water. Washing buffer was pipetted to every well, stilled for 30 seconds then drained, repeated 5 times. 50 µl of HRP-Conjugate reagent was pipette to each well. Incubation: Operation with 2. Washing: Operation with 4. 50 µl of Chromogen Solution A and Chromogen Solution B were pipetted to each well. 50 µl of Stop Solution was pipette to each well. Absorbance at 450 nm was read within 15minute after pipetting Stop Solution.

Ethical Consideration:

An approval of the study was obtained from Zagazig University Academic and Ethical Committee. Written informed consent of all the participants was obtained. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Statistical analysis:

Data collected and analyzed using Microsoft Excel software. Data were then imported into Statistical Package for the Social Sciences (SPSS version 20.0) software for analysis. According to the type of data qualitative represent as number and percentage, quantitative continues group represent by mean ± SD. Differences between quantitative independent multiple by ANOVA. P value was set at <0.05 for significant results &<0.001 for high significant result.

RESULTS

The mean age of the studied group was 49.7 ± 11.5 years, with male to female ratio1:1 ;45 cases were males and 45 cases were females. A significant difference between cases and controls in sex distribution ($p<0.05$) and in presence of hypertension ($p<0.001$) **(Table 1).**

There was a highly statistically significant differences between diabetic subgroups in duration of DM and in type of treatment (pvalue <0.001). Macroalbuminuria group had the longest duration of DM and the highest proportion of patients on insulin therapy **(Table 2).**

There were highly statistically significant differences among diabetic and control groups regarding fasting blood sugar level, HbA1c, Hb, hematocrit, serum albumin and urinary creatinine. However, no statistically significant differences were found regarding other laboratory parameters **(Table 3).**

There was a statistically significant difference between diabetic group and control group regarding plasma α-klotho and urinary albumin/creatinine ratio **(Figure 1)**.

A highly statistically significant differences among the studied groups regarding urinary albumin/creatinine ratio. On the other hand, no significant difference was observed regarding plasma soluble α-klotho between the studied groups **(Table 4).**

Regarding albumin/creatinine ratio, there were highly statistically significant differences between all groups and each other except between control group versus normoalbuminuria group which was not significant **(Figure 2)**.

Table (1): Demographic and clinical findings among diabetic and control groups:

Variables		Controls		DM		Test	Pvalue
		Mean±SD		Mean±SD			
Age,years*		43.7±11.7		51.7±10.7		1.818	0.061[1]
		N	%	N	%	Test	P
Sex	Female	6	30.00	39	55.70	4.28	**0.039**[2]
	Male	14	70.00	31	44.30		
Smoking	No	16	80.00	60	85.70	0.52	0.48[3]
	Yes	4	20.00	10	14.30		
Hypertension		0	0	59	83.20	42.51	**<0.001[3]**

1-Independentt-test. 2-Chi-squareX² test; 3- Fisher exacttest. All variables were expressedusing their No.(%) except

(*)byMean±SD

Table (2): Comparison of duration of DM, hypertension &type of treatment among diabetic subgroups:

Variables		Group			Total N=70	Test	P value
		Macroalbuminuria N=23	Microalbuminuria N=23	Normoalbuminuria N=24			
Duration of DM, years*		17.1±5.4	7.9±2.9	3.8±2.6	7.2±7.2	62.13	**<0.001[1]**
Treatment	Insulin	21(91.3%)	5(21.7%)	2(8.3%)	28 (40%)	**37.4**	**<0.001[2]**
	Oral	2(8.7%)	18(78.3%)	22(91.7%)	42 (70%)		
Hypertension	No	3(13.0%)	5(21.7%)	4(16.7%)	12 (18.5%)	**0.82**	0.604[3]
	Yes	20(87.0%)	18(78.3%)	20 (83.3%)	85 (81.5%)		

1-One-wayANOVA test; 2-Fisherexacttest,3- Chi-squareX2 test.
All variables were expressed using their No. (%) except (*) by Mean ±SD

Table (3): Laboratory parameters among diabetic and control groups:

Laboratory parameters	Controls	DM	t-test*	P value
	Mean ±SD	Mean ±SD		
Blood Sugar				
FBS,mg/dl	91.9±5.5	173.8±43.7	8.35	**<0.001**
HbA1c,%	5.3±0.1	9.2±1.8	9.1	**<0.001**
Complete blood count				
WBC,x10^9/L	8±2.6	8.3±1.8	0.63	0.531
Hb,g/dl	13.9±1.5	12.2±1.6	4.01	**<0.001**
HCT,%	41.3±4	37.4±3.6	4.07	**<0.001**
Platelets,x10^9/L	252 ±72.8	261.4±73.5	0.49	0.622
Lipid Profile				
T.Cholesterol,mg/dl	194 ±36.5	205.2±44	1.02	0.312
LDL,mg/dl	128.8±33.2	125.5±30.3	0.41	0.684
HDL,mg/dl	28.5±11.7	33.8±13.4	1.58	0.118
TG,mg/dl	183.7±78.6	229.8± 118.2	1.62	0.108
Liver Function Tests				
S.Albumin,g/dl	4.4±0.3	3.7±0.6	4.05	**<0.001**
ALT,IU/L	15.8±7	15.4±6.8	0.21	0.833
AST,IU/L	21.9±8.5	21.7±7.5	0.11	0.913
T.Bilirubin,mg/dl	0.4±0.1	0.5±0.2	1.38	0.171
Kidney Function Tests				
Creatinine,mg/dl	0.9±0.1	0.9±0.2	0.93	0.353
BUN,mg/dl	14.4±3.3	14.5±4.2	0.05	0.963
eGFR ml/min/1.73m^2	90.6±13.9	86.9±25.9	0.61	0.543
Urinary creatinine gm/dl	0.17±0.056	0.05±0.054	7.69	**<0.001**

**All variables were expressed by Mean ± SD and compared using Independent t-test. (ALT: alanine transaminase, AST: aspartate transaminase, FBS: fasting blood sugar,eGFR: estimated glomerular filtration rate, HbA1C: Glycosylated hemoglobin, Hb: hemoglobin , HCT; hematocrit,HDL: highdensitylipoprotein,LDL: low densitylipoprotein,TG: triglycerides, WBCs: white blood cells).*

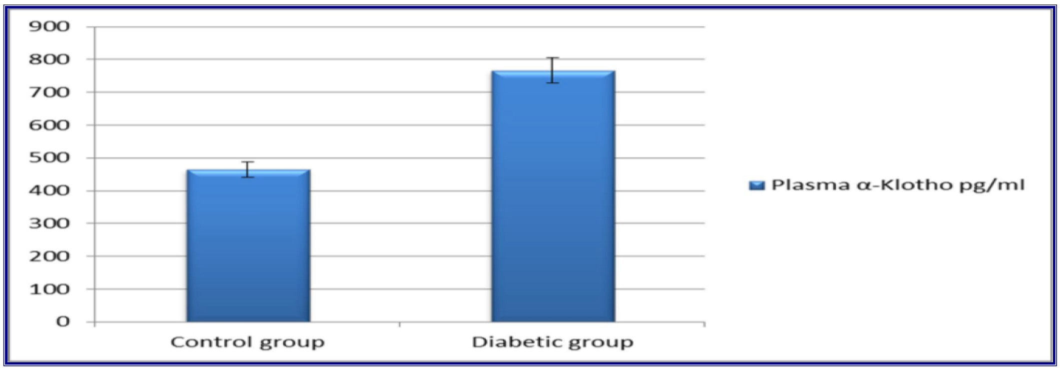

Fig. (1): Mean plasma α-klotho among diabetic and control groups.

Table (4): Comparison of urinary albumin/creatinine ratio and plasma α- klotho values among the studied groups:

Variables	Group				ANOVA test	P-value
	Control	Normo-albuminuria	Micro-abuminuria	Macro-abuminuria		
Urinary albumin/creatinine ratio, mg/g Mean Median (range)	21.6±5.67 21.2 (8.3-29.4)	19.7±9.28 22.5 (1.7-29.9)	92±21.9 86.9 (58-141.7)	1505.84±666.4 1690 (423-2606.1)	94.755	**<0.001**
Plasmaα-Klotho, pg/ml Mean Median (range)	464.6±140.9 446.76 (251-706.1)	1021.6±118.6 630.5 (219.8-5287)	748.34±42 599.5 (263.1-2942.5)	530.3±197.5 559.9 (192.8-900.5)	2.035	0.140

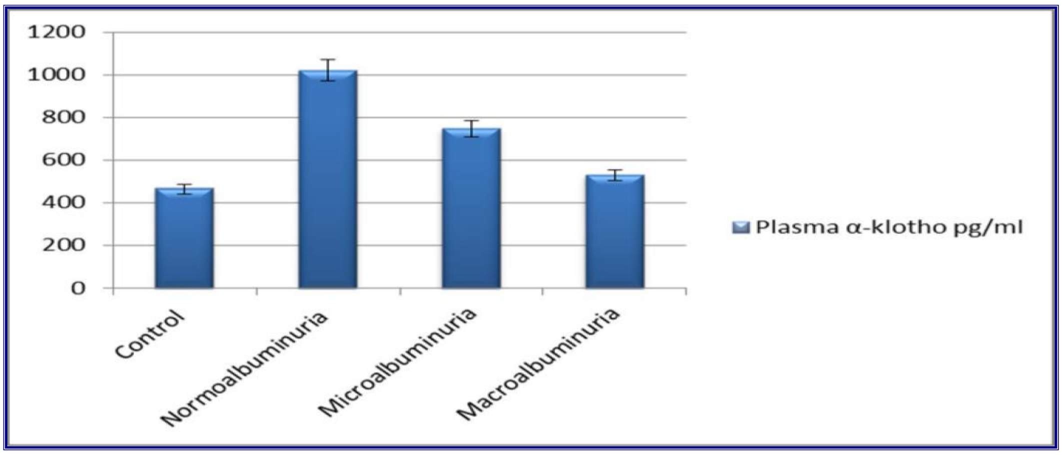

Fig. (2): Plasmaα-klotho in the studied groups.

Discussion:

Diabetes mellitus (DM) refers to “a group of common metabolic disorders which share the phenotype of hyperglycemia” **(9)**. Most patients with diabetic nephropathy will die from cardiovascular disease before they reach ESRD and start on dialysis **(10)**. Therefore, early diagnosis of DN is essential to improve outcomes.

Conventionally, detection of microalbumin in urine is considered the hallmark of early diagnosis of DN. However, a considerable renal injury arises among normoalbuminuric diabetic patients that are accompanied with more advanced glomerular lesions **(11)**. Furthermore, once microalbuminuria is detected, significant glomerular lesion has already developed **(12)**.

Although diabetic nephropathy is classically believed to be a glomerulardisease, tubulointerstitial injury is suggested to precede obvious glomerulopathy **(13)**. This is evidenced by increased levels of tubular injury markers like KIM-1 and NAG in type 1 diabetic patients **(14,15)**.

Consistent with this, numerous glomerular and tubular biomarkers for early detection of DN have been recognized and are becoming of increasing value in diagnosis. Hence, more sensitive and specific markers, along with albuminuria, are required for prediction of diabetic nephropathy even at the very early stage (normoalbuminuria).

The aim of this study is to assess the value of plasma soluble α-klotho level as early marker for diabetic nephropathy.

α-Klotho is a single-pass transmembrane protein which is highly expressed in renal distal convoluted tubules **(16)**. Soluble α- klotho is derived from the proteolytic cleavage of the extracellular domain ofthe membrane-bound α-klotho; alternatively, it can be generated directly by the alterative splicing of the α-klotho transcript **(17)**.

Unlike membrane α-klotho, which functions as a co-receptor for fibroblast growth factor-23 down regulating phosphate metabolism, soluble α-klotho acts as a hormonal factor and plays important roles in anti-aging, anti-oxidation, ion transport modulation, and Wnt signaling **(8)**. Meanwhile, we know little about circulating α-klotho levels in diabetic nephropathy. Recent studies in diabetic patients showed conflicting data. Some studies reported increased levels in diabetic patients, while others observed decreased levels or even no significant difference.

Regarding demographic data, there was statistically significant difference between diabetic and control groups and between diabetic subgroups and each other regarding gender.

Regarding the clinical findings, there was highly statistically significant difference between diabetic and control groups regarding presence of hypertension and between the 3 diabetic subgroups regarding duration of DM and type of the antidiabetic therapy. Macroalbuminuria group had the longest duration of DM and the highest proportion of patients on insulin therapy. However, no statistically significant difference was found regarding presence of hypertension between diabetic subgroups.

We found an interesting decrease in hemoglobin concentration from 12.9 g/dl in normoalbuminuria group and 13.1 g/dl in microalbuminuria group to 11.2 g/dl in macroalbuminuria group (p value <0.001). The decreased level of hemoglobin can be explained by erythropoietin deficiency, which plays a major role in the anemia associated with chronic kidney disease. This anemia may begin even before there is any evidence of renal impairment **(8)**. In the present study, eGFR was significantly lower in normoalbuminuria and microalbuminuria groups compared to macroalbuminuria group (pvalue <0.001).

Other possibility includes eryptosis. Enhanced eryptosis, suicidal erythrocytedeath, is noticed in diabetes **(18)** and eryptosis is further enhanced in klotho-deficient mice (**19)**.

In our study, we found that plasma soluble α-klotho was significantly higher in diabetic group compared to control group (p value <0.05) and the highest level was in normoalbuminuria group (1021±118.6 pg/ml) and tended to decrease with increasing degree of albuminuria to be lower in microalbuminuria group (748.34±42 pg/ml) and the lowest in macroalbuminuria group (530.3±197.46 pg/ml). It was surprising that plasma α-klotho level in macroalbuminuria group was still comparable with that in controls (464.6± 140.9 pg/ml). Our results are in line with Lee et al. and with Inci et al. in 2 studies **(1,8; 20)**.

Kim et al. reported that plasma α-klotho was negatively correlated with the annual decline in eGFR and with the development of albuminuria in the early stage of diabetic nephropathy with preserved GFR (eGFR ≥60 mL/min/1.73 m^2) **(21)**.

During early stages of diabetic nephropathy, it seems that some factors stimulate the secretion of soluble α-klotho into the blood. Haruna and his colleagues previously reported that by reducing mitochondrial oxidative stress, the antiaging klotho protein acts as a potential renoprotective humoral factor, and in that way modulating glomerular and tubulointerstitial patho-biology irrespective of the disease process (**22)**. Soluble α-klotho is secreted from many sources other than kidneys and for renal protection; it may be produced by these extrarenal tissues **(1)**.

Conclusion:

Serum klotho concentrations are negatively associated with renal function. Serum klotho could serve as a biomarker for predicting the risk of developing diabetic nephropathy. Klotho may be involved in the pathogenesis of kidney disease, not only DN. Future studies performed in patients with another kidney disease are needed to explain the precise role of klotho in kidney disease. Our findings that urine klotho correlates with nephropathy markers in type 2 diabetic subjects, suggest that elevation of urine klotho could be an independent predictive marker for the progression of diabetic nephropathy.

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