ORIGINAL ARTICLE

Serum soluble α-Klotho levels in patients with diabetic nephropathy

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Abstract

Background The study aimed to determine the variability in the stages of diabetic nephropathy by examining specific biochemical functions associated with the target organ. As a result, various biochemical parameters were assessed in all of the groups under investigation.

Material and methods These parameters encompassed soluble α-Klotho and serum insulin, which were determined through ELISA. Additionally, spectrophotometric methods were employed to assess other parameters such as blood levels of urea in all groups. Instead of using HPLC method, HbA1c levels were determined. Blood and urine samples were obtained from a total of 90 participants, who were aged between 37 and 70 years. A total of 70 patients were categorized into three groups according to their ACR. The first group consisted of patients with an ACR value of less than 30 mg/g. The second group included patients with an ACR value ranging from 30 to 300 mg/g. The third group comprised patients with an ACR value greater than 300 mg. Additionally, the study also involved 20 healthy individuals.

Result The serum soluble α-Klotho in the patient group was significantly lower than that of the healthy subjects. There were strong negative correlations between serum soluble α-Klotho and both ACR and HOMA-IR. The AUC value was excellent, measuring at 0.93 with a $p < 0.0001$.

Conclusions Soluble α-Klotho levels in the sera of diabetic patients were shown to be lower and significantly linked to patients with diabetic nephropathy. This implies that klotho levels may be influenced by ACR in addition to playing a significant role in insulin resistance.

Keywords ACR · α-Klotho · Diabetic nephropathy · HOMA-IR

Introduction

The Klotho gene has been identified as a gene that inhibits the aging process and promotes longevity when its expression is enhanced, but accelerates aging when it is disrupted [1]. This gene is primarily expressed as a transmembrane protein in the distal convoluted tubules of the kidney and choroid plexus in the brain. Additionally, it is expressed in various endocrine organs, including the pituitary, pancreas, parathyroid gland, adipocytes, and vascular endothelial cells [2]. Secreted Klotho and membrane Klotho are known for their capacity to regulate the activity of various glycoproteins found on cell surfaces. These glycoproteins include ion channels and growth factor receptors, such as insulin and insulin-like growth factor 1 receptor [3, 4]. It has been reported that individuals with chronic kidney disease exhibit lower levels of α-klotho in both plasma and urine during the early stages of the disease. These levels further decline as the disease progresses to more advanced phases [1, 5]. The precise role of α-klotho in the development of diabetic nephropathy is uncertain. Nonetheless, our recent cross-sectional study demonstrated that diabetic patients with relatively preserved renal function (GFR ≥ 60 ml/min/1.73 m2) exhibited significantly elevated levels of plasma and soluble α-klotho in urine when compared to individuals without any known health conditions [6]. In addition, the α-klotho levels in plasma decreased proportionally to urinary albumin excretion, whilst the α-klotho levels in urine remained stable with urinary albumin excretion increased. However, it is still unclear whether the levels of α-klotho in plasma and urine occur prior to the decrease in glomerular filtration rate (GFR) and albuminuria during the initial phases of diabetic nephropathy [7].

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Materials and methods

Diabetic patients with CKD

Seventy diabetic patients with nephropathy, whose ages ranged from 37 to 70 years, were categorized into three groups according to their microalbumin value (ACR). The first group (G1) consisted of patients with an ACR level below 30 mg/g, indicating normoalbuminuria. The second group (G2) included patients with an ACR level between 30 and 300 mg/g, indicating microalbuminuria. The third group (G3) comprised patients with an ACR level exceeding 300 mg, indicating macroalbuminuria.

Healthy subjects

The study involved twenty healthy control participants who were selected to match the patients in terms of age (37–70 years) and gender (male and female). The average age of the control group was 60.85 ± 1.14 years. The controls were chosen according to specific criteria set by the physicians.

Sample collection

Ten milliliters of blood was obtained from each patient and control group. The blood samples were obtained between 8.00 and 11.00 A.M. following a fasting period of 12–15 h. Each blood sample was divided into two portions. The first portion was treated with ethylene diamine tetra-acetic acid (EDTA) (1.5 mg/ml) to measure HbA1c within a timeframe of less than 3 h. The second portion was used to obtain serum by transferring the sample into a regular tube and allowing it to clot at room temperature (22 °C). Subsequently, the serum was extracted by centrifuging the sample at 3000 r.p.m.

Anthropometries measurements

Including age, weight, and height. The body mass index (BMI) is determined using a formula that contains the basic equation of weight divided by the square of height.

Assessment of the homeostasis model assessment (HOMA-IR)

Various methods were employed to measure insulin resistance (IR) in the study, with the most commonly used approach being the calculation of homeostasis model assessment (HOMA). This calculation involved the use of fasting insulin (U/ml) and glucose (mg/dl), as shown

in the following equation. Insulin resistance is a critical topic to investigate since it affects the balance of various metabolic [8]:

$$\textbf{HOMA-IR} = [\textbf{g(mg\textbackslash dl)} \times \textbf{fasting insulin}(\boldsymbol{\mu}\textbf{U/ml})]/\textbf{405}$$

Determination of glomerular filtration rate (eGFR)

Glomerular filtration rate (GFR) was calculated using the modification of Diet in Renal Disease (MDRD) study formula and expressed in milliliters per minute per 1.73 m^2 [9]. The MDRD equation is widely used in the estimation of GFR. It incorporates factors such as age, gender, and race to account for muscle mass. Weight is not necessary in this equation as the result is adjusted to a standardized body surface area of 1.73 m2, which represents the average surface area of adults [10].

$$\textbf{eGFR} = \textbf{186} \times (\textbf{serum creatinine mg/dl})^{-1.154}$$
$$\times (\textbf{age years})^{-0.203} \times (\textbf{0.742 if female})$$

Determination albumin creatinine ratio (ACR)

Albumin creatinine ratio was estimated by dividing the value of urine microalbumin in (mg/L) to urine creatinine (mg/dl), as shown below:

$$[\textbf{Microalbumin}(\textbf{mg/L})/\textbf{urine Creatinine (mg/dl})]$$
$$\times \textbf{100} = \textbf{mg MALB/g CR}$$

Microalbumin in urine was estimated by the MALB method used on Dimension® clinical chemistry system, in vitro diagnostic test for quantitative measurement of microalbumin (mg/L) in human urine by particle-enhanced turbidimetric inhibition immunoassay (PETINIA). Urine creatinine (mg/dl) was measured by Flex reagent cartridge using the CREA method used on the Dimension® clinical chemistry system and analyzer an in vitro diagnostic test for the quantitative determination of creatinine in urine.

Clinical laboratory analysis of groups

An ELISA plate reader was used to determine the quantities of soluble α-Klotho (Human Alpha Klotho kit, Ybiont, China) and Insulin (Demeditec Diagnostic GmbH, Germany) (ROCH Cobas Integra 400 Plus from Human GmbH, Germany). The serum fasting blood sugar, blood urea, serum creatinine, and lipid profile tests were assessed using an automated chemical analyzer (COBSe 411, Germany).

Table 1 The clinical characteristics of the study groups

Parameter	Control	Normoalbuminuria (G1)	Microalbuminuria (G2)	Macroalbuminuria (G3)	P (value)
Age (year)	60.85 ± 1.14	55.25 ± 9.25	65.2 ± 8.30	65.68 ± 10.70	$P = 0.230$
BMI (kg/m^2)	23.91 ± 3.10	27.38 ± 4.14	30.80 ± 9.94	27.27 ± 5.58	$P = 0.07$
FBS mg/dL	88.5 ± 2.47	169.95 ± 0.72	174.23 ± 8.06	204 ± 14.08	$P = 0.001$
HbA1C mmol/mol	3.855 ± 0.23	8.809 ± 0.49	8.933 ± 0.533	9.933 ± 0.40	$P = 0.001$
Insulin µU/mL	9.10 ± 2.53	10.81 ± 1.52	11.55 ± 2.17	14.48 ± 1.58	$P = 0.001$
HOMA-IR	1.40 ± 0.96	5.41 ± 1.52	5.96 ± 1.39	8.66 ± 1.92	$P = 0.001$
Creatinine mg/dL	0.390 ± 0.18	$0.690 \pm 0.17^*$	$0.69 \pm 0.144^{**}$	$1.573 \pm 0.437^{***}$	$P = 0.001$
Blood urea mg/dL	11.45 ± 3.619	$29.25 \pm 2.13.83$	43.040 ± 12.07	$73.68 \pm 23.22^{***}$	$P = 0.001$
GFR mL/min	299 ± 47.98	$117.9 \pm 27.62^*$	$87.84 \pm 10.08^{**}$	$44.040 \pm 9.409^{**}$	$P = 0.001$

Values are presented as mean $\pm$ SEM

*Significance of G1 to control group

**Significance of G2 to G1

***Significance of G3 to G2

Statistical analysis

The statistical analysis was conducted using SPSS. The numerical variables with normally distributed data were analyzed by calculating the mean and standard deviation, while categorical variables were analyzed by determining the frequency and percentage. To examine potential significant differences between the numerical variables, independent t-tests and ANOVA tests were utilized. A significance level of 0.05 was employed in this study. The Pearson correlation and t-test were utilized to determine the significance of the correlation between the two quantitative variables. A receiver operating characteristic (ROC) curve method was used to assess the utility of soluble α-Klotho as a diagnostic marker or disease screening tool.

Result

Anthropometries and biochemical parameters of patient groups and healthy subjects

The biochemical results among study groups are given in Table 1. No significant differences were detected among the renal function tests, metabolic parameters (including, FSG, HbA1c, HOMA-IR), and BMI in any of the study groups. The insulin levels were significantly higher in patient groups compared with the control group. In addition, in the diabetic groups, there were significant differences observed in insulin levels ($p = 0.001$) (Table 1).

Serum soluble α-klotho in patient groups and healthy subjects

The serum soluble α-Klotho levels in healthy subjects and patients with diabetic nephropathy are presented in Fig. 1. The soluble α-Klotho levels for the control group were 1.54 ± 0.07 ng/ml. Although it was lower in patients with normoalbuminuria (0.737 ± 0.03 ng/ml) and microalbuminuria (0.539 ± 0.06 ng/ml), the differences in soluble α-Klotho levels between the control and two early-stage groups were not significant ($p = 0.07$ and $p = 0.05$, respectively) (Fig. 1). However, we found a statistically significant difference in the levels of microalbuminuria between patients (0.362 ± 0.02 ng/ml) and the healthy group ($p = 0.007$) (Fig. 1).
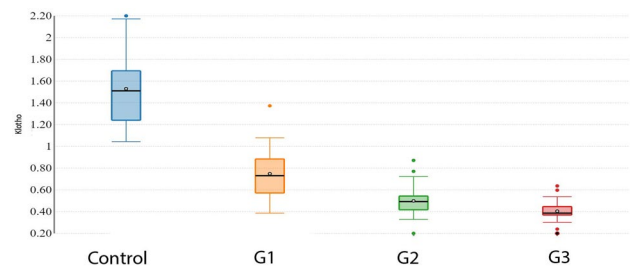

Fig. 1 Soluble α-Klotho levels in response to the control and patient groups. Values are presented as mean $\pm$ SE

Table 2 Simple linear regression analysis between Klotho and other parameters in diabetic patients with nephropathy

Variable	α- Klotho	
	r	P value
Age, year	− 0.311**	0.003
Duration, year	− 0.282*	0.018
GFR, ml/min	0.862**	0.001
S.Cr, mg/dL	− 0.768**	0.001
Urea, mg/dL	− 0.701**	0.001
ACR	− 0.270*	0.010
FBS, mg/dL	− 0.442**	0.001
HbA1C, mmol/mol	− 0.616**	0.001
HOMA-IR	− 0.717**	0.001
Insulin, μU/mL	− 0.488**	0.001

*Significant at the 0.05 level (2-tailed)

**Significant at the 0.01 level (2-tailed)

Simple linear regression

Table 2 shows the correlation coefficient of α-Klotho level with each of age, duration of diabetes, FBS, insulin, HOMO-IR, HbA1C, S.Cr, urea, ACR, and GFR in diabetic patients with nephropathy. The findings from the correlation coefficient analysis indicated a significant negative correlation ($P < 0.01$) between α-Klotho and age ($r = -0.311$, $P = 0.003$), S.Cr ($r = -0.768$, $P = 0.001$), blood urea ($r = -0.701$, $P = 0.001$), FBS ($r = -0.442$, $P = 0.001$), insulin ($r = -0.488$, $P = 0.001$), HOMO-IR ($r = -0.717$, $P = 0.001$), and HbA1C ($r = -0.616$, $P = 0.001$). The study demonstrates a negative correlation ($P < 0.05$) between α-Klotho and ACR ($r = -0.270$, $P = 0.011$) as well as duration ($r = -0.282$, $P = 0.018$). In addition, a strong positive correlation ($P < 0.01$) is observed between α-Klotho and eGFR ($r = 0.862$, $P = 0.001$).
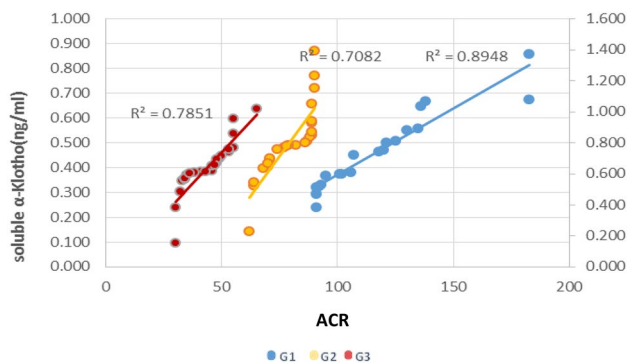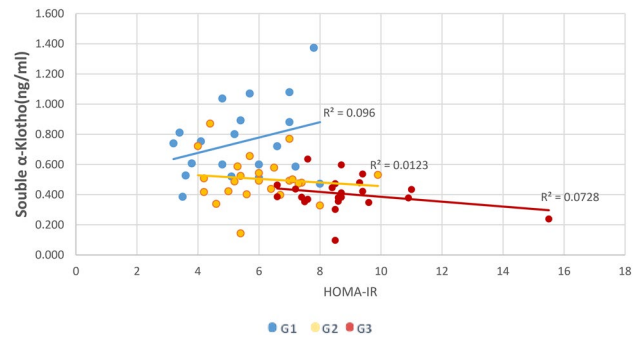

Fig. 3 Linear regression of α-Klotho and HOMA-IR in diabetic patients with nephropathy

Multiple regression analysis

To determine the impact of a set of explanatory variables on the baseline serum Klotho level, a multiple linear regression model was employed. All potential explanatory variables were initially included in the model. The equation resulting from this analysis can be found in Figs. 2 and 3, while Table 3 demonstrates a positive correlation between several factors, including age (measured in years), gender (male versus female), index, BMI, baseline HbA1c%, and baseline FBS, with the average serum α-Klotho level. However, none of these variables demonstrated statistical significance. Conversely, only two variables, namely, ACR ($r = 0.510$, $p = 0.000$) and HOMA-IR ($r = -0.149$, $p = 0.04$), independently exerted an influence on Klotho levels. The resulting equation was found to be statistically significant ($P = 0.001$), accounting for 89% of the observed variability in the response variable.

Fig. 2 Linear regression of α-Klotho and ACR in diabetic patients with nephropathy

Table 3 Multivariate linear regression analysis between Klotho and other parameters in diabetic patients with CKD

Model		Unstandardized coefficients		Standardized coefficients	Sig.
		B	Std. error	Beta	
1	Age	− .005	.002	− .101	.108
	GFR	− .007	.003	− .097	.15
	Urea	− .003	.001	− .141	.054
	Creatinine	− .209	.065	− .221	.102
	ACR	.002	.000	.510	.000
	FSG	.000	.000	− .047	.296
	Insulin	.012	.008	.071	.126
	HOMA-IR	− .025	.008	− .149	.004
	HbA1C%	− .012	.011	− .075	.246

Table 4 Receiver operator curve (ROC) analysis for the investigated soluble α-Klotho in studied groups

Area	Std. error[a]	Asymptotic Sig.[b]	Asymptotic 95% confidence interval	
			Lower bound	Upper bound
0.993	0.006	0.000	0.982	1.005

The test result variable(s): soluble α-Klotho has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased

[a]Under the nonparametric assumption

[b]Null hypothesis: true area $= 0.5$

Receiver operator characters curve analysis (ROC)

The analysis of the ROC curve categorizes the parameters based on their placement within the ROC area and determines whether this placement holds significance. In this study, the ROC curve was used to distinguish between the T2DM with different stages of nephropathy and the control group. Based on the ROC curve analysis, the optimal threshold for soluble α-Klotho is determined to have a sensitivity of 98.0% and a specificity of 100.0% as a marker. Analysis reveals the area under the curve (AUC) of ROC in a value of 0.993 for soluble α-Klotho as shown in Table 4 and Fig. 4.

Discussion

Persistently elevated blood glucose levels have the potential to harm various kidney cell types, leading to the advancement of kidney failure. Consequently, it becomes
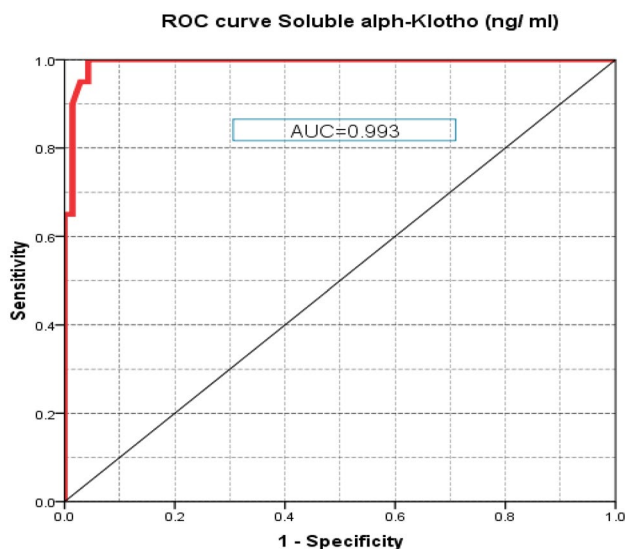

Fig. 4 ROC analysis for the investigated soluble α-Klotho in studied groups

challenging to identify the hazards linked to early-stage kidney disease, highlighting the difficulties in successfully preventing diabetic nephropathy in individuals diagnosed with type 2 diabetes mellitus [11]

Our study findings align with previous research that has observed elevated fasting blood sugar levels in individuals with diabetes and chronic kidney disease (CKD) compared to healthy individuals without any medical conditions [12]. Multiple studies have provided evidence of a direct correlation between elevated serum creatinine levels and an accompanying increase in serum urea levels [13, 14]. During our study, we discovered that as diabetes progressed, there were increased levels of urea and creatinine. This resulted in glomerular injury, a reduction in glomerular filtration capacity, and the buildup of metabolic byproducts. Among these by-products, urea, and creatinine are acknowledged as noteworthy indicators of alterations in renal function [15].Therefore, sustained hyperglycemia can cause irreparable damage to the kidneys.

The results of our study showed a notable decrease in serum levels of s-Klotho compared to the control group used as a reference. Furthermore, we investigated the relationship between serum Klotho levels and both ACR and insulin resistance in individuals with diabetic nephropathy. Additionally, the average values observed in our study for individuals with diabetic nephropathy and those in a healthy control group align with the results reported in previous literature that utilized the same assay method [16–18]. The decrease in serum levels, coupled with the progression of renal damage, is likely attributed to a reduction in renal synthesis of Klotho. If renal Klotho production remained stable, reduced excretion due to renal damage would result in higher serum levels. Conversely, if renal excretion were to remain normal, a predictable decrease in serum levels would not be expected. Furthermore, the likelihood of increased renal excretion appears highly improbable, as it contradicts available clinical evidence [1] and experimental data indicating reduced urinary s-Klotho in CKD [19].

The statistical analysis demonstrated a significant negative correlation between soluble alpha klotho and each of the variables creatinine, glucose, urea, HbA1c, ACR, and age. Conversely, a positive correlation was observed between soluble alpha klotho and insulin as well as eGFR according to the data presented in Table 2. This finding aligns with studies [20, 21]. After transferring the variables to the multiple regression model, we observed that the relationship between Klotho and other parameters was no longer significant. It was found that Klotho primarily correlated with ACR. Our findings align with previous studies, further substantiating the observed correlation. In our study, we found that ACR was the strongest predictor of s-Klotho levels, which confirms the hypothesis we have previously discussed [1, 19].

Additionally, the multiple regression model suggests a connection between Klotho and insulin resistance, which aligns with outcomes from other studies [22, 23]. These studies have indicated that Klotho levels can influence insulin metabolism by inhibiting tyrosine phosphorylation of insulin and IGF1 receptors, as well as enhancing glucose-induced insulin secretion through the upregulation of TRPV2 membrane retention. Considering the apparent deficiency of Klotho observed in CKD stages, it is plausible to hypothesize that low Klotho levels could lead to excessive insulin release, resulting in a state of insulin resistance.

ROC analysis for serum α-Klotho demonstrated an AUC value of 0.993, with a sensitivity of 98.0% and a specificity of 100% at a cutoff value of 0.82. The findings of this study suggest that serum α-Klotho can be a valuable tool for differentiating individuals with diabetic nephropathy from those without the condition. These findings align with Drew et al. [24], although Seiler et al. [25] did not support the hypothesis of reduced plasma levels of secreted Klotho in CKD. It should be noted that this discrepancy in hypothesis may be attributed to Seiler et al.'s omission of measuring α-Klotho in healthy controls or stage I CKD patients. The ROC analysis for α-Klotho at a cutoff value of ≤ 9.9 yielded a sensitivity of 92%, a specificity of 100%, a positive predictive value of 100%, a negative predictive value of 88.2%, and an accuracy of 97% in discriminating between individuals with CKD and those without. The area under the curve for α-Klotho was 0.970.

Conclusions

Lower levels of soluble α-Klotho were observed in the blood samples of diabetic patients, and these reduced levels were found to be significantly associated with diabetic nephropathy patients. This suggests that the levels of klotho may be influenced by ACR also play crucial roles in insulin resistance.

Declarations

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