RESEARCH ARTICLE

Increased oxidative stress in diabetic nephropathy and its relationship with soluble Klotho levels

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Abstract

Background: In the present study, we aimed to assess the relationship between the levels of soluble Klotho (s-Klotho) and oxidative stress markers in diabetic nephropathy patients with different stages of chronic kidney disease (CKD) and albuminuria levels.

Methods: We enrolled 109 patients with type 2 diabetes (mean age, 61.63 ± 9.77 years) and 32 healthy controls (mean age, 49.53 ± 7.32 years) between January and June 2014. Patients were classified into three groups based on their urinary albumin/creatinine ratio (UACR). Blood samples were collected to measure the levels of s-Klotho, serum creatinine, calcium, phosphorus, 25-hydroxyvitamin D3, and parathyroid hormone (PTH). We used the total oxidant status (TOS), total antioxidant status (TAS), ischemia-modified albumin (IMA), and ischemia-modified albumin ratio (IMAR) values to measure the oxidative status. Moreover, the oxidative stress index (OSI) was estimated as the percentage ratio of TOS/TAS values.

Results: The TOS, TAS, and OSI values were significantly greater in the diabetic nephropathy patients compared to controls ($p < 0.001$). When patients were classified based on their UACR, we noted that the TOS, OSI, and IMA values did not significantly differ, although the TAS ($p < 0.001$), and IMAR ($p = 0.002$) values significantly differed between the groups. The s-Klotho levels also significantly differed ($p = 0.031$) between the groups. These s-Klotho levels exhibited a significant positive correlation with TOS ($r = 0.186$, $p = 0.034$) and OSI ($r = 0.207$ $p = 0.018$), but showed no correlation with the estimated glomerular filtration rate; UACR; HbA1c, calcium, phosphorus, and PTH levels; and TAS, IMA, and IMAR values.

Conclusion: Oxidative stress is greater in patients with diabetic nephropathy, and the TOS was positively correlated with s-Klotho levels in diabetic patients. The therapeutic reduction of oxidative stress in patients with diabetic nephropathy could improve the renal and cardiovascular outcomes. Hippokratia 2016, 20(3): 198-203.

Keywords: Diabetes, diabetic nephropathy, oxidative stress, s-Klotho, soluble Klotho

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Introduction

Diabetes is the most common cause of chronic kidney disease (CKD) and is associated with an increased risk for cardiovascular morbidity and mortality[1]. However, the underlying pathogenic mechanism linking diabetic nephropathy with cardiovascular disease (CVD) remains unclear. In addition to traditional risk factors such as hypertension, hyperglycemia, and dyslipidemia, inflammation/oxidative stress and endothelial dysfunction may also contribute to the pathogenesis of atherosclerosis and increased cardiovascular risk among these patients[2].

Klotho is an aging suppressor gene that was first discovered as a membrane protein. Membrane Klotho forms a complex with the fibroblast growth factor 23 (FGF23) receptor and serves as a mediator for the actions of FGF23, including urinary phosphate (P) excretion, inhibition of calcitriol [1,25(OH)2D] secretion, and inhibition of parathyroid hormone (PTH) synthesis and secretion[3-5]. Klotho may also be released into circulation via ectodomain shedding, after which it transforms into soluble Klotho (s-Klotho) and functions as a humoral factor. s-Klotho is involved in the regulation of nitric oxide production in the endothelium, preservation of endothelial integrity and permeability, calcium (Ca) homeostasis in the kidneys, and the inhibition of intracellular insulin and insulin-like growth factor-1 signalling[6].

Oxidative stress is defined as a disturbance in the balance between the production of reactive oxygen species (ROS) and antioxidants. Certain clinical and laboratory markers can be used to detect the oxidative stress and

antioxidant status. In particular, the measurement of total antioxidant status (TAS) and total oxidant status (TOS) can provide useful information on the overall serum antioxidative status of an individual[7]. Albumin is a major determinant of the antioxidant capacity of human serum. The molecule has the ability to bind and carry radical scavengers, and sequesters transition metal ions with pro-oxidant activity, in addition to its direct antioxidant capabilities. The generation of ROS and free radicals can transiently modify the N-terminal region of albumin and produce an increase in the concentration of ischemia-modified albumin (IMA). Some previous studies have described IMA as a marker of ischemia and oxidative stress[8,9]. A defect in Klotho gene expression can lead to premature aging. In fact, Yamamoto et al showed that the Klotho-induced inhibition of insulin/insulin-like growth factor 1 signaling is associated with an increased resistance to oxidative stress, which may potentially contribute to the anti-aging properties of Klotho[10]. Recently, we investigated the s-Klotho and FGF23 levels in diabetic nephropathy with different stages of albuminuria in the same study population, and we intended to investigate the factors affecting the levels of s-Klotho[11]. In the present study, we investigated the relationship between the levels of s-Klotho and oxidative stress markers in diabetic nephropathy patients with different stages of CKD and albuminuria levels.

Methods:

Between January and June 2014, we enrolled 109 patients with diabetic nephropathy (mean age: 61.63 ± 9.77 years) and 32 healthy controls (mean age: 49.53 ± 7.32 years). Patients were recruited at the outpatient clinic of the Nephrology Unit of Antalya Research and Training Hospital while healthy controls from the hospital staff. The healthy group had no chronic illness or drug use, and the age did not match with that of the diabetic group. We excluded from the study patients aged <18 years, pregnant women, those with hepatic diseases, other kidney diseases, clinically apparent infections, or active malignancy, and those using vitamin D or phosphate binders to eliminate possible conditions that influence oxidative stress parameters and s-Klotho levels. We conducted this study according to the Declaration of Helsinki and the guidelines of Good Clinical Practice and it was approved by the Ethics Committee of Antalya Training and Research Hospital (No: 215/01.01.2014). All the patients and healthy controls provided written informed consent.

Patients were classified into three groups based on their urinary albumin/creatinine ratio (UACR): the normoalbuminuria group (UACR <30 mg/g), microalbuminuria group (UACR =30-300 mg/g), and macroalbuminuria group (UACR >300 mg/g). The CKD epidemiology collaboration (CKD-EPI) equation for the glomerular filtration rate was used to calculate the estimated glomerular filtration rate (eGFR).

Blood and urine samples were collected in the morning after an 8h fast. The serum was stored at −80°C. Blood was analyzed for fibroblast growth factor 23 (FGF23), s-Klotho, PTH, P, Ca, creatinine, and 25-hydroxyvitamin D3 levels. The urinary protein-to-creatinine ratio (UPCR) and UACR were calculated via spot urine protein, albumin, and creatinine measurements.

The serum s-Klotho levels were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (YH Biosearch, Shanghai, China), with a coefficient of variation of <10 % for both parameters; the detection range of serum soluble α-Klotho level assay ranged from 0.05 to 20 ng/mL. These assays used the quantitative sandwich enzyme immunoassay technique. To avoid variability within each assay, measurements were performed simultaneously, in duplicate, using the same ELISA kit.

We used the TOS, TAS, IMA, and IMA/serum albumin ratio (IMAR) values to measure the oxidative status. The serum TAS was measured using an automated colorimetric measurement method developed by Erel[7] and a commercially available reagent kit (Relassay®, Turkey). In this method, antioxidants in the sample reduce the dark blue-green-coloured 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical to the colorless reduced ABTS form. The change in absorbance at 660 nm is related to the total antioxidant level in the sample. The results are expressed as micromolar trolox equivalent per liter.

The TOS of the plasma was measured using an automated colorimetric measurement method developed by Erel[12] and a commercially available reagent kit (Relassay®, Turkey). In this method, the oxidants present in the sample oxidize the ferrous ion chelator complex to ferric ion, which produces a colored complex with a chromogen in an acidic medium. The results are expressed in terms of micromolar hydrogen peroxide equivalent per liter (μmol H_2O_2 Equiv/L).

The percentage ratio of the TOS to TAS level yields the oxidative stress index (OSI). For calculation, the resulting micromolar unit of TAS is changed to millimoles per liter, and the OSI value is calculated using the following formula: OSI (arbitrary unit) = TOS (micromolar hydrogen peroxide equivalent per liter)/TAS (micromolar trolox equivalent per liter).

The IMA level (reflecting the reduced cobalt–albumin-binding capacity) was measured using the rapid and colorimetric method developed by Bar-Or et al[8]. In brief, 200 μl of patients' serum was transferred into glass tubes and 50 μl of 0.1 % $CoCl_2$*$6H_2O$ (lot S38901-248, Sigma-Aldrich, St Louis, MO, USA) was added. After gentle shaking, the mixture was incubated for 10 min to ensure sufficient cobalt-albumin binding. Thereafter, 50 μl of 1.5 mg/ml dithiothreitol (DTT) (lot D5545-1G, Sigma-Aldrich) was added as a coloring agent. After 2 min, we added 1 ml of 0.9 % NaCl to stop the binding between cobalt and albumin. For every specimen a blank was prepared; at the DTT addition step, we used 50 μl of distilled water instead of 50 μl of 1.5 mg/ml DTT to obtain a blank without DTT. The absorbance values were recorded at

470 nm using a spectrophotometer (UV1201, Shimadzu, Kyoto, Japan). The color formation in specimens with DTT was compared to color formation in the blank tubes, and the results are expressed as absorbance units. The IMAR was also estimated and was used to eliminate the effect of reduced albumin concentrations.

Statistical Analysis

Continuous variables are presented as means ± standard deviation and categorical variables are presented as percentages. The Kolmogorov-Smirnov test was used to verify the normality of the distributions of continuous variables. Statistical analysis of the clinical data between the two groups was performed using unpaired t-tests for parametric data and the Mann-Whitney U test for non-parametric data. Moreover, one-way analysis of variance (ANOVA) or the Kruskal- Wallis test was used to evaluate comparisons between ≥3 groups. Bonferroni correction was applied to posthoc analyses. Correlations were assessed with Pearson or Spearman correlation coefficients, whereas the chi-square test was used for categorical variables. The factors related to the serum soluble α-Klotho levels in patients were evaluated using multiple linear regression analysis. The analyses were performed with PASW 18 software (SPSS/IBM, Chicago, IL, USA), and two-tailed p values of <0.05 were considered statistically significant.

Results

The clinical and demographic characteristics of the patients with diabetic nephropathy (n =109) and the controls (n =32) are shown in Table 1. The levels of creatinine, s-Klotho, and PTH were significantly greater, whereas the eGFR was significantly lower in the patient group compared to the healthy controls (p <0.001). The TOS, TAS, and OSI values were also significantly greater in the patient group (p <0.001). However, the IMA and IMAR values did not differ significantly between the groups (Table 1).

UACR values were obtained from 107 patients, who were then assigned into three groups based on their UACR; accordingly, 28, 29, and 50 patients were included in the normoalbuminuria, microalbuminuria, and macroalbuminuria groups, respectively. Table 2 presents the main parameters of the patients in these groups. One-way ANOVA indicated significant differences between these groups in terms of the creatinine (p <0.001), PTH (p =0.002), Ca (p =0.002), glycated haemoglobin (HbA1c) (p <0.001), and albumin levels (p <0.001). Moreover, the s-Klotho levels were also significantly different between the groups (p =0.031). The TOS, OSI, and IMA values did not significantly differ between the groups, although the TAS (p <0.001) and IMAR (p =0.002) values were significantly different.

Correlation analyses were performed between s-Klotho levels and age, UACR, other bone mineral metabolism parameters, and oxidative stress marker levels (Table 3). A significantly positive correlation was observed between the s-Klotho levels and TOS (r =0.186, p =0.034) and OSI (r =0.207, p =0.018). However, no significant correlation was found between the s-Klotho levels and eGFR; UACR; HbA1c, Ca, P, and PTH levels; and TAS, IMA, and IMAR values.

Regression analysis was performed to determine the effect of age; UACR; Ca, P, and PTH levels; eGFR; and TOS, TAS, OSI, IMA, and IMAR values on the s-Klotho levels in patients with CKD. A significant relationship was only observed between the serum s-Klotho levels and the TOS (β =0.187, p =0.034) and IMAR (β = -0.230, p =0.014) values (Table 4).

Discussion

CVD, rather than progression to end-stage renal

Table 1: Baseline characteristics of the 109 patients with diabetic nephropathy and the 32 healthy controls that were recruited in the cross-sectional study.

Parameter	Diabetes (n =109)	Control (n =32)	p
Age (years)	61.63 ± 9.77	49.53 ± 7.32	**<0.001**
Gender (F/M)	47/62	20/12	0.054
Creatinine (mg/dl)	1.57 ± 0.75	0.88 ± 0.12	**<0.001**
GFR (ml/dak)-CKD-EPI	51.71 ± 23.11	90.15 ± 20.71	**<0.001**
s-Klotho (ng/mL)	5.69 ± 4.64	3.62 ± 4.27	**<0.001**
PTH (pg/mL)	96.33 ± 111.52	57.79 ± 22.28	**<0.001**
Calcium (mg/dL)	9.36 ± 0.55	9.37 ± 0.39	0.974
Phosphorus (mg/dL)	3.43 ± 0.64	3.28 ± 0.67	0.136
TOS (μmol H2O2 Equiv./L)	2.95 ± 2.65	1.73 ± 1.17	**<0.001**
TAS (nmol Troloks/L)	1.16 ± 0.18	0.97 ± 0.15	**<0.001**
OSI	3.57 ± 5.81	1.83 ± 1.29	**0.006**
IMA	0.47 ± 0.07	0.45 ± 0.06	0.179
IMAR	0.14 ± 0.03	0.13 ± 0.02	0.083

n: number, F: female, M: male, GFR (ml/dak)-CKD-EPI: chronic kidney disease epidemiology collaboration equation assessment of the glomerular filtration rate, s-Klotho: soluble Klotho, PTH: parathyroid hormone, TAS: total anti-oxidative status, TOS: total oxidative status, OSI: oxidative stress index = ratio TOS/TAS, IMA: ischaemia-modified albumin, IMAR: ischaemia-modified albumin rate (IMA level/albumin level).

Table 2: Comparison of the 32 healthy controls and the 109 patients with diabetic nephropathy according to their urinary albumin creatinine ratio (UACR) value.

Parameter	Control	Normoalbuminuria	Microalbuminuria	Macroalbuminuria	p
Age (years)	49.53 ± 7.32*	62.75 ± 12.07	63.72 ± 8.03	59.83 ± 8.78	**<0.001[a]**
Male/Female (n)	12/20	11/17	18/11	31/19	0.053
Creatinine (mg/dl)	0.88 ± 0.12*	1.04 ± 0.29*	1.42 ± 0.46*	1.90 ± 0.79*	**<0.001[b]**
GFR (ml/dak)-CKD-EPI	90.15 ± 2 0.71*	69.38 ± 19.28*	52.86 ± 20.02*	42.02 ± 21.06*	**<0.001[b]**
s-Klotho (ng/mL)	3.62 ± 4.27*	7.00 ± 6.34	5.98 ± 4.56	4.87 ± 3.41	**0.031[a]**
PTH (pg/mL)	57.79 ± 22.28	55.73 ± 23.08	69.05 ± 41.44*	142.54 ± 176.05*	**0.002[c]**
Calcium (mg/dL)	9.37 ± 0.39	9.59 ± 0.38	9.50 ± 0.49	9.17 ± 0.61*	**0.002[d]**
Phosphorus (mg/dL)	3.28 ± 0.67	3.38 ± 0.52	3.26 ± 0.52	3.59 ± 0.75	0.058
Albumin (mg/dL)	4.22 ± 0.36	4.20 ± 0.24	4.05 ± 0.38	3.70 ± 0.55*	**<0.001[d]**
HbA1c (%)		7.6 ± 1.73	7.90 ± 1.70	9.66 ± 1.21*	**<0.001[d]**
TOS (μmol H_2O_2 Equiv./L)	1.73 ± 1.17	2.63 ± 2.52	3.16 ± 2.90	2.99 ± 2.57	0.166
TAS (nmol Troloks/L)	0.97 ± 0.15*	1.09 ± 0.20*	1.21 ± 0.19*	1.17 ± 0.16*	**<0.001[b]**
OSI	1.83 ± 1.29	3.20 ± 4.18	2.51 ± 2.00	4.59 ± 8.11	0.540
IMA	0.452 ± 0.065	0.468 ± 0.070	0.479 ± 0.074	0.469 ± 0.064	0.150
IMAR	0.127 ± 0.018	0.125 ± 0.017	0.138 ± 0.027	0.144 ± 0.029*	**0.002[d]**

n: number, GFR (ml/dak)-CKD-EPI: chronic kidney disease epidemiology collaboration equation assessment of the glomerular filtration rate, s-Klotho: soluble Klotho, PTH: parathyroid hormone, HbA1c: glycated haemoglobin, TOS: total oxidative status, TAS: total anti-oxidative status, OSI: oxidative stress index = ratio TOS/TAS, IMA: ischaemia-modified albumin, IMAR: ischaemia-modified albumin rate (IMA level/albumin level), *,[a]: significant difference between the control group and others, *,[b]: significant difference between each group, *,[c]: significant difference between the patients with microalbuminuria, macroalbuminuria, and others, *,[d]: significant difference between the patients with macroalbuminuria and others.

Table 3: Correlation between the levels of s-Klotho and clinical parameters of the 109 patients with diabetic nephropathy.

	s-Klotho	
Age	r =0.080	p =0.410
UACR	r = -0.083	p =0.459
HbA1c	r =0.028	p =0.784
Calcium	r =0.169	p =0.083
Phosphate	r =0.032	p =0.745
PTH	r = -0.055	p =0.586
eGFR	r =0.160	p =0.097
Creatinine	r = -0.129	p =0.180
TOS	**r =0.186**	**p =0.034**
TAS	r =0.110	p =0.211
OSI	**r =0.207**	**p =0.018**
IMA	r =0.049	p =0.622
IMAR	r = -0.143	p =0.152

s-Klotho: soluble Klotho, UACR: urinary albumin creatinine ratio, HbA1c: glycated haemoglobin, PTH: parathyroid hormone, eGFR: estimated glomerular filtration rate, TOS: total oxidative status, TAS: total anti-oxidative status, OSI: oxidative stress index = ratio TOS/TAS, IMA: ischaemia-modified albumin, IMAR: ischaemia-modified albumin rate (IMA level/albumin level).

Table 4: Regression analysis of the serum s-Klotho levels in the 109 patients with diabetic nephropathy.

	s-Klotho	
	β value	p value
Age	0.033	0.709
eGFR	0.074	0.270
Calcium	0.058	0.367
Phosphate	0.000	0.999
PTH	-0.009	0.889
UACR	-0.060	0.554
TOS	**0.187**	**0.033**
TAS	0.094	0.287
OSI	0.138	0.118

s-Klotho: soluble Klotho, eGFR: estimated glomerular filtration rate, PTH: parathyroid hormone, UACR: urinary albumin creatinine ratio, TOS: total oxidative status, TAS: total anti-oxidative status, OSI: oxidative stress index = ratio TOS/TAS.

disease, is the leading cause of death in CKD patients. Moreover, oxidative stress is a non-traditional risk factor of CVD. Oxidative stress induces endothelial dysfunction and atherosclerosis progression by reducing nitric oxide availability. In fact, oxidative stress and changes in cellular function play a key role in the development and progression of diabetic nephropathy[13]. Previous studies indicated an increase in ROS generation in diabetic patients, which is a major contributor to the pathogenesis of diabetic nephropathy[14,15]. Nitric oxide production and nitric oxide synthase isoform expression in the kidney are upregulated during the early phase of diabetic nephropathy; as the renal function declines, the nitric oxide levels decrease[16]. In experimental studies, renal nitric oxide levels decreased due to increased oxidative stress, primarily as a result of the enhanced expression of superoxide dismutase (SOD) and catalase[17]. In fact, superoxide radi-

cals play an important role in diabetic complications by causing vascular dysfunction; these radicals are primarily cleared by copper/zinc superoxide dismutase. Moreover, extracellular SOD levels are higher in patients with diabetes, possibly as a compensatory effect that reflects the presence of increased oxidative stress and vascular injury. Liu et al observed an increase in extracellular SOD levels in diabetic patients and found that SOD levels were positively correlated with s-Klotho levels in diabetic patients[18]. In the present study, we observed elevated TOS, TAS, and OSI values in patients with diabetic nephropathy, as compared to those in healthy controls, consistent with the current literature. We also used IMA as a marker of oxidative stress. A 1 g/dl change in albumin has been found to produce a contrasting change of 2.6 % in the IMA levels, thus exhibiting a negative correlation. To avoid the impact of the differences in albumin concentration between the groups, we evaluated the IMA values along with those of albumin, and the formula 'IMA value/individual serum albumin concentration' was used to maintain the IMAR.

In diabetic patients, microalbuminuria is the first predictive marker that shows the presence of extensive endothelial damage and progression to macrovascular disease. Ukinc et al showed that elevated IMA levels may indicate an underlying subclinical vascular disease in type 2 diabetes mellitus patients; in their study, the IMA levels in type 2 diabetic patients with microalbuminuria were markedly higher as compared to those in normoalbuminuric diabetic individuals[19]. In another study in diabetic nephropathy patients, the IMA levels progressively increased with the degree of albuminuria[20]. In the present study, the IMA and IMAR values did not differ significantly between diabetic nephropathy patients and healthy controls; however, when the patients were divided based on their UACR values, the IMAR value was found to be significantly different among the groups. As the renal disease progressed, the IMAR values increased.

Klotho is expressed in multiple tissues, with particularly high levels in the kidney. In CKD, Klotho tissue expression begins to decrease during the early stages of the disease[21]. In the present study, patients with CKD secondary to diabetic nephropathy exhibited higher s-Klotho levels as compared to healthy controls. As we mentioned before in the same study population, we investigated the s-Klotho and FGF23 levels in patients with Stage 1–4 diabetic nephropathy with different stages of albuminuria, and found an increase in the s-Klotho levels, in comparison with the control group; however, there was no significant correlation between the s-Klotho levels and eGFR[11]. Consistent with these findings, the serum levels of s-Klotho were not associated with kidney function in patients with CKD Stages 2, 3a, and 4 in another cohort study[22]. Lee et al[23] also described increased s-Klotho levels in patients with diabetes, although the kidney function was normal in those patients.

When patients were grouped according to their UACR values, we found that the s-Klotho levels were greatest in those with normoalbuminuria and decreased as albumin excretion increased, despite the reduction in eGFR. As we mentioned before, nitric oxide production is upregulated during the early phase of diabetic nephropathy. We believed that s-Klotho production is also similarly upregulated during the early stages of diabetic nephropathy. In the present study, an increase in s-Klotho levels was associated with an increase in the TOS and OSI values, as s-Klotho has anti-inflammatory functions in the kidney, in addition to its ability to increase resistance to oxidative stress. However, with the decline in renal function, the renal s-Klotho expression also decreased, which was followed by a reduction in plasma s-Klotho levels. In a previous study, Adema et al assessed the effect of antioxidative therapy on α-Klotho concentrations in patients with mild-moderate CKD, and found that the s-Klotho concentrations did not differ in the treatment group[24]. In addition, Antoniadi et al showed that SOD levels did not significantly differ between hemodialysis patients and healthy volunteers, and these findings indicate a lack of adaptation to increased oxidative stress, which a common characteristic among patients on dialysis[25]. Prior to the decline in renal function among patients with normoalbuminuria or microalbuminuria, antioxidant therapy may be useful. Moreover, the expression of the *Klotho* gene reduces following the activation of the renin angiotensin aldosterone (RAS) system[26]. The RAS blockade with the angiotensin-converting enzyme inhibitor or angiotensin receptor blocker may upregulate the s-Klotho levels. However, in the present study, we did not assess the effect of these drugs on the levels of oxidative stress markers and s-Klotho.

The present study has certain limitations. Due to its cross-sectional nature, the cause and effect relationship cannot be easily established. Moreover, the diabetic and control group were not matched in terms of age and this may have affected our results. Also, additional plasma markers of oxidative stress should be assessed in further studies. In fact, a future study with a larger sample size would be able to clarify the relationship between s-Klotho levels, oxidative stress, and albuminuria in diabetic nephropathy patients.

In conclusion, oxidative stress is greater in patients with diabetic nephropathy, and we found that oxidative status is an important factor affecting s-Klotho levels. Moreover, the therapeutic reduction of oxidative stress during the early stages of diabetic nephropathy can improve the renal and cardiovascular outcomes in these patients.

Conflict of interest

The authors declare no conflict of interest.

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