Klotho levels: association with insulin resistance and albumin-to-creatinine ratio in type 2 diabetic patients

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Abstract
Purpose The present study aimed at evaluating the relationship between Klotho levels and insulin resistance and albumin-to-creatinine ratio (ACR) in type 2 diabetic patients with CKD.

Methods We conducted an observational, cross-sectional study in our outpatient diabetic nephropathy clinic from 2014 to 2016, enrolling a total of 107 type 2 diabetic patients with stage 2–3 CKD, with a mean age of 59 years. Several clinical and laboratorial parameters were evaluated, including those related to mineral and carbohydrate metabolism.

Results The mean eGFR at baseline was 53.2 mL/min, and the mean levels of ACR and Klotho were 181.9 µg/mg and 331.1 pg/ml, respectively. In the simple linear regression model, Klotho levels were correlated with age, phosphorus, PTH, ACR, HOMA, IL-6, FGF-23, OxLDL, eGFR and vitamin D levels. Applying a multivariate linear regression model, only the ACR, HOMA-IR, FGF-23 and vitamin D independently influenced the Klotho levels. In the generalized linear model, only the Klotho groups were statistically significant as independent variable (p = 0.007). The results show that the group 1 (<268) compared with group 3 (>440) had higher odds in the higher ACR (≥181), ORa = 3.429, p = 0.014. There were no statistically significant differences between Klotho groups 2 and 3, and the HOMA-IR obtained showed that group 1 (<268) had greater odds of HOMA-IR ≥2 when compared with group 3 (>440), ORa = 21.59, p = 0.017.

Conclusions Our results showed that Klotho levels are influenced by FGF23, vitamin D and insulin resistance. This suggests that Klotho levels might be affected by renal function as well as having a relevant role on insulin metabolism and ACR homeostasis.

Keywords Klotho · Chronic kidney disease · Diabetes · Mineral metabolism

Introduction
Klotho is a protein expressed in several organs and tissues, with greater predominance in the kidney and brain. It has been implicated in multiple organic processes, being able to regulate growth factors signaling pathways, ion channels and transporters [1].

Klotho plays a particularly relevant role within the bone- kidney endocrine axis. In association FGF-23, it mediates the phosphate excretion and homeostasis through the inhibition of 1.25(OH)2 vitamin D3 synthesis and the induction of phosphaturia [2].

Recently, increasing evidence links Klotho levels with chronic kidney disease (CKD), as basic and clinical research have shown the link between Klotho deficiency and CKD [3–5] stages. Moreover, Klotho also seems to play a renoprotective role through its anti-oxidation properties: protection of vasculature, promotion of vascularization and inhibition of fibrinogenesis [6]. Therefore, it appears reasonable to consider Klotho as a potential surrogate...
biomarker and target for early intervention and treatment of CKD.

Despite this, the association between Klotho and CKD is still far from being fully understood, as some studies have arrived at divergent conclusions [7, 8]. The discrepancy of results may well be explained by the multifactorial pathophysiology of CKD, especially in patients with comorbidities, such as diabetics.

In this group, some studies argued that Klotho levels may be able to mediate insulin metabolism through the inhibition of intracellular insulin and IGF1 signaling [9], as well as to enhance glucose-induced insulin secretion [10].

Furthermore, different studies have speculated that Klotho levels might also be correlated with the albumin-to-creatinine ratio (ACR) both by direct [11, 12] and indirect mechanisms, with low Klotho levels being associated with proteinuria.

In the present study, our objective was to evaluate the relationship between Klotho levels and insulin resistance and ACR. We intended to understand Klotho’s role in insulin resistance, as well as to examine whether it played a renoprotective role in type 2 diabetics with chronic renal disease.

**Methods**

**Subjects**

An observational, cross-sectional study was conducted in our outpatient diabetic nephropathy clinic from 2014 to 2016, enrolling a total of 107 type 2 diabetic patients with stage 2–3 CKD. The diabetes classification was based on the American Diabetes Association guidelines [13].

The exclusion criteria were: age > 75 years, previous CVD (defined as history of one or more of the following: non-fatal myocardial infarction, (stable or unstable) angina pectoris, stroke or transient ischemic attacks, peripheral vascular disease or congestive heart failure); uncontrolled hypertension (BP ≥ 140/90 mmHg), albumin-to-creatinine ratio (ACR) >500, estimated glomerular filtration rate (eGFR) ≤29 or >90 mL/min, type 1 diabetes, known neoplastic or infectious diseases, non-diabetic renal disease (patients without previous history of diabetes, with diagnosis of glomerulopathies associated with other pathologies like systemic diseases, IgA nephropathy, kidney disease of unknown etiology, chronic interstitial nephritis, vasculitis, component complement 3 pathologies or renal hereditary diseases. Patients with parathyroid hormone (PTH) ≥350 pg/mL were excluded to avoid bias from anti-hyperparathyroidism medication that can affect Klotho and FGF-23 levels. The same happened with patients with phosphorus >5.5 and the phosphorus-chelating agents that can affect the Klotho-FGF-23 axis. Patients undergoing therapy with vitamin D and vitamin D receptor activators and phosphate binders were also excluded.

**Blood measurements**

Fasting samples were drawn from all subjects, and plasma was frozen at −80 °C in order to measure eGFR, phosphorus (P), calcium (Ca), PTH, ACR, insulin resistance degree, interleukin-6 (IL-6), fibroblast growth factor-23 (FGF 23), 1,25(OH)2D3 (vitamin D), oxidized low density lipoprotein (oxLDL) and soluble α-Klotho (Klotho). Serum levels of FGF-23 were quantified using an enzyme-linked immunosorbent assay, Human FGF-23 (C-Term) ELISA kit (Cat. #60-6100 Immunotopics Inc, San Clemente, CA, USA). Serum levels of vitamin D were determined with the help of a radioimmunoassay (IDS, Boldon, UK). Phosphorus and calcium were assayed by the ARCHITECT Systems and the AEROSET System (Abbott Diagnostics Division, Abbott Laboratories Abbott Park, IL). IL-6 and oxLDL determination was done with a sandwich enzyme-linked immunoassay (ELISA) kit (eBioscience, San Diego, California). PTH levels were measured using an electrochemiluminescent immunoassay (ECLIA). PTH concentrations were measured on an Immulite 2000 Intact PTH assay (Cat. #L2KPP2, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). Soluble α-Klotho serum levels were determined by enzyme-linked immunosorbent assay using the “Human soluble α-Klotho ELISA kit” (Catalog number: 27998, IBL—Immuno-Biological Laboratories Co., Ltd, Gunma, Japan), according to manufacturer’s instructions and adapted to Triturusmicroplate automatic system (Grifols S.A., Barcelona, Spain). The degree of insulin resistance was estimated using the homeostasis model assessment (HOMA-IR) described by Matthews et al. [14]. Serum creatinine was assayed by the enzymatic method, using the ARCHITECT® device (Abbott Diagnostics Division, Abbott Laboratories Abbott Park, IL). We estimated the GFR according to the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) Equation [15].

**Statistical analysis**

Descriptive quantitative data were presented as mean and standard deviation. Simple linear regressions were used to investigate possible correlations between these variables and Klotho. Only the variables with a statistically significant relationship were introduced in a multiple regression model. The generalized linear model (GLM) for binary dependent variables was used (binomial distribution) with a logistic link function. The exponentials of the model parameters were the adjusted odds ratio (ORa) to other variables of the model. The test was used to determine whether
Klotho was an important predictive factor in the determination of the albumin-to-creatinine event and the resistance to insulin. Prior to conducting the GLM, the 25th, 50th and 75th percentiles of serum Klotho level in the current study population were determined. The subjects were categorized as follows: group 1 (Klotho < 268), group 2 (Klotho: 268–440) and group 3 (Klotho > 440).

The null hypothesis was rejected below the level of 5%. Statistical analysis was performed using SPSS (version 17.0; SPSS, Chicago, IL).

The institutional Ethics Committee approval (reference 207/2010) was obtained for this study.

### Results

One hundred and seven (107) consenting patients with stage 2–3 CKD were included in the study after confirming they did not meet any of the exclusion criteria.

The mean age was 59 ± 8.6 [range 41–72] years, and the mean Klotho level was 331.10 ± 117.06 [range 70–663 pg/mL]. The demographic and clinical parameters are presented in Table 1.

Simple linear regression (Table 2) demonstrated that Klotho levels were inversely correlated with age \((r = -0.232, p = 0.016)\), phosphorus \((r = -0.381, p < 0.001)\), PTH \((r = -0.606, p < 0.001)\), ACR \((r = -0.336, p < 0.001)\), HOMA-IR \((r = -0.482, p < 0.001)\), IL-6 \((r = -0.571, p < 0.001)\), FGF-23 \((r = -0.695, p < 0.001)\), OxLDL \((r = -0.598, p < 0.001)\) and directly correlated with eGFR \((r = 0.234, p = 0.021)\) and vitamin D \((r = 0.661, p < 0.001)\) levels.

Applying the multivariate linear regression (Table 3), only the ACR \((r = -0.636, p = 0.036)\), HOMA \((r = -0.322, p = 0.018)\), FGF-23 \((r = -0.668, p < 0.001)\) and vitamin D \((r = 8.465, p = 0.010)\) independently influenced Klotho levels.

In the generalized linear model (GLM), only the Klotho groups were statistically significant as independent variable \((p = 0.007)\). The results show that group 1 (<268) compared to group 3 (>440) had higher odds in the higher ACR \((≥ 181)\), ORa \(= 3.429, p = 0.014\). Group 2 had no statistical significant difference compared to group 3. The optimized model as function of Klotho groups, clearance values, age, phosphorus, PTH, IL6, vitamin D, OxLDL and FGF-23 allowed for a final and statistical significant model \((p < 0.001)\) which included Klotho groups and vitamin D variables. The area under the ROC curve for this optimized model was 0.772

### Table 1 Patients demographic and clinical characteristics at baseline

| Characteristics | Values                        |
|-----------------|-------------------------------|
| Number of patients (n) | 107                           |
| Gender (f/m) | 40/67                         |
| Age (years) | 59.00 ± 8.57                  |
| Hb (g/dL) | 12.97 ± 1.83                  |
| Albumin (g/dL) | 4.27 ± 0.48                   |
| eGFR (mL/min) | 53.20 ± 10.15                 |
| ACR (µg/mg) | 181.89 ± 123.83               |
| Phosphorus (mg/dL) | 3.99 ± 0.85                   |
| PTH (pg/mL) | 113.11 ± 74.65                |
| Calcium (mg/dL) | 9.43 ± 0.92                   |
| FGF-23 (RU/mL) | 135.04 ± 35.23                |
| [1.25(OH)2D3] (pg/mL) | 21.18 ± 7.37                 |
| IL-6 (pg/mL) | 5.71 ± 3.80                   |
| OxLDL (U/L) | 39.91 ± 19.55                 |
| α-Klotho (pg/mL) | 331.10 ± 171.06               |
| HOMA-IR | 1.84 ± 1.67                   |
| HbA1c | 7.8 ± 2.0                     |

Values are presented as mean ± standard deviation

| Variable | Coefficient | SE     | p value |
|----------|-------------|--------|---------|
| Age      | 0.222       | 1.599  | 0.890   |
| eGFR     | 0.090       | 0.626  | 0.886   |
| Phosphorus | 11.909     | 21.277 | 0.577   |
| Calcium  | 2.424       | 10.701 | 0.821   |
| PTH      | -0.291      | 0.253  | 0.252   |
| ACR      | -0.636      | 0.105  | 0.036   |
| HOMA-IR  | -0.322      | 0.588  | 0.018   |
| IL-6     | -7.889      | 6.098  | 0.199   |
| FGF-23   | -0.668      | 0.135  | <0.001  |
| Vit.D    | 8.465       | 3.225  | 0.010   |
| OxLDL    | 1.856       | 1.223  | 0.132   |

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

### Table 3 Multivariate linear regression analysis between Klotho and other parameters in diabetic patients with CKD
(p < 0.001), revealing a sound discriminant capacity of the model. The estimates obtained showed that group 1 (<268) compared with group 3 (>440) had greater odds of ACR ≥181 (ORa = 1.324, p = 0.024) and that higher levels of vitamin D were statistically associated with lower values of ACR (ORa = 0.808, p < 0.001) (Table 4).

No statistical significant difference was found between groups 2 and 3, but the HOMA-IR obtained showed that group 1 (<268) had greater odds of HOMA-IR ≥2 (ORa = 21.59, p = 0.017) when compared with group 3 (>440). The area under the ROC curve for this optimized model was 0.978 (p < 0.001), revealing an excellent discriminant capacity of the model (Table 5).

Discussion

In the last few years, Klotho has become an ineluctable topic of discussion among nephrologists, due to its known contribution to mineral metabolism homeostasis in general and phosphate handling in particular. For this reason, Klotho has been recurrently seen as a promising target for the management and treatment of patients with CKD. Nevertheless, contradictory recent evidence has raised questions on whether Klotho actually plays any active role in CKD.

CKD is a well-known multifactorial disease, with several contributing factors and associated triggering agents. Most of these seem to be linked in a well-arranged web, and a single deregulation is able to affect the whole chain, producing different pathological outcomes. The addition of other comorbidities such as diabetes often leads to an extreme complex puzzle of interconnections which may be difficult to understand.

The current study investigated the correlations between serum Klotho levels and ACR and insulin resistance, in type 2 diabetic patients with stage 2–3 CKD.

Results showed that Klotho levels were correlated with FGF23, vitamin D and insulin resistance, suggesting that Klotho levels might be affected by renal function. This seems to be in accordance with most of the studies that describe CKD as a state of FGF-23 resistance caused by the deficiency of Klotho [16]. Nevertheless, this is still a debatable subject, as conflicting results have reported that Klotho levels were significantly correlated with age, but not with eGFR or other parameters of mineral metabolism in patients with CKD [8].

| Table 4 | GLM for the albumin-to-creatinine ratio (≥181) |
|---------|-----------------------------------------------|
|         | Initial model                                  | Optimized model                      |
|         | ORa   | 95% CI for ORa | p value | ORa   | 95% CI for ORa | p value |
| Klotho groups |       |               |        |       |               |        |
| <268    | 3.429 | 1.288–9.125  | 0.014  | 1.324 | 1.061–1.721  | 0.024  |
| 268–440 | 0.839 | 0.302–2.328  | 0.736  | 0.586 | 0.182–1.886  | 0.370  |
| >440    | Ref.  |               |        | Ref.  |               |        |
| Vitamin D | –    | –             |        | 0.808 | 0.725–0.901  | <0.001 |
| p value (model) | 0.007 | <0.001        |        |       |               |        |
| Area under ROC (p value) | –     | 0.772 (p < 0.001) |        |       |               |        |

GLM generalized linear model, ORa adjusted odds ratio, 95% CI for OR 95% confidence interval for the odds ratio, Ref category versus the one is making comparisons.

| Table 5 | GLM for the HOMA-IR (≥2) |
|---------|--------------------------|
|         | Initial model             | Optimized model             |
|         | ORa   | 95% CI for ORa | p value | ORa   | 95% CI for ORa | p value |
| Klotho groups |       |               |        |       |               |        |
| <268    | 30.000 | 8.256–109.006 | <0.001 | 21.590 | 1.733–268.903 | 0.017  |
| 268–440 | 2.640  | 0.809–8.616   | 0.108  | 7.264  | 0.847–62.332  | 0.071  |
| >440    | Ref.   |               |        | Ref.   |               |        |
| p value (model) | <0.001 | <0.001        |        |       |               |        |
| Area under ROC (p value) | <0.001 | 0.978 (p < 0.001) |        |       |               |        |

GLM generalized linear model, ORa adjusted odds ratio, 95% CI for OR 95% confidence interval for the odds ratio, Ref category versus the one is making comparisons.
In this study, Klotho levels were associated with insulin resistance inasmuch as being a predictive factor for insulin resistance, as indicated by the optimized generalized model (Table 5). The association between Klotho levels and insulin resistance also seems to be in accordance with other studies [9, 10]. These studies reported that Klotho levels can mediate insulin metabolism through the inhibition of tyrosine phosphorylation of insulin and IGF1 receptors, as well as enhancing glucose-induced insulin secretion via up-regulating membrane retention of TRPV2. If the apparent Klotho deficiency seen in CKD stages is taken into consideration, we might hypothesize that low Klotho levels could result in excessive insulin release, leading to a state of insulin resistance.

When our variables were transferred to the multiple regression model, the link between Klotho and eGFR was lost. Klotho was mainly associated with ACR. In the generalized linear model, used to investigate the relationship of possible predictors, only Klotho was a predictive factor in the determination of the album-to-creatinine event (Table 4). This association seems, once again, to be in line with other recent studies. These have hypothesized and investigated direct and indirect mechanisms that could possibly explain how these two variables influence each other. On a direct level, it has been argued that interstitial inflammation induced by proteinuria may downregulate Klotho expression. According to Moreno et al. [11], the release of inflammatory cytokines such as tumor necrosis factor (TNF)-like weak inducer of apoptosis' (TWEAK) and TNF-α was responsible for the downregulation of Klotho expression through a nuclear factor kappa-B-dependent mechanism. This seems to be a valid hypothesis to support the correlation between Klotho levels and ACR found in our study. The observations from Moreno et al. were supported by another study that reported an increased Klotho circulating level in type 2 diabetic patients whose proteinuria was ameliorated with losartan [12]. According to the authors, the use of renin-angiotensin system antagonists with angiotensin-converting enzyme inhibitors or angiotensin receptor blockers in patients with diabetic nephropathy was able to reverse Klotho’s downregulation induced by angiotensin II, therefore retarding disease progression.

Another direct mechanism thought to be part of the relationship Klotho/ACR is the one involving the vascular endothelial growth factor A (VEGF-A). It is believed that VEGF-A plays a pathogenic role in diabetic nephropathy, particularly on angiogenesis and vascular permeability. In their study on diabetic patients, Kacso and colleagues [17] observed a consistent positive relationship between soluble Klotho and VEGF-A (both factors having low levels in the presence of microalbuminuria). It is still to be understood whether they both respond to the same pathogenic trigger or whether it is a reactive action to the other. Nevertheless, we may hypothesize that the downregulation Klotho/VEGF-A can lead to ACR worsening through their impact on endothelial dysfunction.

In addition to these mechanisms, Klotho has also an endogenous anti-fibrotic function via antagonism of Wnt/β-catenin signaling, which promotes fibrinogenesis. Therefore, it is reasonable to argue that a loss of Klotho may be associated with the progression of diabetic nephropathy and ACR worsening by accelerated fibrinogenesis [18, 19].

Some studies have also speculated that the correlation between Klotho and ACR may be due to indirect mechanisms as well. Within the Klotho/FGF-23/vitamin D axis, it is already known that low Klotho levels are associated with increased FGF-23 and decreased vitamin D levels. FGF-23 is then able to indirectly increase proteinuria by diminishing calcitriol synthesis [20] and inducing endothelium dysfunction [21]. It is also negatively correlated with vitamin D levels in diabetic nephropathy patients with microalbuminuria [22, 23]. Thus, it is not unreasonable to hypothesize that Klotho and ACR levels might be correlated through indirect, albeit unclear but possibly FGF-23-mediated, mechanisms.

Despite some limitations, namely the small size of the sample and the limited statistical power of the applied, this study offers an added value by generating hypotheses. Notwithstanding this, further studies are needed to determine the degree of correlation between plasma levels of soluble Klotho and ACR.

Conclusions

The current study found that Klotho levels were influenced by FGF-23, vitamin D and insulin resistance, variables that are affected by the renal function. Despite this, the eGFR lost its relationship with Klotho in the multiple regression model. The generalized linear model revealed that Klotho was the sole predictive factor in the determination of the album-to-creatinine event and insulin resistance. Additional studies are required in clarifying the meaning of the relationship between Klotho and ACR, and the links between Klotho and insulin resistance also demands further analysis.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All performed procedures were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.
Informed consent

Informed consent was obtained from all individual participants included in the study.

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