Relationships Between Serum α-Klotho Concentration and Inflammation-Related Cytokines in Hemodialysis Patients

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**Abstract**

We investigated the relationship between α-Klotho and cytokines related to inflammation in HD patients. We analyzed levels of α-Klotho with ELISA and inflammatory cytokines with CBA in the serum of HD patients. There was a significant negative correlation between the concentration of serum α-Klotho and patients’ age and the serum concentration of PTH. No correlation has been found between α-Klotho and Ca or Pi. HD time, creatinine or eGFR. However, there were significant positive correlations between the concentration of α-Klotho and the serum concentration of IL-12p70, IL-10, and IL-1β. Furthermore, the concentration of IL-10 and IL-1β was significantly lower in HD patients with low α-Klotho concentrations compared with HD patients with high α-Klotho. However, in a multivariable linear regression analysis, only patients’ age was associated independently with α-Klotho level. While these results draw our attention to potential relationships between α-Klotho proteins and inflammatory markers in HD patients, our cross-sectional study could not fully explain the pathogenic link between α-Klotho and inflammation in these patients. Therefore, further studies are necessary to clarify these relationships. However, this observation aligns with previous studies that confirm a significant relationship between Klotho concentration and human aging.

**Introduction**

The KLOTHO gene encoding the protein of the same name discovered at the end of the 20th century is one of the genes affecting animal and human life span. Its knock-out mutations in mice induced osteoporosis, vascular calcification, muscle atrophy, hypoglycemia, and hyperphosphatemia and promoted a reduced activity, hearing impairment, generally leading to accelerated aging. Many of the animal studies were later partially confirmed in humans. One of the best-known functions of Klotho, which belongs to the β-glucuronidase family, is its role in regulating calcium and phosphate metabolism, among others, by regulating pathways dependent on fibroblast growth factor 23 (FGF23) and IL-12p70, IL-10, and IL-1β. Furthermore, the concentration of IL-10 and IL-1β was significantly lower in HD patients with low α-Klotho concentrations compared with HD patients with high α-Klotho. However, in a multivariable linear regression analysis, only patients’ age was associated independently with α-Klotho level. While these results draw our attention to potential relationships between α-Klotho proteins and inflammatory markers in HD patients, our cross-sectional study could not fully explain the pathogenic link between α-Klotho and inflammation in these patients. Therefore, further studies are necessary to clarify these relationships. However, this observation aligns with previous studies that confirm a significant relationship between Klotho concentration and human aging.

**Baseline demographic, clinical, and laboratory findings**

The clinical information about 67 HD patients, including serum concentrations of parathormone (PTH), calcium (Ca), phosphorus (Pi), levels of cytokines IL-12p70, TNF, IL-10, IL-6, IL-1β, IL-8, and α-Klotho as shown by median values in Table 1. 63 (94%) patients were suffering from hypertension. In addition, 36 (54%) patients were diagnosed with DM. 36 (54%) of patients received alfacalcidol 0,25 µg daily.
### Table 1
HD patients’ characteristics. eGFR – estimated glomerular filtration rate, PTH – parathormone, Ca – calcium, Pi – phosphorus.

|                      | Median (minimum and maximum values) |
|----------------------|-------------------------------------|
| Age (years)          | 69 (36; 89)                         |
| HD time (months)     | 28 (0,5; 169)                       |
| Creatinine (mg/dl)   | 6,3 (2,71; 10,38)                   |
| eGFR (min/ml/1.73 m2)| 7 (4; 22)                           |
| Kt/V                 | 1,5 (0,74; 2,37)                    |
| PTH (pg/ml)          | 356 (44,5; 4276)                    |
| Ca (mg/dl)           | 8,7 (6,5; 10,8)                     |
| Pi (mg/dl)           | 5,5 (2,4; 10,5)                     |
| IL-12 (pg/ml)        | 2,45 (0; 108,95)                    |
| TNF (pg/ml)          | 2,5 (0; 127,65)                     |
| IL-10 (pg/ml)        | 2,8 (1,4; 21,88)                    |
| IL-6 (pg/ml)         | 10,3 (3,45; 805,45)                 |
| IL-1 (pg/ml)         | 1,75 (0; 18,24)                     |
| IL-8 (pg/ml)         | 38,8 (10,4; 5000)                   |
| α-Klotho (pg/ml)     | 6,96 (0,45; 127,1)                  |

#### Factors associated with α-Klotho levels

There was a significant negative correlation between the concentration of serum α-Klotho and patients’ age ($r = -0.268, p = 0.028$) and the serum concentration of PTH ($r = -0.290, p = 0.017$) (Tab. 2). No correlation has been found between α-Klotho and Ca or Pi. HD time, creatinine or eGFR. The serum concentration of α-Klotho was positively correlated with serum IL-12p70 ($r = 0.250, p = 0.041$), IL-10 ($r = 0.333, p = 0.006$), and IL-1β ($r = 0.436, p < 0.001$). However, in a multivariable linear regression analysis, only age was associated independently with patients’ Klotho level (Tab. 3).
Table 2
Correlations between serum concentrations of α-Klotho, patients' clinical parameters, and serum cytokines. Bivariate Spearman Rank Correlation test, the results in bold are statistically significant. Cr – creatinine, α-KL – α-Klotho.

| Variable   | Age  | HD time | Cr   | eGFR | Kt/V | PTH  | Ca  | Pi   | IL-12 | TNF  | IL-10 | IL-6  | IL-1 | IL-8 |
|------------|------|---------|------|------|------|------|-----|------|-------|------|-------|-------|------|------|
| **Age**    | 1,000| 0,186   | -0,358| 0,192| -0,194| -0,253| 0,106| -0,330| -0,138| -0,076| -0,178| 0,047| -0,048| 0,053|
| **HD time**| 1,000| 0,178   | -0,286| 0,415| 0,449| 0,036| -0,106| -0,003| 0,042| 0,045| 0,028| -0,004| -0,278|
| **Cr**     | 1,000| -0,668 | 0,294| 0,143| -0,223| 0,408| 0,122| -0,064| 0,119| -0,004| -0,080| 0,189|

Table 3
Multivariable linear regression analyses for α-Klotho, the results in bold are statistically significant. CI – confidence interval.

| Variable   | Estimate | Standard error | 95% CI          | P value |
|------------|----------|----------------|-----------------|---------|
| Age        | -0,5510  | 0,2715         | -1,094 to -0,008166 | 0,0468  |
| PTH        | -0,007788 | 0,004907      | -0,01760 to 0,002024 | 0,1176  |
| IL-12      | -0,06474 | 0,1672         | -0,3990 to 0,2696  | 0,6999  |
| IL-10      | -0,5447  | 0,9972         | -2,539 to 1,449   | 0,5869  |
| IL-1       | 1,706    | 1,154          | -0,6012 to 4,014  | 0,1444  |

As for other relationships between inflammatory markers and clinical parameters, the concentration of IL-8 was negatively correlated with HD time (r = -0.278, p = 0.023) (Tab. 2). Other correlations are shown in Table 2.

Demographic, clinical, and laboratory parameters by median α-Klotho level

The primary cause of CKD or the type of DM did not influence the serum concentration of α-Klotho. Likewise, the vitamin D supplementation did not influence the serum concentrations of PTH, Ca, Pi, or α-Klotho.

Next, patients were divided into two subgroups according to the median Klotho value: those with α-Klotho concentrations below (34 people) or above (33 people) the median (6,96 pg/ml). The subdivision was somehow artificial, but there are no clear ranges of serum values for α-Klotho. HD patients with low α-Klotho concentrations were characterized by higher PTH concentrations (the median value of 425 pg/ml) compared to patients with high α-Klotho (the median value of 320 pg/ml) (Tab. 4, Fig. 1A). No difference was found in calcium or phosphorus level. HD patients with low α-Klotho had lower IL-10 levels (the median value of 2,45 pg/ml) compared to patients with high α-Klotho (the median value of 3,44 pg/ml) (Tab. 4, Fig. 1B). The concentration of IL-1β was significantly lower in HD patients with low α-Klotho (the median value of 1,5 pg/ml) compared with HD patients with high α-Klotho (the median value of 2,34 pg/ml) (Tab. 4, Fig. 1C). No significant differences were observed between the two groups in age, hemodialysis duration, eGFR, dialysis adequacy, or other serum biomarkers.
Discussion

The history of Klotho is associated with the discovery of its relationship between its expression and aging in mice. Among three isoforms, the most important is α-Klotho, which occurs in the kidney in high concentrations. It is produced mainly in the distal nephron tubules and is associated with calcium-phosphate metabolism. CKD patients present a gradual decrease in serum sKlotho, accompanied by an increase in FGF23. In the kidney, decreased α-Klotho expression occurs as early as in stage 2 of CKD. However, even in those with ESRD, we can still detect some level of Klotho in blood. α-Klotho in these patients is secreted compensatively from other places, e.g., from choroid plexus or parathyroid glands. However, studies show that the parathyroid tissue of patients with more advanced stages of CKD has also reduced the expression of Klotho. Even the vascular smooth muscle of CKD patients displays reduced Klotho content.

Over the years, many publications have emerged showing significant correlations between the concentration of α-Klotho in the blood of CKD patients and the progression of the disease, and cardiovascular complications, especially in HD patients. However, we do not have data on whether there is any relationship between α-Klotho concentration and inflammatory markers in HD patients. According to some animal studies, α-Klotho exerts some anti-inflammatory effects.

ESRD is well characterized by increased oxidative stress and inflammation, which seem to be a central component of the uraemic phenotype and are linked with cardiovascular mortality among HD patients. HD patients have high serum IL-1, IL-6, IL-8, and TNF-α, produced mainly by mononuclear cells. IL-6, in particular, is recognized as a strong predictor of poor outcomes in ESRD patients. In our study, α-Klotho positively correlated with IL-12p70, IL-10, and IL-1β, but not with TNF or IL-6. Downregulation of renal α-Klotho expression increases kidney inflammation and leads to renal fibrosis. These processes are related to reduced production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), suppressed TNF-induced NF-kappaB activation, and upregulated anti-inflammatory IL-10. Thus, the anti-inflammatory role of α-Klotho could explain a positive correlation with anti-inflammatory IL-10 in our study group. From human studies, such an association was only confirmed in patients with established cardiovascular disease (CVD), whose low Klotho concentrations accompanied decreased IL-10 levels. We did a similar observation for our patients – those with low α-Klotho concentrations had significantly lower IL-10 compared with HD patients with high α-Klotho.

However, what is surprising is the positive correlation between Klotho and IL-1β and IL-12p70, which have a pro-inflammatory effect. IL-12 is a pro-inflammatory cytokine that induces the production of interferon-γ (IFNγ) by T helper 1 (Th1) cells associated with cell-mediated immunity. Studies showed that HD patients exhibit an increased percentage of Th1 cells compared with healthy controls, and their monocytes produce high levels of IL-12. IL-1β, also known as leukocytic pyrogen, is an important mediator of the inflammatory response associated with acute symptoms such as fever and hypotension in HD patients. Both cytokines are associated with attenuated inflammatory responses in ESRD, aggravated by high concentrations of uremic toxins and repeated contact with the dialysis membrane. Their positive correlation with a high level of α-Klotho draws attention to its potential, not yet described, role in promoting inflammation. As mentioned above, α-Klotho protects cells from oxidative stress, inflammation, and fibrosis. Klotho depletion increases nuclear

### Table 4

|                        | α-Klotho <6.96 pg/ml | α-Klotho >6.96 pg/ml | P   |
|------------------------|----------------------|----------------------|-----|
| Age (years)            | 71 (39; 89)          | 63 (36; 84)          | 0.068 |
| HD time (months)       | 29.5 (0.5; 169)      | 18 (0.5; 167)        | 0.760 |
| Creatinine (mg/dl)     | 6.67 (2.77; 169)     | 6.12 (2.71; 10.38)   | 0.473 |
| eGFR (min/ml/1.73 m2)  | 7 (4; 22)            | 8 (4; 16)            | 0.469 |
| Kt/V                   | 1.59 (1.13; 2.14)    | 1.44 (0.74; 2.37)    | 0.179 |
| PTH (pg/ml)            | 425 (67; 4276)       | 320 (445; 2665)      | 0.045 |
| Ca (mg/dl)             | 8.65 (7.10; 9.9)     | 8.7 (6.5; 10.8)      | 0.393 |
| Pi (mg/dl)             | 5.5 (2.4; 8.8)       | 5.6 (2.6; 10.5)      | 0.995 |
| IL-12 (pg/ml)          | 2.24 (0; 108.95)     | 2.6 (0; 59.61)       | 0.156 |
| TNF (pg/ml)            | 2.52 (0.56; 127.65)  | 2.4 (0; 31.1)        | 0.554 |
| IL-10 (pg/ml)          | 2.45 (1.4; 19.77)    | 3.44 (1.95; 21.88)   | 0.002 |
| IL-6 (pg/ml)           | 8.36 (3.45; 805.45)  | 11.37 (3.79; 120.28) | 0.078 |
| IL-1 (pg/ml)           | 1.5 (0; 4.65)        | 2.34 (0; 18.24)      | 0.002 |
| IL-8 (pg/ml)           | 43.05 (10.4; 5000)   | 30.2 (11.8; 4111.76) | 0.087 |
factor NFkB activation and the subsequent transcription of pro-inflammatory genes. The positive correlation between α-Klotho and pro-inflammatory cytokines could be ultimately not of much importance because, in a multivariable linear regression analysis, only age was associated independently with patients’ α-Klotho level.

On the other hand, HD patients with low α-Klotho concentrations had low both IL-10 and IL-1. Thus, the relationship between IL-10 and IL-1 levels is most likely the result of monocytes and macrophages responding to high levels of pro-inflammatory cytokines. This is related to the anti-inflammatory role of IL-10. In particular, macrophages produce IL-10 in a negative feedback loop to reduce the uncontrolled inflammatory cytokine production during, for example, infection. By binding with its receptor on cells of innate immunity, IL-10 inhibits the release of pro-inflammatory cytokines, decreases antigen presentation and phagocytosis. Accordingly, it can be expected that high IL-1 levels will be accompanied by the higher IL-10 level.

The correlation matrix showed no relationships between serum α-Klotho and any variables associated with CKD, including creatinine, eGFR, or calcium and phosphorus concentrations, except for PTH. However, some studies show that α-Klotho concentrations strongly correlate with eGFR in different stages of CKD. The exception is the study by Seiler et al., in which the authors demonstrated that serum level of Klotho in CKD patients correlates only with the patient’s age. In their opinion, therefore, Klotho concentration is not related to kidney function and cannot predict adverse outcomes in CKD patients. Also, serum α-Klotho in CKD patients does not necessarily reflect Klotho deficiency at the tissue level. Besides the kidneys, Klotho is also produced in other organs, e.g., choroid plexus or parathyroid glands. Moreover, Yildirim et al. have recently demonstrated that HD and PD patients have higher serum α-Klotho compared to healthy people. The presented results underline the assumptions made so far that the level of α-Klotho decreases with CKD progress. It is also possible that in HD patients, the eGFR is already too low and the calcium-phosphate balance too disturbed to demonstrate any relationship with Klotho levels. Wei et al. also saw no correlation between serum Klotho and Ca, Pi, or PTH in HD patients despite the larger study group. Finally, in a multivariable linear regression analysis, only age was associated with patients’ serum Klotho level. This observation aligns with previous studies that confirm a significant relationship between Klotho concentration and the aging of the human or animal organism.

In summary, these associations, which we demonstrated in our study, draw attention to the potential relationship between α-Klotho levels and inflammation status of HD patients. However, our cross-sectional study could not fully explain the pathogenic link between α-Klotho, and inflammation. Therefore, further studies are necessary to clarify these relationships.

**Patients And Methods**

**Patients**

The study groups consisted of 67 HD patients of mean age of 67.06 ± 12.73 years (27 men and 40 women). The mean time of HD treatment was 36.47 months (minimum time was 0.5 of the month, and maximum – 169 months) (Tab. 1). All patients had estimated glomerular filtration rate (eGFR) below 15 ml/min/1.73 m2 and underwent 4h sessions of hemodialysis three times a week using low-flux NIPRO PES 150DL, 170DL, 210DL or high-flux ELISIO 15H and 17H dialyzers. In addition, patients had regular dialysis adequacy assessment by measuring urea clearance using equation Kt/V (K – urea clearance, t – time on dialysis, V – volume of distribution); mean Kt/V was 1.52 ± 0.33.

In 13 patients, the primary cause of chronic kidney disease was glomerulonephritis (GN), in 25 – diabetic nephropathy (DN), in 13 – ischemic nephropathy (IN), in 4 – hypertensive nephropathy (HN), in 6 – adult polycystic kidney disease (ADPKD). In 2 patients, the primary cause of CKD was unknown. 2 patients had obstructive nephropathy, and 2 patients had granulomatosis with polyangiitis (GPA). None of the patients suffered from any infection, inflammation, malnutrition, malignancy, or blood loss during the study. 36 patients had diabetes mellitus (DM).

All participants were informed about the purpose of the tests and gave their written informed consent. The Bioethical Committee for Scientific Research at the Medical University of Gdańsk approved the study. We performed all the experiments following the relevant guidelines and regulations.

We collected 5 ml of peripheral venous blood from each patient before the HD session into anticoagulant-free tubes to collect serum to assess concentrations of cytokines and α-Klotho. We stored serum samples at -80°C.

**Cytokine measurement in plasma samples**

BD™ Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit (BD Biosciences, USA) was used according to the manufacturer's protocol to determine the level of different cytokines: IL-12p70, TNF, IL-10, IL-6, IL-1β, IL-8 in the plasma samples of HD patients. We performed quantitative cytometric fluorescence analysis with the FACScan cytometer (Becton Dickinson, USA) in the Department of Pathophysiology, Medical University of Gdańsk. Cytokine concentrations were analyzed with the use of Becton Dickinson software.

**Detection of α-Klotho alpha in plasma samples**

Human KL(Klotho) ELISA Kit (Wuhan Fine Biotech China) was used according to the manufacturer's protocol to determine the level of α-Klotho in the plasma samples of HD patients. Both kits used a sandwich enzyme-linked immune-sorbent assay technology. The density of yellow color was proportional to the target amount of sample captured in the 96-well plate, and the O.D. absorbance was read at 450 nm absorbance in Epoch™ Microplate Spectrophotometer (BioTek, USA).

**Analysis and Statistics**

The concentration of α-Klotho was calculated with GraphPad Prism 9 (GraphPad Software Inc., USA). Statistica (data analysis software system), version 13 (TIBCO Software Inc. 2017), and GraphPad Prism 9 were used to perform statistical analysis. The Kolmogorov-Smirnov and Lilliefors tests were used for
testing normality. The significance tests were chosen according to data distribution with the level of significance p<0.05.

Declarations

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All cytometric analyses were performed using the instruments acquired for the Cellular Function and Pathology Imaging Network of the Medical University of Gdańsk and the University of Gdańsk.

Authors contributions

Authors K.A.L., and H.S. conceived and designed experiments. K.A.L., M.S.C, and M.M. performed the experiments. K.A.L. wrote the paper. H.S. and A.D.-Ś. were responsible for selecting the study group, collecting clinical data about patients, and interpreting those data. Finally, K.A.L. reviewed the final manuscript.

Financial Disclosures

All co-authors reported no biomedical financial interests or potential conflicts of interest.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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**Figures**

![Figure 1](image)

Comparison of PTH (A), IL-10 (B), and IL-1 (C) depending on α-Klotho levels. The line represents the median, Mann-Whitney U test, * p<0.05, ** p< 0.01.