Soluble alpha klotho and its relation to kidney function and fibroblast growth factor-23

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Context: Relations between fibroblast growth factor-23 (FGF-23), soluble-alpha klotho (s-α klotho), and kidney function in chronic kidney disease (CKD) are still unclear. Especially the role of s-α klotho requires further study.

Objective: To analyze the relation of s-α klotho to estimated glomerular filtration rate (eGFR), FGF-23 and other parameters of calcium-phosphate metabolism, and to investigate the response of s-α klotho to cholecalciferol.

Design and setting: Eight-week RCT (Vitamin D and Chronic Renal Insufficiency).

Patients: Twenty-four CKD patients stage 1–5.

Interventions: 40000 IU cholecalciferol or placebo weekly.

Main outcome measure: S-α klotho determined by ELISA with anti-human klotho antibodies 67G3 and 91F1.

Results: For all patients, s-α klotho concentrations did not differ between CKD stages. When patients were subdivided based on FGF-23 concentrations a positive association of s-α klotho with eGFR became apparent in patients with lower than median FGF-23 concentrations but not in those above median value. Patients with s-α klotho below 204 pg/ml showed higher age, lower phosphate clearance, and lower bone-specific alkaline phosphatase (b-ALP) compared to patients with higher s-α klotho. Treatment with cholecalciferol significantly increased 1,25-diOH. The increase of FGF-23 had only borderline significance. There was no significant effect of high-dose cholecalciferol administration for 8 weeks on plasma s-α klotho.

Conclusions: CKD patients with s-α klotho below 204 pg/ml had higher age, lower phosphate clearance, and lower b-ALP. An association of s-α klotho with eGFR was observed only in presence of close to normal, but not high, FGF-23 concentrations. Cholecalciferol treatment did not change s-α klotho concentrations.

Abbreviations:

Soluble alpha klotho (s-α klotho) is the protein product of the putative aging-suppressor gene klotho, which can be observed in plasma, urine, and cerebrospinal fluid (CSF) (1, 2). Two forms of s-α klotho have recently been described; a secreted 70 kDa protein resulting from alternative splicing and a 130 kDa protein consisting of the large extracellular domain of transmembrane alpha klotho (2). Evidence from investigations in human serum using two different s-α klotho immunoassays supports the notion of coexisting s-α klotho variants (3). There exists a pronounced interest in the soluble form of the klotho protein. First, while transmembrane alpha klotho acts as a coreceptor for fibroblast growth factor 23 (FGF23), s-α klotho has also several paracrine and endocrine functions (4).

Second, alpha klotho is an important player in mineral and calcium-phosphate metabolism and their disturbances in chronic kidney disease (CKD) (5). Soluble-alpha klotho inhibits the expression of renal sodium phosphate cotransporter NaPi-2a and intestinal sodium phosphate co-transporter NaPi-2b (6, 7).

Alpha klotho is expressed in kidney tubulus cells, parathyroid gland, and choroid plexus, but also in the pituitary gland, aorta and several other tissues (2, 8–11). The main source for s-α klotho has not been defined. Moreover, the origin of upregulated s-α klotho after vitamin D receptor agonist therapy remained unclear (12).

Soluble-alpha klotho has been welcomed as early biomarker and new therapeutic target in CKD; but it’s relation to kidney function in CKD was also refuted (13, 14). In the present study we investigated the relation between plasma concentrations of s-α klotho and estimated glomerular filtration rate (GFR) in CKD patients. We determined s-α klotho during an 8-week randomized, placebo-controlled, double-blind, parallel intervention study with cholecalciferol.

**Materials and Methods**

The study was performed at the Department of Nephrology at Odense University Hospital, Denmark (latitude 55° north). The protocol was in accordance with the ethical standards of the Declaration of Helsinki, approved by the regional ethics committee (reference number: S-20090061), the Danish Medicines Agency (EudraCT: 2008–006438–82), reported to the Danish Data Protection Agency (reference number: 11–88–37–29) and ClinicalTrials.gov (Identifier: NCT00968877).

**Study population**

The subjects in this study represent the subgroup of patients with CKD which participated in an 8-week randomized, placebo-controlled, double-blind, parallel intervention study with cholecalciferol, except one subject for whom no blood material was stored in the biobank. Patients were subdivided according to gender and CKD status (conservatively treated or transplanted CKD patients). Random allocation to cholecalciferol and placebo treatment was undertaken in consecutive pairs of 2 within each of these subgroups (balanced and stratified randomization) by third part (study manager at the local pharmacy at Odense University Hospital) using a computer-generated list of random numbers. Investigators and participants were both blinded to treatment until study completion. The study has been described elsewhere (15). Briefly, potential study participants had their vitamin D status screened in summer (June-August). They were invited to participate in the study if plasma 25-OHD was below 50 nmol/L at screening. Exclusion criteria were: supplementary intake of a total of more than 10000 IU ergo- or cholecalciferol within the last 3 months, hypercalcemia, and severe hyperphosphatemia. The group of CKD patients included one patient CKD stage 1, three patients stage 2, nine patients stage 3, nine patients stage 4, and two patients stage 5. P-s-α klotho concentrations from hemodialysis patients (n = 27) included in the initial study (15) are also given in the current publication for the reason of comparison (Supplemental Figure 1).

The intervention period lasted from end of summer till late fall (September 14th till November 25th), participants were excluded if they met any of the exclusion criteria, or if they developed acute illness leading to hospital admission, if they initiated dialysis therapy, were kidney transplanted, or died.

The treated group received 40000 IU of vitamin D₃ (corresponding to two capsules of Dekristol® from MIBE, Brehna, Germany) weekly for 8 weeks. The control group received placebo capsules of identical size, color and shape.

**Blood analyses**

All analyses were performed at the local laboratories of the participating departments according to current laboratory standards. The procedures have been described in detail (15). Briefly, fasting blood samples were performed between 7 and 10 am. Serum phosphate was determined by photometric endpoint measurement using an ammonium-phosphomolydbdate complex, measured at 340 nm on the automated analyzer Modular P (Roche Diagnostics, Mannheim, Germany). The relative standard deviation for the day-to-day precision was ≤ 2.1%. Serum ionized calcium was measured by direct potentiometry on a Nova 2 automatic instrument (Nova Biomedical, Newton, Massachusetts, USA). Anerobiosis was maintained for this analysis. The relative standard deviation for the day-to-day precision was ≤ 1.1%. Plasma parathyroid hormone (PTH) was assessed by an electrochemiluminescence immunoassay (ECLIA) on Immulite 2000 (Siemens, Munich, Germany), using polyclonal AP-labeled capturing antibody (1–34) and a monoclonal detecting antibody (44–84) with an interassay CV of 5.8% and an intra-assay CV of 2.5%. Samples for determination of 25-OHD, 1,25-dihydroxyvitamin D₃, and FGF-23 were stored at −80°C until completion of the study, and then analyzed in batches. Plasma 25-OH-D₃ and 25-OH-D₃ were analyzed by mass spectrometry (LCMSMS 1, Applied Biosystems, Dionex, Sunnyvale, California, USA) with an interassay CV of < 10%, whereas plasma 1,25-dihydroxyvitamin D₃ was analyzed with a competitive immunometric method (Immuno-Diagnostic Systems Nordic a/s, Herlev, Denmark) on Wizard 1470 γ-counter (Perkin Elmer, Waltham, Massachusetts, US) with an interassay CV of < 15%. Serum FGF-23 concentrations...
were measured with an ELISA (Kainos Laboratories Inc., Tokyo, Japan), which only detects the biologically active intact FGF-23 using a combination of two monoclonal antibodies directed towards epitopes on either side of the cleavage site of FGF-23. The intra- and interassay CV was < 5.0%.

Determination of plasma soluble-alpha klotho (P-s-α klotho) has been described in detail elsewhere (14). A sandwich ELISA with anti-human klotho (67G3) mouse IgG and anti-human klotho (91F1) mouse IgG Fab, both affinity purified, was used (Immuno-Biological Laboratories, Fijjoka-shi, Gunma, Japan). According to recent literature the antibodies used in this sandwich ELISA were shown to immunoprecipitate recombinant human (rh) soluble-alpha klotho and an about 130 kDa protein from human plasma (16). A 130 kDa form is found in plasma and CSF produced by proteolytic cleavage of the glycosylated 135 kDa full-length alpha klotho (2).

Bone-specific alkaline phosphatase (b-ALP) in plasma was determined using a chemiluminescence immunoassay; the access-Ostase-immunoassay (Beckman-Coulter Inc., Munich, Germany) run on the fully automated ACCESS immunoassay system. The interassay coefficient of variation was < 6.5%.

Statistics

Continuous data are reported as median and interquartile range. Categorical variables were reported as numbers and percentages. Normal distribution of continuous variables was tested by D’Agostino & Pearson omnibus normality test. Nonparametric tests were applied where necessary. Nonparametric bivariate correlation analysis (Spearman) was performed. Selected variables were analyzed using linear regression. Analyses were performed with GraphPad prism software (version 5.0, GraphPad Software, San Diego, CA, USA). All statistical tests were two-sided and p-values less than 0.05 were considered to indicate statistical significance.

Results

Baseline data of study participants are presented in Tables 1. Median plasma s-α klotho was 282 pg/mL (IQR 229 - 305 pg/mL, n = 4) in CKD stage 1/2, 223 pg/mL (IQR 197 - 282 pg/mL, n = 9) in CKD stage 3, and 203 pg/mL (IQR 186 - 293 pg/mL, n = 11) in CKD stage 4/5, respectively. Plasma s-α klotho concentrations did not differ significantly between CKD stages (P = .484, Kruskal-Wallis test).

Spearman correlation analyses performed for parameters of calcium – phosphate metabolism and age only showed a significant association of plasma s-α klotho with bone-specific alkaline phosphatase (Table 2).

It was suggested that klotho expression can be influenced by FGF-23 (17, 18). Hence, as shown in Table 3, we grouped our data according to FGF-23 concentrations below and above the median FGF-23 concentration (73 pg/mL). Median s-α klotho concentrations were not significantly different between these groups (236 pg/mL; IQR, 191–287 pg/mL) vs 236 pg/mL; IQR 194–319 pg/mL; P = .665). It should be noted that patients with higher FGF-23 had significantly lower GFR, higher phosphate, and lower 1,25-dihydroxyvitamin D.

As shown in Table 2 we observed a significant positive association of s-α klotho with eGFR and phosphate clearance, and a negative association with age in patients showing lower than median FGF-23 concentrations (IQR, 25 – 52 pg/mL), which was close to the FGF-23 reference interval in healthy control subjects (18 – 55 pg/mL). In patients showing higher than median FGF-23 concentrations (IQR, 94 – 640 pg/mL) these correlations were absent.

Figure 1 shows the distribution of s-α klotho in each group. We also performed the same kind of analysis for other circulating parameters of calcium-phosphate metabolism. Spearman analyses for s-α klotho and eGFR for the respective values above and below the median of calcium, phosphate, PTH, 25-OHD and 1,25-OHD were performed. We did not observe a significant influence of

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Gender, male/female</th>
<th>Body mass index, kg/m²</th>
<th>Systolic blood pressure, mmHg</th>
<th>Diastolic blood pressure, mmHg</th>
<th>Diuresis, mL/24 h</th>
<th>Functioning kidney graft, n (%)</th>
<th>Underlying kidney disease, n (%)</th>
<th>Diabetic nephropathy</th>
<th>Nephrosclerosis</th>
<th>Glomerulonephritis</th>
<th>Others</th>
<th>Disease prevalence, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>21 / 3</td>
<td>26.3 (24.1 – 30.4)</td>
<td>142 (129 – 159)</td>
<td>75 (68 – 83)</td>
<td>1583 (1583 – 2666)</td>
<td>8 (33)</td>
<td>10 (42)</td>
<td>22 (92)</td>
<td>12 (50)</td>
<td></td>
<td>11 (46)</td>
<td>3 (6.5%)</td>
</tr>
</tbody>
</table>
| Abbreviations: ACE = angiotensin converting enzyme, AT = angiotensin.
these parameters on the relation between s-α klotho and eGFR (Supplementary Table 3).

Next, we investigated differences between patients with reduced and normal s-α klotho concentrations. We orientated towards an s-α klotho reference interval (2.5th – 97.5th percentile) that had been determined in a local population of healthy subjects (3). Therefore an s-α klotho concentration of 204 pg/mL was chosen as the lowest normal value for our analyses. Table 4 shows the results for the comparisons between patients with s-α klotho below and above 204 pg/mL. As expected the age was significantly higher in the group with lower s-α klotho concentrations. Although eGFR and FGF-23 did not differ between the groups the phosphate clearance was significantly higher in the group with higher s-α klotho. Moreover, in accordance with the positive correlation between bone-specific alkaline phosphatase (b-ALP) and s-α klotho shown above we also found significantly higher values of b-ALP in the group with normal s-α klotho concentrations.

We analyzed s-α klotho concentrations during an 8-week intervention period with cholecalciferol compared to placebo (Figure 2). Supplementary Tables 1 and 2 show baseline values and eight-week changes for P-s-α klotho, S-phosphate clearance, and P-b-ALP. Baseline values and eight-week changes for other calcium-phosphate parameters are given in reference (15). The intervention group showed a median 24% increase of s-α klotho under cholecalciferol treatment that was not significantly different from a 10% increase in the placebo group. Median change of s-α klotho concentration in the placebo group was 32 pg/mL (IQR, 6 – 66 pg/mL) while in the cholecalciferol group klotho changed in median 66 pg/mL (IQR, 26 – 86 pg/mL).

### Discussion

Soluble-α klotho has received much attention in recent nephrological research activity. When we compared s-α klotho according to GFR between the CKD stages we did not find a significant difference between CKD 1/2, CKD 3, and CKD 4/5. Previous studies gave discrepant results concerning s-α klotho and CKD stages. A significant reduction of s-α klotho has been shown in hemodialysis patients (19, 20). Pavik et al showed a progressive decrease of s-α klotho with increasing CKD stage (21). However, in a recent large cohort study Seiler et al did not find a significant difference of s-α klotho concentrations between CKD stage 2, 3a, 3b and 4 patients, a finding that is in accordance with our present results (14). We analyzed the biologically active intact FGF-23 in our study and performed correlation analyses separately in patients above and below the median FGF-23 concentration. It has been suggested that FGF-23 can activate klotho gene transcription via mitogen-activated protein kinase (17, 18).
Patients showing lower than median FGF-23 concentrations had a positive association between s-α/klotho and eGFR, as well as the negative association with age, a finding that has repeatedly been described in healthy subjects (3, 16). These patients showed FGF-23 concentrations around the reference interval of a healthy local control population described by Pedersen et al (2) (fifth – 97.5th percentile; 18 – 55 pg/mL) (3). This means that the aforementioned correlations were present in subjects with FGF-23 close to normal. In those subjects the s-α/klotho values declined linear with reduced eGFR. On the other hand, in the high FGF-23 group these correlations were missing. Half of the patients in the high FGF-23 group showed s-α/klotho concentrations higher than expected according to eGFR levels. In these patients pathologically elevated FGF-23 concentrations might activate klotho gene transcription as proposed (17, 18). Taken the above results together we suggest that the relation between eGFR and s-α/klotho found in a certain CKD population depends on the degree by which FGF-23 concentration is raised in that population. If FGF-23 is directly responsible for that observation or is just an indicator for a certain uremic state cannot be concluded from our study.

We also compared calcium-phosphate metabolism between patients with reduced (< 204 pg/mL) and normal (> 204 pg/mL) s-α/klotho concentrations. While eGFR and FGF-23 were not different between the groups the phosphate clearance was significantly higher in the group with

### Table 3. Baseline characteristics. Data are median and interquartile range. Groups were compared using Mann Whitney test.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>All patients (n = 24)</th>
<th>Patients with FGF23 &lt; 73 pg/mL (n = 12)</th>
<th>Patients with FGF23 &gt; 73 pg/mL (n = 12)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-s-α/klotho, pg/mL</td>
<td>236</td>
<td>236</td>
<td>236</td>
<td>0.665</td>
</tr>
<tr>
<td>eGFR, ml/min/1.73 m²</td>
<td>(193 – 291)</td>
<td>(191 – 287)</td>
<td>(194 – 319)</td>
<td>0.015</td>
</tr>
<tr>
<td>S-Phosphate, mmol/liter</td>
<td>1.05</td>
<td>1.01</td>
<td>1.17</td>
<td>0.011</td>
</tr>
<tr>
<td>S-Phosphate clearance, ml/min/1.73 m²</td>
<td>(0.98 – 1.25)</td>
<td>(0.75 – 1.09)</td>
<td>(1.04 – 1.48)</td>
<td>0.032</td>
</tr>
<tr>
<td>S-FGF-23, pg/mL</td>
<td>(8 – 20)</td>
<td>(11 – 27)</td>
<td>(6 – 15)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>P-1,25-diOHD, pmol/liter</td>
<td>67</td>
<td>92</td>
<td>56</td>
<td>0.061</td>
</tr>
<tr>
<td>Age, years</td>
<td>68</td>
<td>61</td>
<td>70</td>
<td>0.132</td>
</tr>
<tr>
<td>P-b-ALP, U/liter</td>
<td>35.0</td>
<td>36.9</td>
<td>31.1</td>
<td>0.112</td>
</tr>
<tr>
<td>P-PTH, pmol/liter</td>
<td>10.9</td>
<td>9.6</td>
<td>12.4</td>
<td>0.312</td>
</tr>
<tr>
<td>S-Calcium ion, mmol/liter</td>
<td>1.22</td>
<td>1.22</td>
<td>1.22</td>
<td>0.706</td>
</tr>
<tr>
<td>P-25-OHD, nmol/liter</td>
<td>33.0</td>
<td>28.4</td>
<td>36.4</td>
<td>0.436</td>
</tr>
<tr>
<td>(A) FGF-23 &lt; 73 pg/mL</td>
<td>(1.18 – 1.24)</td>
<td>(1.19 – 1.25)</td>
<td>(1.16 – 1.23)</td>
<td></td>
</tr>
<tr>
<td>(B) FGF-23 &gt; 73 pg/mL</td>
<td>(19.5 – 49.6)</td>
<td>(17.3 – 50.3)</td>
<td>(26.8 – 46.3)</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 1.** Association of soluble-alpha klotho and eGFR according to FGF-23 concentrations (A) below and (B) above the median (73 pg/mL). In the group with lower FGF-23 values (A) analysis showed a significant correlation between s-α/klotho and eGFR. Regression line and 95% confidence interval (CI) are shown. (B) S-α/klotho concentrations in the group with FGF-23 above median were normally distributed after logarithmical transformation. There was no significant association to eGFR.
higher s-α klotho. This underlines the well known phosphaturic function of klotho protein (5, 22). We also found a significant difference of b-ALP concentrations between patients with s-α klotho below and above 204 pg/ml; with higher b-ALP in subjects with higher s-α klotho. This finding is in accordance with a positive correlation between b-ALP and s-α klotho that we found in our CKD patients. Bone-specific alkaline phosphatase is released from osteoblasts and reflects synthetic activity of bone-forming cells (23). In osteoblasts of klotho-deficient mice a significantly reduced alkaline phosphatase activity has been described (24).

Furthermore, we investigated the effect of 8-weeks of cholecalciferol treatment on s-α klotho concentrations compared with placebo. It had been published that in a mice CKD model with partial renal ablation and hyperphosphatemia and also in a bovine cell system an increase of klotho concentration was reached by calcitriol and paricalcitol treatment (12, 25). The 25-OHD concentrations reached by our cholecalciferol treatment were in median 163.3 nmol/L compared to median 24.3 nmol/L in the placebo group at the end of the intervention period. Nevertheless, although the increase of s-α klotho in the cholecalciferol treatment group seemed to be higher it did not reach statistical significance compared to the placebo group. First, the patients in our study were not hyperphosphatemic. Second, effects of cholecalciferol and active vitamin D treatment might differ even if a significant rise in 1,25-diOHD is achieved by cholecalciferol. The rise in serum calcium that we observed during cholecalciferol treatment (15) might inhibit klotho expression as indicated by several publications (10, 25). Also, the rise in 25-OHD that accompanies the rise in 1,25-diOHD during cholecalciferol treatment might modify 1,25-diOHD actions.

Some limitations of the study shall be discussed. The size of the study population is small. Also, although the design of the study aimed at prevention of bias it cannot be excluded that selection bias occurred in our study. The study is therefore suitable for the generation of hypotheses. Especially concerning the effect of a cholecalciferol intervention on s-α klotho a larger sized study is desirable.

### Table 4. Baseline characteristics. Data are median and interquartile range. Groups were compared using Mann Whitney test.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patients with P-s-α Klotho &lt; 204 pg/mL (n = 9)</th>
<th>Patients with P-s-α Klotho &gt; 204 pg/mL (n = 15)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-s-α Klotho, pg/mL</td>
<td>189 (175 – 200)</td>
<td>281 (249 – 310)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>eGFR, ml/min/1.73 m²</td>
<td>25 (20 – 44)</td>
<td>37 (22 – 60)</td>
<td>0.245</td>
</tr>
<tr>
<td>S-Phosphate, mmol/liter</td>
<td>1.09 (1.04 – 1.39)</td>
<td>1.05 (0.84 – 1.25)</td>
<td>0.221</td>
</tr>
<tr>
<td>S-Phosphate clearance, ml/min/1.73 m²</td>
<td>8 (5 – 12)</td>
<td>16 (10 – 23)</td>
<td>0.020</td>
</tr>
<tr>
<td>S-FGF-23, pg/mL</td>
<td>76 (54 – 144)</td>
<td>54 (29 – 268)</td>
<td>0.612</td>
</tr>
<tr>
<td>P-1,25-diOHD, pmol/liter</td>
<td>66 (45 – 73)</td>
<td>74 (41 – 95)</td>
<td>0.371</td>
</tr>
<tr>
<td>Age, years</td>
<td>69 (68 – 79)</td>
<td>62 (55 – 72)</td>
<td>0.034</td>
</tr>
<tr>
<td>P-b-ALP, U/liter</td>
<td>30.1 (26.7 – 32.3)</td>
<td>37.7 (34.1 – 48.8)</td>
<td>0.012</td>
</tr>
<tr>
<td>P-PTH, pmol/liter</td>
<td>11.0 (7.0 – 20.5)</td>
<td>10.4 (4.8 – 17.7)</td>
<td>0.592</td>
</tr>
<tr>
<td>S-Calcium ion, mmol/liter</td>
<td>1.22 (1.19 – 1.24)</td>
<td>1.22 (1.18 – 1.24)</td>
<td>0.928</td>
</tr>
<tr>
<td>P-25-OHD, nmol/liter</td>
<td>29.2 (13.7 – 51.0)</td>
<td>33.2 (20.1 – 50.0)</td>
<td>0.633</td>
</tr>
</tbody>
</table>
On the other hand, according to current understanding a rise of s-α klotho would be expected after a rise of 1,25-diOHD (12, 25). It is therefore of importance that in our study with cholecalciferol no significant increase of s-α klotho was observed compared to placebo although the patients showed a pronounced and highly significant increase of 1,25-diOHD. Possible reasons for this were discussed above.

Taken together, our study showed that the association between s-α klotho and eGFR is not uniform at differing FGF-23 concentrations. Furthermore, we found a significantly reduced phosphate clearance and bone-specific alkaline phosphatase activity in patients with low s-α klotho. An intervention with cholecalciferol significantly increased 1,25-diOHD but the increase of plasma s-α klotho in the cholecalciferol group did not reach significance compared to placebo.

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The authors declare that they have no competing interests.

**Authors’ Contributions:** AS, LMR, MT designed research; AS, LP, HJR, YL, SX, BH, LMR, and MT conducted research; AS, HJR, YL, SX, BH, LP, LMR, and MT wrote the paper; AS had primary responsibility for final content. All authors read, critically revised and approved the final manuscript.

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