Laboratory aspects of circulating α-Klotho

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ABSTRACT

Background. α-Klotho is a protein mainly produced in the kidney. Its circulating form has been suggested to link renal damage and distant tissue pathology. As three assays to measure α-Klotho became commercially available, we performed an evaluation of these commercially available Klotho assays.

Methods. We studied within-run variation, between-run variation, matrix effects, linearity, and recovery of added

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recombinant human Klotho in the α-Klotho assays of IBL (IBL International GMBH, Hamburg, Germany), Cusabio (Cusabi Biotech, Wuhan, China) and USCN (USCN life Science, Inc., Wuhan, China) using both serum and ethylenediaminetetraacetic acid plasma.

**Results.** Within run variation was 4, 13 and 32% for the IBL, Cusabio and USCN assay, respectively. Agreement between serum and EDTA plasma was good in the IBL assay, but poor in the USCN and Cusabio assays however improved after modifications in the Cusabio assay. Standardization and agreement between assays was poor.

**Conclusions.** The commercially available methods for the measurement of α-Klotho differ in quality. Some of the manufacturers should improve their assays in order to produce accurate results so that reliable conclusions can be drawn from studies in which these assays are used.

### INTRODUCTION

Apart from being an obligate cofactor for classical fibroblast growth factor 23 (FGF23) signal transduction via the FGF-receptor type 1 [1], the α-Klotho protein shows FGF23-independent effects, such as anti-oxidant and vasculoprotective effects [2]. In the kidney, α-Klotho is involved in the regulation of both calcium [3] and phosphate handling [4], while in the vasculature, it inhibits calcification [5] and improves endothelial integrity [6]. There are two forms of the α-Klotho protein, membrane-bound Klotho and secreted Klotho. The extracellular domain of the membrane-bound Klotho is shed and subsequently released in the circulation. As α-Klotho is mainly produced in the kidney, its circulating form has been suggested as a link between renal damage and distant tissue pathology. Importantly, the circulating α-Klotho proteins can be either the shed-product of the ectodomain of the membrane bound form, or a Klotho protein that originates from alternate splicing of the Klotho gene.

Recently, three immunoassays to measure α-Klotho became commercially available. The quality of commercially available immunoassays is not guaranteed (by example shown for FGF23 assays and vitamin D assays [7, 8]), and therefore, assays should be evaluated carefully. We thus performed an evaluation of commercially available Klotho assays.

### SUBJECTS AND METHODS

We evaluated the α-Klotho assays of IBL (α-KlothoIBL, IBL International GMBH, Hamburg, Germany), Cusabio (α-KlothoCusabio, Cusabi Biotech, Wuhan, China) and USCN (α-KlothoUSCN, USCN life Science Inc, Wuhan, China). Both Cusabio and USCN do not provide information on the epitopes against which their antibodies are directed in their respective Klotho assays. The IBL assay makes use of the antibodies described by Yamazaki et al. [9]. According to Yamazaki et al. both antibodies specifically recognize a tertiary protein structure of an extracellular domain of αKl. As a consequence, with the IBL assay both forms of circulating Klotho might be measured.

We studied within-run variation, between-run variation, matrix effects, linearity and recovery of added recombinant human Klotho (rhKlotho, Sigma-Aldrich, St Louis, MO, USA) in the three commercially available α-Klotho assays. Analyses were performed according to the instructions of the manufacturers (Table 1). We used leftover coupled serum and EDTA plasma samples of healthy individuals and patients with chronic kidney disease. Within-run variation (within run %CVs) was calculated from duplicate variation (n = 36–40) using the formula:

\[
CV\% = \sqrt{\frac{\sum (a-b)^2}{2N}} \left(\frac{N}{\sum X} \right),
\]

whereby \(\sum\) is sum, \(a\) and \(b\) are the duplicate Klotho concentrations, \(N\) is the total number of duplicates and \(X\) is the mean [Klotho] of \(a\) and \(b\). Between run variation was studied using two samples that were measured every run and calculated as \(CV\% = (\text{standard deviation/mean [Klotho]}) \times 100\%\). Matrix effects were studied by comparing Klotho measured in EDTA plasma and serum, calculated with Pearson’s correlation. Recovery of rhKlotho was measured by adding rhKlotho to two serum samples. The amount of the added rhKlotho was chosen based on the concentration range of the respective assay, 20 000 and 40 000 pg in the α-KlothoCusabio and 200 and 400 pg in the α-KlothoIBL. Recovery was calculated using the following formula:

\[
\text{Recovery}\% = \left(\frac{[Klotho_{spiked\ sample} - [Klotho]_{sample\ without\ addition}}{[Klotho]_{spiked\ sample}}\right) \times 100\%.
\]

### Table 1. Instructions of the manufacturers of the three klotho assays used in the evaluation

<table>
<thead>
<tr>
<th></th>
<th>α-KlothoIBL</th>
<th>α-KlothoCusabio</th>
<th>α-KlothoUSCN</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Matrix</strong></td>
<td>Serum, plasma</td>
<td>Serum, plasma (citrate, EDTA, heparin)</td>
<td>Serum, plasma (EDTA, heparin)</td>
</tr>
<tr>
<td><strong>Sample dilution</strong></td>
<td>2–4</td>
<td>200</td>
<td>–</td>
</tr>
<tr>
<td><strong>Concentration range of standard curve (pg/mL)</strong></td>
<td>94–6000</td>
<td>7.8–500</td>
<td>156–10 000</td>
</tr>
</tbody>
</table>

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Linearity was measured by performing 2-, 4- and 8-time dilutions and calculating the % of expected values. All analyses were performed using MedCalc 9 (MedCalc Software, Mariakerke, Belgium) and Microsoft Excel 2007.

**RESULTS**

Within- and between-run variation is shown in Table 2. Within-run variation of the \(\alpha\)-Klotho\textsubscript{IBL} consists of only 18 observations, as the other 18 samples measured were either above or below the range of the standard curve. Impressive differences between EDTA plasma and serum were observed. As we judged the within run variation of the \(\alpha\)-Klotho\textsubscript{USCN} unacceptably high (32%), we did not analyse the other parameters using this assay.

Using the \(\alpha\)-Klotho\textsubscript{Cusabio}, about half of the samples measured read above the standard curve. Even more samples were above the standard curve measuring the same samples in a second run. Within-run variation was 13%. The agreement between serum and plasma was poor \((R^2 = 0.65; n = 11)\). Linearity of the \(\alpha\)-Klotho\textsubscript{Cusabio} was moderate, as a 2-, 4- and 8-time dilutions led to values that are 44–80% of expected. Addition of 20 000 and 40 000 pg rhKlotho to serum samples with a basal concentration of 9510 and 8240 pg/mL as measured in the \(\alpha\)-Klotho\textsubscript{Cusabio} was not detected by the \(\alpha\)-Klotho\textsubscript{Cusabio}. The within- and between-run variation of the \(\alpha\)-Klotho\textsubscript{IBL} was <5 and <8%, respectively. Measurements in serum and EDTA plasma were in agreement \((R^2 = 0.99; n = 20)\). Linearity was tested by dilution in two samples with a concentration of 1929 and 2864 pg/mL. In one sample, 2-, 4- and 8-time dilutions gave results as expected (100–117% of expected values). However, the 4- and 8-time dilutions in the other sample led to results that were higher than expected (129 and 142%). Undiluted measurements were not possible. Addition of 400 and 200 pg of rhKlotho to serum samples with a basal concentration of 571 and 338 pg/mL as measured with the \(\alpha\)-Klotho\textsubscript{IBL} led to a recovery of 138 and 160%.

**DISCUSSION**

Standardization of the Klotho assays should improve. All three assays have the same units (pg/mL), but differ in the concentration range of their standard curves. In addition to the standardization problem that might lead to different absolute values yet a high correlation between assays, the question arises what exactly is measured by these assays. Almost no information is available on the antibodies used in the various assays; thereby, it is not certain which forms of Klotho are detected. Moreover, cross reactivity with other analytes cannot be excluded. A comparison of 20 samples measured using both \(\alpha\)-Klotho\textsubscript{IBL} and \(\alpha\)-Klotho\textsubscript{Cusabio} led to a correlation between these assays of \(R^2 = 0.003\), neither serum nor EDTA plasma correlated between assays.

Recently, Devaraj et al. \[10\] published an evaluation of the \(\alpha\)-Klotho\textsubscript{Cusabio}, using three modifications to improve the performance of this Klotho assay. One of the modifications is a 2000- instead of 200-fold dilution of the samples. Although this seems to improve the assay, with probably less samples reading above the standard curve, such a high dilution step is a design mistake of the manufacturer as it either leads to imprecision or to waste of sample buffer. In the study of Deveraj et al. only serum was tested, without comparison with EDTA plasma. We, therefore, repeated part of our measurements with these modifications. The intra-assay variation of the \(\alpha\)-Klotho\textsubscript{Cusabio} did not improve in our hands (15%, \(n = 24\)). However, the agreement between EDTA plasma and serum significantly improved by the additional dilution, \(R^2 = 0.92\) (\(n = 15\)).

Despite the above-mentioned important limitations of the current assays, several publications report on results of circulating forms of Klotho in clinical cohorts, as summarized in Table 3. Some of the inconsistencies between these cohorts might be due to the limitations of the assays used.

In conclusion, the commercially available methods for the measurement of \(\alpha\)-Klotho differ in quality. Some of the manufacturers should improve their assays in order to produce accurate results so that reliable conclusions can be drawn from studies in which these assays are used.

### Table 2. Coefficients of variation (CV) of the three commercially available Klotho assays

<table>
<thead>
<tr>
<th></th>
<th>(\alpha)-Klotho\textsubscript{IBL}</th>
<th>(\alpha)-Klotho\textsubscript{Cusabio}</th>
<th>(\alpha)-Klotho\textsubscript{USCN}</th>
</tr>
</thead>
<tbody>
<tr>
<td>(N)</td>
<td>Conc (pg/mL)</td>
<td>CV%</td>
<td>Conc (pg/mL)</td>
</tr>
<tr>
<td>Within-run CV%</td>
<td>40</td>
<td>787</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Between-run CV%</td>
<td>24</td>
<td>796</td>
<td>7.3</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>188</td>
<td>5.8</td>
</tr>
</tbody>
</table>

ND, not determined.

*a*In order not to have many samples above the standard curve, our protocol changed over the runs, trying to improve the assay. Not more than two runs were determined using the same protocol, therefore no reliable between run CV can be calculated.
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Angiotensin-converting enzyme 2 activity in patients with chronic kidney disease

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Keywords: angiotensin-converting enzyme 2, cardiovascular disease, chronic dialysis, chronic kidney disease, end-stage kidney disease

ABSTRACT

Background. Angiotensin-converting enzyme 2 (ACE2) is a novel regulator of the renin–angiotensin system that counteracts the adverse effects of angiotensin II. In heart failure patients, elevated plasma ACE2 activity predicted adverse events and greater myocardial dysfunction. We aimed to describe plasma ACE2 activity and its clinical associations in patients with kidney disease.

Methods. Patients recruited from a single centre comprised of chronic kidney disease Stage III/IV (CKD), haemodialysis patients and kidney transplant recipients (KTRs). Plasma ACE2 enzyme activity was measured using a fluorescent substrate assay in plasma, collected at baseline and stored at −80°C. Linear regression was performed in both males and females separately to determine the covariates associated with log-transformed ACE2.

Results. The median (interquartile range) plasma ACE2 activity in pmol/mL/min was 15.9 (8.4–26.1) in CKD (n = 59),