Soluble Klotho protein as a novel serum biomarker in patients with acromegaly

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Acromegaly is a chronic disease caused by overproduction of growth hormone, in most cases due to excessive secretion from a pituitary adenoma [1, 2]. The incidence of acromegaly is approximately 5 cases per million per year, and the prevalence is estimated to be about 60 cases per million [3]. Growth hormone (GH) induces the synthesis of insulin-like growth factor-1 (IGF-1), which leads to severe metabolic complications resulting in significant morbidity and mortality [2, 4, 5]. It has been suggested that the mortality associated with acromegaly is at least two-fold higher compared to the general population, especially due to the higher prevalence of hypertension, diabetes, cardiovascular complications, and sleep apnoea, and it may be reduced after sufficient control of GH and IGF-1 levels [4, 6, 7]. Approximately 70% of acromegalic subjects have macroadenomas, and the majority of them will not be cured by surgery alone and will require adjuvant medical therapy or radiotherapy [2, 5, 8].

Klotho protein (α-Klotho), named after one of the three Fates in Greek mythology - the goddess who spins the thread of life, was first investigated in 1997 by Kuro-o et al., who found that mice with a defective Klotho gene had phenotypes of accelerated aging including atherosclerosis, osteoporosis, ectopic calcification, skin atrophy, and pulmonary emphysema, conditions resembling human premature aging syndrome [9–12]. It is well known that overexpression of Klotho leads to aging suppression and lifespan extension [9, 11, 13, 14].

There are studies which indicate that soluble Klotho (sKlotho) levels are associated with GH and IGF-1 production and thus are markedly increased in patients with acromegaly and return to normal after resection of GH-producing pituitary adenoma at least as quickly as IGF-1 [14, 15]. Moreover, acromegaly is found to be the only acquired disease characterised by excessively elevated serum soluble Klotho (sKlotho) concentrations [16].

In this paper we focus on sKlotho used in monitoring patients with acromegaly, based on the three studies conducted in the Zurich cohort [9, 15, 16].

In acromegaly, IGF-1 appears to be more closely related to disease activity and better correlates with morbidity than GH levels do [9]. The criteria for a biochemical cure of acromegaly include GH levels < 0.4 μg/l after oral glucose tolerance test (OGTT) and IGF-1 concentrations within the normal age range and gender-adjusted range [2, 7, 17]. Determinations of GH and IGF-1 are known to have biological and technical limitations [18, 19]. Similarly, results in oral glucose tolerance tests may be false negative, and elevated levels of IGF-1 may be connected with nor-
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In humans, variants of Klotho are associated with aging, including atherosclerosis, endothelial dysfunction, ectopic calcification (e.g. vascular calcifications), low bone mineral density, emphysema, sarcopenia, skin atrophy, and impaired cognition [11, 16, 30]. Moreover, several single nucleotide polymorphisms (SNPs) in the human Klotho gene are linked not only with lifespan but also osteoporosis, stroke, and coronary artery disease, and thus the Klotho gene takes part in the regulation of human aging and age-related diseases [14, 31].

Swiss authors suggest that determining sKlotho in the serum may be a useful supplementary tool to IGF-1 in monitoring somatotropinomas [9]. In the last 2 years Swiss researchers performed prospective analyses in patients with acromegaly before and after transsphenoidal surgery [9, 15, 16].

In 2012 Sze et al. [15] first reported that serum sKlotho concentrations were excessively high in acromegalic subjects. This study was conducted in 24 patients who were referred to the University Hospital in Zurich between 2006 and 2009 with newly diagnosed disease (9 women and 15 men, aged 28–76 years), before and after 22–124 days following transsphenoidal surgery. Preoperatively, the patients with acromegaly had elevated sKlotho concentrations (4.2 ±0.7 ng/ml) compared to the control group of healthy volunteers (0.6 ±0.2 ng/ml) and the results were positively correlated with GH (r = 0.64, p = 0.0007) and IGF-1 (r = 0.57, p = 0.003) levels and tumour size (r = 0.5, p = 0.01) as well. After the resection of somatotropinomas the levels of GH, IGF-1, and sKlotho decreased, and sKlotho concentrations returned to a level comparable to that of normal control subjects (0.7 ±0.1 ng/ml). The authors observed that, similarly to IGF-1, sKlotho correlated with GH (r = 0.66, p < 0.001). The results of the study by Sze et al. [15] are shown in Table I.

The second study was carried out by Neidert et al. [16]. They compared a group of patients with GH-secreting pituitary adenomas (14 patients with active acromegaly: 8 females and 6 males) to subjects with clinically non-functioning pituitary adenomas (22 participants: 13 females and 9 males). Serum sKlotho concentrations were not only significantly higher in the patients with acromegaly (median 4217 pg/ml vs. 532 pg/ml) but also rapidly decreased to normal levels following successful surgical removal of the GH-secreting adenoma after 2–6 days and 2–3 months following the operation (median 4217 pg/ml vs. 646 pg/ml and 902 pg/ml). This study showed for the first time that the preoperative sKlotho excess is specific only to patients with GH-producing adenomas. Moreover, immunohistochemical stainings have been performed in adenoma tissue of acromegalic and controls. Klotho expression seemed to be equal or slightly increased in the control

| Parameter | Before surgery (mean ± SEM) | After surgery (mean ± SEM) | Value of p |
|-----------|-----------------------------|-----------------------------|------------|
| GH [μg/l] | 26.3 ±5.2                   | 2.6 ±0.6                    | < 0.0001   |
| IGF-1 [μg/l] | 588 ±35                   | 193 ±12                     | < 0.0001   |
| sKlotho [ng/ml] | 4.2 ±0.7                   | 0.7 ±0.1                    | < 0.0001   |
There are some uncertainties concerning the usefulness of the determination of sKlotho in serum as a biomarker in patients with acromegaly.

Although Klotho was discovered more than a decade ago, huge differences remain in estimating its content in human blood [14, 25, 26]. On the one hand, Hu et al. [26] reported Klotho ranging from 10 nM to 50 nM, and on the other hand, Maeda et al. [25] found that the concentration of sKlotho to be approximately 10 pM. In the Zurich area, the median value of healthy adults was found to be around 0.6 ng/ml [14].

Both ELISA and Western blot assays were used to determine sKlotho protein in human serum [15, 25, 27, 28, 32]. At present, the ELISA system seems to be the most suitable method to measure circulating sKlotho levels in the blood, but the commercially available assays (IBL, Cusabio, USCN) differ in quality. Standardisation and convergence of their results are poor. Only IBL assay measurements in the serum and EDTA plasma produce close results [33]. What is more, the IBL method, in which antibodies described by Yamazaki et al. [34], enables the determination of both forms of circulating sKlotho: the shed product of the ectodomain of the membrane-bound form and Klotho protein that originates from alternate splicing of the Klotho gene [29, 33]. Neither Cusabio nor USCN methods provide information on the epitopes against which their antibodies are directed, and therefore they cannot precisely determine which forms of Klotho are detected [33]. Pedersen et al. [23], who measured serum sKlotho using a time-resolved fluorescence immunoassay (TRF) based on the antibodies provided in the ELISA kit from Cusabio and compared this method to the IBL assay, suggest that each form of sKlotho should be considered as an independent factor whose role as a biomarker must be evaluated separately. A discrepancy of reference ranges for sKlotho was also found by other authors using the same assay [14].

### Table II. GH, IGF-1, and sKlotho concentrations in the study by Neidert et al. [16]

| Parameter | Acromegaly group: | Control group |
|-----------|-------------------|---------------|
|           | Before surgery (median) | 2–6 days after surgery (median) | Value of p | 2–3 months after surgery (median) | Value of p |
| GH [ng/ml] | 10 (7–43) | 1.9 (0.6–2.5) | < 0.001 | – | – |
| IGF-1 [ng/ml] | 483 (367–640) | 182 (144–229) | < 0.001 | – | – |
| sKlotho [pg/ml] | 4217 (1813–6624) | 646 (550–1303) | < 0.001 | 902 (498–1341) | < 0.001 |

### Table III. IGF-1 and sKlotho concentrations in the study by Kohler et al. [9]

| Parameter | Before surgery (mean ± SEM) | After surgery (mean ± SEM) |
|-----------|-----------------------------|-----------------------------|
| IGF-1 [ng/ml] | 579 ±32 | 198 ±10 |
| sKlotho [pg/ml] | 4113 ±415 | 779 ±63 |
It should be noted that Sze et al. [15] presented the results of sKlotho in ng/ml but other authors and IBL kit instructions used pg/ml. Similarly, the levels of GH and IGF-1 were presented in two different units: μg/l and ng/ml [9, 16, 33, 34]. Obviously, it would be easier to compare sKlotho concentrations (and data shown in Tables I–III) if all the authors used the same units. Taking copyright into consideration, we have to present the results in the units used by the researchers, and we are not allowed to convert them. To compare the results of all authors, it should be mentioned that 1 ng/ml corresponds to 1000 pg/ml and 1 μg/l corresponds to 1 ng/ml.

It is noteworthy that the sandwich ELISA, first described by Yamazaki et al. [34] in measuring circulating sKlotho, was used not only by the Zurich researchers but also by other authors [29, 35, 36].

Many factors such as gender, age, and BMI should be considered in the analysis of sKlotho concentration. In healthy subjects, sKlotho declines with age, but the effect of gender remains inconclusive [11, 23, 27, 34]. Previous studies found no influence of BMI on sKlotho levels [11], but a small study indicated that both anorexia nervosa and morbid obesity decrease sKlotho concentrations [37]. Furthermore, its serum levels are expected to be considered as a biomarker for kidney function [34].

Although GH is the strongest predictor for both sKlotho and IGF-1, Sze et al. [38] recently found that serum sKlotho concentrations are higher and IGF-1 levels are lower in women than in men. Thus, gender needs to be considered if sKlotho is to be used for monitoring acromegalic patients. In untreated acromegals, age had no impact on sKlotho concentrations, which was explained by the autonomous GH secretion. As for BMI, an effect on sKlotho cannot be statistically excluded [38].

Soluble Klotho was detected not only in the serum, but also in the urine and the cerebrospinal fluid (CSF), where its levels are particularly high [14, 25]. In their comprehensive review on growth hormone and Klotho, Schmid et al. [14] reported that some patients with newly diagnosed acromegaly had increased urinary sKlotho, and it could have a high positive predictive value for the diagnosis of the disease. However, before urinary sKlotho as a supplementary tool to serum measurements for estimating GH excess is used, the sensitivity and standardisation of the analysis should be improved.

At present, it is not clear which mechanism leads to the excess in sKlotho concentration in acromegaly. On the one hand, it is well known that FGF-23, phosphate, and calcitriol levels are increased in acromegalic patients and that mKlotho plays a role as a coreceptor for FGF-23 [9, 16, 39]. In addition, renal FGF-23 resistance is typical for acromegaly [15]. On the other hand, based on immunohistochemical analysis, the rise in serum sKlotho is not due to higher pituitary transmembrane Klotho expression, but to increased pituitary GH secretion [14, 16]. In acromegalic patients, sKlotho levels may be elevated due to GH-mediated enzymatic shedding of mKlotho probably mainly in the kidneys, which would also explain the FGF-23 resistance [14, 16, 40, 41].

Preliminary findings of Swiss cohort studies indicate that serum sKlotho levels are markedly increased in relation to GH excess and decline to normal after surgery in acromegalic patients. Data suggest that soluble Klotho responds to GH excess to an extent comparable to IGF-1 and may reflect the disease activity in acromegaly. It can be postulated that the determination of serum sKlotho could be a novel, useful biomarker in the long-term follow-up of GH-producing pituitary adenomas. However, to confirm and generalise these results, further studies performed on a larger population of individuals with acromegaly are required. The mechanisms leading to excessive sKlotho concentrations should be clarified as well. Both the effect of other factors such as gender, age, and BMI and improvement of the standardisation of Klotho assays should be considered in the future. Moreover, additional studies are needed to determine the usefulness of urinary sKlotho in monitoring acromegaly.

Conflict of interest
The authors declare no conflict of interest.

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