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

Anna M. Dąbrowska, Jerzy S. Tarach

Chair and Department of Endocrinology, Medical University of Lublin, Poland

Submitted: 3 March 2014
Accepted: 28 May 2014

Arch Med Sci 2016; 12, 1: 222–226
DOI: 10.5114/aoms.2014.45050

Corresponding author:
Anna M. Dąbrowska MD, PhD
Chair and Department
of Endocrinology
Medical University of Lublin
8 Jaczewskiego St
20-954 Lublin, Poland
Phone: +48 604 676 091
E-mail: dabrowska.ania.m@
gmail.com

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-

mal GH concentrations and suppression on OGTT [9, 20, 21].

Considering these facts, long-term follow-up of acromegalic patients as well as the diagnosis of late recurrence can be difficult, and an additional specific and sensitive biomarker is needed to diagnose and monitor acromegaly [16, 22].

The *Klotho* gene is abundantly expressed in the kidneys (in the distal tubules), choroid plexus in the brain and placenta, and in many endocrine tissues such as parathyroid cells, adipocytes, endothelial cells, pituitary, testis, ovary, and pancreas [10, 15, 23, 24]. There are at least two forms of Klotho protein, namely membrane-bound Klotho (mKlotho) and secreted soluble Klotho (sKlotho), and each of them has different functions [11–13, 25]. What is more, some studies suggest that an additional shorter 60 kDa form originating from alternative splicing of *Klotho* mRNA exists, as well as the 130 kDa form of sKlotho [23, 26–28]. Whereas it is thought that mKlotho is a co-receptor for fibroblast growth factor-23 (FGF-23) – a bone derived phosphaturic hormone that inhibits renal phosphate reabsorption and calcitriol production, sKlotho works as a hormone and is involved in the regulation of nitric oxide production in the endothelium, calcium homeostasis in the kidney, ion channels on the cell surface, and attenuation of intracellular insulin/IGF-1 signalling [9, 14, 16, 25]. Soluble Klotho also possibly acts as an enzyme that modifies glycans of cell surface glycoproteins [14, 26]. It is suggested that sKlotho comes from either a distinct transcript or from secretase-catalysed ecto-domain (by α- and β-secretases) shedding of mKlotho [9, 26, 29].

In humans, variants of Klotho are associated with aging, including atherosclerosis, endothelial dysfunction, ectopic calcification (e.g. vascular calcifications), low bone mineral density, emphysema, sarcopaenia, 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

Table I. GH, IGF-1 and sKlotho concentrations in acromegalic patients before and after transsphenoidal surgery in the study by Sze *et al.* [15]

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

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

	Before surgery (median)	2–6 days after surgery (median)	Value of *p*	2–3 months after surgery (median)	Value of *p*
Acromegaly group:					
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
Control group					
GH [ng/ml]	0.3 (0.1–0.7)	–	–	–	–
IGF-1 [ng/ml]	86 (53–136)	–	Not significant	–	–
sKlotho [pg/ml]	532 (400–678)	404 (320–635)	Not significant	524 (359–621)	Not significant

group compared to the subjects with GH-positive adenomas, and immunofluorescence analysis revealed that Klotho was independently expressed from GH-positive cells, suggesting that elevated sKlotho is due to systemic actions of GH rather than local sKlotho expression by the adenoma.

The results of the study by Neidert *et al.* [16] are presented in Table II.

The third study was performed by Kohler *et al.* [9]. They examined 50 patients admitted to the University Hospital in Zurich between 2000 and 2009, with newly diagnosed acromegaly, before and 1–3 months after removing GH-adenomas. The IGF-1 and sKlotho concentrations are shown in Table III**.** Both IGF-1 and sKlotho levels dropped after surgery. Based on reduced IGF-1 and sKlotho levels and GH suppressible to < 1 ng/ml, the researchers noted that more than half of the patients (60%) had no biochemical evidence of residual disease activity after transsphenoidal surgery. Two of the acromegalic patients developed recurrent symptoms of the disease during follow-up, and increased IGF-1 and sKlotho levels were found.

The results of the three Swiss studies discussed above indicate that the main advantage of ordering serum sKlotho tests in patients with acromegaly is the fact that this marker is a good indicator of disease activity because its level is markedly increased in newly diagnosed patients and a significant decrease in its concentration after surgery is noted. Therefore, sKlotho is likely to be a very useful tool, supplementary to IGF-1 and GH, in monitoring acromegalic subjects. However, there are some uncertainties concerning the usefulness of the determination of sKlotho in serum as a biomarker in patients with acromegaly.

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

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.* are used [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].

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 acromegalics, 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.

References

1. Dąbrowska AM, Tarach JS, Kurowska M, Nowakowski A. Thyroid diseases in patients with acromegaly. Arch Med Sci 2014; 10: 837-45.
2. Thanabalasingham G, Grossman AB. Acromegaly: beyond surgery. Indian J Endocrinol Metab 2013; 17: 563-67.
3. Pita-Gutierrez F, Pertega-Diaz S, Pita-Fernandez S, et al. Place of preoperative treatment of acromegaly with somatostatin analog on surgical outcome: a systematic review and meta-analysis. PLoS ONE 2013; 8: e61523.
4. Kauppinen-Mäkelin R, Sane T, Reunanen A, et al. A nationwide survey of mortality in acromegaly. J Clin Endocrinol Metab 2005; 90: 4081-6.
5. Grasso LFS, Pivonello R, Colao A. Investigational therapies for acromegaly. Expert Opin Investig Drugs 2013; 22: 955-63.
6. Suda K, Inoshita N, Iguchi G, et al. Efficacy of combined octreotide and cabergoline treatment in patients with acromegaly: a retrospective clinical study and review of the literature. Endocr J 2013; 60: 507-15.
7. Melmed S, Casanueva FF, Klibanski A, et al. A consensus on the diagnosis and treatment of acromegaly complications. Pituitary 2013; 16: 294-302.
8. Nomikos P, Buchfelder M, Fahlbusch R. The outcome of surgery in 668 patients with acromegaly using current criteria of biochemical 'cure'. Eur J Endocrinol 2005; 152: 379-87.

9. Kohler S, Tschopp O, Sze L, et al. Monitoring for potential residual disease activity by serum insulin-like growth factor 1 and soluble Klotho in patients with acromegaly after pituitary surgery: Is there an impact of the genomic deletion of exon 3 in the growth hormone receptor (d3-GHR) gene on "safe" GH cut-off values? Gen Comp Endocrinol 2013; 188: 282-7.
10. Kuro-o M, Matsumura Y, Aizawa H, et al. Mutation of the mouse klotho gene leads to a syndrome resembling ageing. Nature 1997; 390: 45-51.
11. Semba RD, Cappola AR, Sun K, et al. Plasma Klotho and cardiovascular disease in adults. J Am Geriatr Soc 2011; 59: 1596-601.
12. Matsumura Y, Aizawa H, Shiraki-Iida T, Nagai R, Kuro-o M, Nabeshima Y. Identification of the human klotho gene and its two transcripts encoding membrane and secreted klotho protein. Biochem Biophys Res Commun 1998; 242: 626-30.
13. Dewaraj S, Syed B, Chien A, Jialal I. Validation of an immunoassay for soluble Klotho protein. Decreased levels in diabetes and increased levels in chronic kidney disease. Am J Clin Pathol 2012; 137: 479-85.
14. Schmid C, Neidert MC, Tschopp O, Sze L, Bernays RL. Growth hormone and Klotho. J Endocrinol 2013; 219: R37-57.
15. Sze L, Bernays RL, Zwimpfer C, Wiesli P, Brändle M, Schmid C. Excessively high soluble Klotho in patients with acromegaly. J Intern Med 2012; 272: 93-7.
16. Neidert MC, Sze L, Zwimpfer C, et al. Soluble alpha-Klotho: a novel serum biomarker for the activity of GH-producing pituitary adenomas. Eur J Endocrinol 2013; 168: 575-83.
17. Giustina A, Chanson P, Bronstein MD, et al. A consensus on criteria for cure of acromegaly. J Clin Endocrinol Metab 2010; 95: 3141-8.
18. Bidlingmaier M, Strasburger CJ. Growth hormone assays: current methodologies and their limitations. Pituitary 2007; 10: 115-9.
19. Clemmons DR. Consensus statement on the standardization and evaluation of growth hormone and insulin-like growth factor assays. Clin Chem 2011; 57: 555-9.
20. Giustina A, Barkan A, Casanueva FF, et al. Criteria for cure of acromegaly: a consensus statement. J Clin Endocrinol Metab 2000; 85: 526-9.
21. Subbarayan S, Fleseriu M, Gordon M, et al. Serum IGF-1 in the diagnosis of acromegaly and the profile of patients with elevated IGF-1 but normal glucose-suppressed growth hormone. Endocr Pract 2012; 18: 817-25.
22. Neggers SJ, Biermasz NR, Van der Lely AJ. What is active acromegaly and which parameters do we have? Clin Endocrinol (Oxf) 2012; 76: 609-14.
23. Pedersen L, Pedersen SM, Brasen CL, Rasmussen LM. Soluble serum Klotho levels in healthy subjects. Comparison of two different immunoassays. Clin Biochem 2013; 46: 1079-83.
24. Lim SC, Liu JJ, Subramaniam T, Sum CF. Elevated circulating alpha-Klotho by angiotensyn II receptor blocker losartan is associated with reduction of albuminuria in type 2 diabetic patients. J Renin Angiotensin Aldosterone Syst 2013; DOI: 10.1177/1470320313475905.
25. Maeda R, Imura A, Nebeshima Y. Complex regulation and diverse functions of alpha-Klotho. Contrib Nephrol 2013; 180: 25-46.
26. Hu MC, Shiizaki K, Kuro-o M, Moe OW. Fibroblast growth factor 23 and Klotho: physiology and patophysiology of an endocrine network of mineral metabolism. Annu Rev Physiol 2013; 75: 503-33.
27. Xiao NM, Zhang YM, Zheng Q, Gu J. Klotho is a serum factor related to human aging. Chin Med J (Engl) 2004; 117: 742-7.
28. Kato Y, Arakawa E, Kinoshita S, et al. Establishment of the anti-Klotho monoclonal antibodies and detection of Klotho protein in kidneys. Biochem Biophys Res Commun 2000; 267: 597-602.
29. Navarro-González JF, Donate-Correa J, Muros de Fuentes M, Pérez-Hernández H, Martínez-Sanz R, Mora-Fernández C. Reduced Klotho is associated with the presence and severity of coronary artery disease. Heart 2014; 100: 34-40.
30. Asai O, Nakatani K, Tanaka T, et al. Decreased renal α-Klotho expression in early diabetic nephropathy in humans and mice and its possible role in urinary calcium excretion. Kidney Int 2012; 81: 539-47.
31. Ceppioglu SK, Yurdun T, Canbakan M. Assessment of matrix Gla protein, Klotho gene polymorphism and oxidative stress in chronic kidney disease. Ren Fail 2011; 33: 866-74.
32. Zhang R, Zheng F. PPAR-gamma and aging: one link through Klotho? Kidney Int 2008; 74: 702-4.
33. Heijboer AC, Blankenstein MA, Hoenderop J, de Borst MH, Vervloet MG. Laboratory aspects of circulating alpha-Klotho. Nephrol Dial Transplant 2013; 28: 2283-7.
34. Yamazaki Y, Imura A, Urakawa I, et al. Establishment of sandwich ELISA for soluble alpha-klotho measurement: age-dependent change of soluble alpha-klotho levels in healthy subjects. Biochem Biophys Res Commun 2010; 398: 513-8.
35. Matsubara T, Miyaki A, Akazawa N, et al. Aerobic exercise training increases plasma Klotho levels and reduces arterial stiffness in postmenopausal women. Am J Physiol Heart Circ Physiol 2014; 306: H348-55.
36. Kitagawa M, Sugiyama H, Morinaga H, et al. A decreased level of serum soluble Klotho is an independent biomarker associated with arterial stiffness in patients with chronic kidney disease. PLoS One 2013; 8: e56695.
37. Amitani M, Asakawa A, Amitani H, et al. Plasma klotho levels decrease in both anorexia nervosa and obesity. Nutrition 2013; 29: 1106-9.
38. Sze L, Neidert MC, Bernays RL, et al. Gender dependence of serum soluble Klotho in acromegaly. Clin Endocrinol (Oxf) 2014; 80: 869-73.
39. Ito N, Fukumoto S, Taguchi M, et al. Fibroblast growth factor (FGF)23 in patients with acromegaly. Endocr J 2007; 54: 481-4.
40. Bloch L, Sineshchekova O, Reichenbach D, et al. Klotho is a substarate for alpha-, beta- and gamma-secreatase. FEBS Lett 2009; 583: 3221-4.
41. Saftig P, Reiss K. The 'A Disintegrin and Metalloproteinases: ADAM10 and ADAM 17: novel drug targets with therapeutic potential? Eur J Cell Biol 2011; 90: 527-35.