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Original Research

Soluble alpha klotho—impact of biological variables and reference intervals for adults

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Abstract

Objective: Concentrations of soluble alpha klotho (sαKL) are higher in active acromegaly compared with healthy controls. However, reference intervals based on large population-based samples are lacking, and the impact of many biological variables is unclear.

Design: Cross-sectional study

Methods: We measured sαKL concentrations in samples from an adult population (20-89 years, 435 males, 455 females). Associations with sex, age, body mass index, waist-hip-ratio, estimated glomerular filtration rate (eGFR), IGF-I and IGFBP 3, glucose-, lipid-, calcium- and liver-metabolism, fasting, and estrogen status were analyzed. Reference intervals were calculated using LMS quantile regression with a Box-Cox transformation to normality. We also analyzed sαKL in patients with non-functioning pituitary adenoma (NFPA, $n = 18$) and prolactinoma ($n = 65$).

Results: Across all ages, sαKL concentrations (pg/mL, median [interquartile ranges]) were slightly, but significantly higher in females compared with males (678 (537-859) vs. 651 (537-812), $P = .01$), suggesting an impact of estrogens. SαKL exhibited a weak negative correlation with age, and positive correlations with eGFR and IGF-I ($P < .001$ for both). Correlations to other biological factors including glucose, liver and calcium metabolism and duration of fasting were negligible ($P > .05$ for all). Compared with sαKL, IGF-I more often was correlated significantly to other biological variables. SαKL was not different in patients with NFPA, but slightly higher in patients with prolactinoma ($P < .05$).

Conclusion: Our findings suggest sαKL is a stable GH-sensitive biomarker that may be less impacted by biological variables compared with IGF-I and IGFBP 3. Our reference intervals will facilitate the potential use of sαKL in GH-related diseases.

Keywords: biomarker, acromegaly, growth hormone, population-based study

Significance

Soluble alpha klotho (sαKL) concentrations correlated with age and estimated glomerular filtration rate, and might be impacted by estrogen status. However, the impact of other biological variables is negligible, and weaker than that observed for the classical GH-dependent biomarkers IGF-I and IGFBP 3. Our detailed reference intervals for sαKL from a large population-based sample might facilitate its use as a specific GH-sensitive biomarker in GH related diseases.

Introduction

Klotho is a protein first described in mice, where defects in *klotho* gene expression are associated with short lifespan, pituitary abnormalities, growth retardation, and altered calcium and glucose metabolism.[1] *Klotho* is abundantly expressed in the kidney, but expression also occurs in the pituitary.[1,2] The *Klotho* gene might be a tumor suppressor gene,[3-6] and encodes a 130-kDa transmembrane protein called alpha klotho. Its extracellular domain contains 2 internal repeats (KL1 and KL2). Three isoforms of alpha klotho exist: the full-length transmembrane form (mKL), a soluble form consisting of cleaved parts of the extracellular domain (KL1 attached to KL2 [KL1-KL2] or KL1 alone), and a secreted form resulting from alternative splicing and consisting of KL1 only.[7] While full-length mKL functions as a co-receptor of fibroblast growth factor 23, regulating calcium and phosphorous homeostasis,[8,9] soluble alpha klotho (sαKL) presumably has endocrine functions. Enzymes from a disintegrin and metalloprotease family (ADAM 10 and 17), as well as β APP converting enzyme 1 (BACE-1) lead to shedding of sαKL. Recent studies suggest an association between klotho and the growth

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hormone (GH) and insulin-like growth factor I (IGF-I) axis,[7,10-13] and an association with insulin physiology, suppression of IGF-I receptor phosphorylation and insulin and IGF-I signaling.[12,14] We and others have shown that concentrations of sαKL are particularly high in patients with active acromegaly, and decrease with disease control.[15-18] This suggests it could be an interesting new biomarker in patients with acromegaly. However, the clinical use of biomarkers requires the availability of reference intervals. In our study, the cutoff for sαKL concentrations to distinguish uncontrolled patients from those with controlled acromegaly or healthy controls was higher than the upper limit of the normal range commonly used for the respective immunoassay. This upper limit was derived from the publication first describing the assay, and is based on a small number of healthy individuals.[18,19] Until today, no study has established reference intervals for sαKL in a larger group of healthy subjects. In addition, information about the impact of biological variables on its concentrations is scarce,[19-24] and limited data are available about assay performance characteristics, and pre-analytical stability.[19,25]

Here, we provide reference intervals for sαKL based on a large number of samples from well-characterized adults. Measurements were performed after careful evaluation of assay performance and pre-analytical stability. We also provide information about the impact of biological variables on sαKL concentrations compared with their impact on well-established biomarkers of GH action, and report concentrations of sαKL in patients with NFPA and prolactinoma.

Methods

Ethics

Collection of blood samples in the different studies, use of data, and measurement of biochemical parameters were approved by the ethics vote of the Bavarian Medical Association and Ethics Committee of the Faculty of the Ludwig Maximilians University, Munich, Germany (approval numbers: 08064, 06068, 228-16 and 152-10). All participants signed informed consent. All research was conducted in compliance with the Declaration of Helsinki.

Subjects and samples

The reference population is comprised of individuals representative of the background population from which the cases were derived. Ethnic background was not an inclusion criterion, but most participants had Caucasian origin. Blood was collected in the context of 3 different studies, and all studies have been previously described in detail.[26-29] For the present analysis, we selected a total of 890 samples from adult individuals (20-89 years, 435 males, group A). We excluded pregnant females, and subjects with pituitary, hepatic, or malignant diseases. 64 patients (35 males) had a diagnosis of diabetes type 2, all were controlled (glycated hemoglobin (HbA1c) < 6.5%). Extensive demographic details and laboratory data are provided in table S1. Selection was random, with the exception that we ensured equal representation of males and females, and approximately 120 samples per age decade. 199 serum samples (93 males, age 20-76 years) came from the AcroCut study (Munich, Germany) in healthy volunteers; 574 EDTA plasma samples (283 males, age 32-79 years) from the Cooperative Health Research in the Region of Augsburg study (KORA F4, Augsburg, Germany), and 117 serum samples (58 males, age 80-89 years) from the KORA-Age study. The majority of samples were collected in the morning hours after an overnight fast (>12 h [$n = 573$]). Participants of the KORA-Age study were not requested to be fasting when visiting the study center, but the information on fasting status was recorded (8-12 h ($n = 27$), <8 h ($n = 113$). In the remaining subjects ($n = 177$), information on fasting status was missing.

We also collected serum samples from patients with NFPA (B, $n = 18$, 9 males, 24-87 years) or prolactinoma (C, $n = 65$, 22 males, age 17-82 year, table S2). All patients were included in the Network of Excellence for Neuroendocrine Tumors Munich, a registry alliance of several academic institutions. No patient had medical history, signs, or symptoms of acromegaly or Cushing.

All samples were stored at −20 °C or below before analysis.

Validation of the analytical method and measurement of soluble alpha klotho

SαKL was measured using the commercially available solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) from Immuno-Biological Laboratories (IBL, Hamburg, Germany). We conducted validation experiments for key performance parameters following CLSI guidelines,[30,31] and tested stability of sαKL under different collection and storage conditions. Detailed information on the validation experiments is provided in the supplemental data file attached to this article. As a result, while generally following the manufacturer's instructions, we implemented modifications to the assay protocol as described in the results section below.

A subgroup of the samples (32 controls, 22 patients with acromegaly) was also analyzed using another commercially available assay (total alpha-Klotho ELISA, Immundiagnostik (IDK), Bensheim, Germany).

Biological variables

Biological variables expected to be potentially relevant for interpretation of sαKL concentrations were collected: Sex (male/female), age (in years), BMI (kg/m^2), waist-hip-ratio, estimated glomerular filtration rate (mL/min/1.73 m2), IGF-I (μg/L) and IGFBP 3 (μg/L) concentrations, parameters of glucose, lipid, liver, and calcium metabolism, and estrogen status (defined as pre-menopausal, post-menopausal, or intake of estrogen containing medication).

Other laboratory assessments

Random GH, IGF-I, and IGFBP 3 were measured using automated chemiluminescence immunoassays (IDS-iSYS CLIA, Immunodiagnostic Systems, Boldon, UK). Extensive characterization of these analytical methods and reference intervals has been published elsewhere.[26,32,33] Estimated glomerular filtration rate (CKD-EPI 2009), and parameters of glucose, lipid, liver, and calcium metabolism, prolactin, and estradiol concentrations were measured using standard methods.

Statistics

The Kolmogorov Smirnov test was used to confirm the non-Gaussian distribution. Continuous variables are presented using medians and interquartile ranges (IQR). For comparisons between different groups (population-based studies, patients with NFPA or prolactinoma), we used Kruskal–Wallis test

followed by Dunn's multiple comparisons post-test if significant. Correlations between sαKL and biological variables were calculated by Spearman's rank correlation for non-parametric variables. *P*-values of <.05 were considered significant. Analyses were performed by GraphPad Prism 9.0 (GraphPad Software, La Jolla, CA, USA).

Age- and sex-adjusted reference intervals were calculated by LMS quantile regression with a Box-Cox transformation to normality.[34,35] The estimated percentiles for each age and sex category are provided. Multivariate regression including some of the studied biological variables as independent predictors was performed. These analyses were performed using R 4.2.2 (©The R Foundation), ie, the package VGAM.

Results

Assay characteristics, pre-analytical stability, and comparison to another assay

Our validation generally confirmed appropriate analytical performance of the IBL sαKL immunoassay when following the manufacturer's protocol. Detailed information is provided in the supplements (text and Figures S1-S5).[36] We identified 2 steps in the assay protocol where modifications improved performance:

Following the manufacturer's original protocol, which recommends 1 h incubation, we found acceptable intra-assay variability for repeats done in a row, but if pipetted randomly across assay plates, variability increased significantly. Systematic comparison of 1, 2, and 4 h or overnight incubation revealed that increasing the time beyond 2 h reliably prevents this variability (Figure S2). For logistical reasons we used overnight incubation.

We confirmed good linearity for samples with concentrations below 3000 pg/mL (recovery rate (% mean [range]): 96 [92-107]) but found that samples with concentrations exceeding 3000 pg/mL must be diluted at least 1:4 to allow further dilutions in a linear manner (Figure S3). In the majority of samples from healthy subjects, sαKL remains above the limit of quantification even when diluted 1:4. We therefore routinely used 1:4 dilution, and only repeated without dilution the few samples with very low concentrations ($n = 29$).

Pre-analytical stability was excellent (Figure S4), with no significant differences in concentrations observed between samples stored at room temperature for up to 120 h before (whole blood), for up to 72 h after centrifugation (serum), submitted to up to 4 freeze/thaw cycles, or after long-term storage at −20 °C (31 months). SαKL also did not differ between serum and EDTA plasma taken in parallel from the same individual (Figure S5).

Parallel assessment of samples by the immunoassays from IBL and IDK revealed significant disagreement (Figures S6A and 6B), with concentrations measured by IDK approximately 8 times higher compared with IBL in healthy controls, but only 2.4 times higher in patients with acromegaly. Results from both assays correlated only in patients with acromegaly ($r_s = 0.70$, $P = .0003$), and not in healthy controls ($r_s = 0.04$, $P = .83$).

Establishment of reference intervals and comparison to IGF-I and IGFBP 3

Intake of (estrogen containing) oral contraception (OC) or estrogen containing oral medication (OE) was discussed to affect sαKL concentrations. For analysis of the impact of biological variables, and for establishment of reference intervals, we therefore excluded 66 females (56 pre-menopausal, 10 post-menopausal) on OC/OE. We also excluded 6 female patients with prolactinoma on OC/OE before comparing patients with prolactinoma to the reference cohort.

In the remaining subjects from the reference sample ($n = 824$), sαKL concentrations (pg/mL, median [IQR]) were slightly, but significantly higher in females compared with males (678 [537-859] vs. 651 [537-812], $P = .01$). In both sexes, sαKL was negatively correlated to age, with a decline until the age of 40 years (Figure 1). After the age of 40, concentrations remained unchanged in each sex until old age (89 years), still slightly higher in females (651 [522-824]) vs. 613 [522-751], $P = .04$). Sex-specific reference intervals adjusted for age in 1 year increments are provided in tables S3 (males) and 4 (females).

IGF-I and IGFBP 3 concentrations also differed significantly between age groups. Correlations (r_s [95% confidence interval]) with age were higher for IGF-I ($r_s = -0.58$ [−0.63–−0.54], $P < .0001$), but similar for IGFBP 3 ($r_s = -0.31$ [−0.35-−0.22], $P < .0001$) as compared with sαKL ($r_s = -0.29$ [−0.36-−0.24], $P < .0001$, Figure 2). Across all ages, the median (interquartile range [IQR], µg/L) was 115 (88-153) for IGF-I and 3869 (3318-4390) for IGFBP 3. IGF-I was higher in males (117 [92-155]) vs. females (106 [84-131]; $P < .0001$), while IGFBP 3 was higher in females (3925 [3355-4536] vs. 3797 [3156-4536]; $P = .01$). IGF-I correlated with IGFBP 3 ($r_s = 0.46$ [0.40-0.51], $P < .0001$). SαKL correlated with IGF-I ($r_s = 0.31$ [0.26-0.38], $P < .0001$, Figure 3), but not with IGFBP 3 ($P = .56$).

In the Acrocut cohort, we were able to measure GH (µg/L) and prolactin (µU/mL, $n = 199$, 93 males, 106 females [37 females on OE] for both), and estradiol (pg/mL, $n = 137$, 31 males, 106 females [37 females on OE]). There was no statistically significant correlation between sαKL (868 [720-1122]) and random GH (1.37 [0.13-4.97], $r_s = 0.12$, $P = .09$). This did not change after removing the 37 females on OE ($r_s = 0.14$, $P = .06$). SαKL correlated with prolactin ($n = 199$, 264 [185-381], $r_s = 0.31$, $P < .0001$ ($n = 162$, $r_s = 0.21$, $P = .0006$, if excluding females on OE) and with estradiol concentrations ($n = 137$, 27.5 [14.4-49.9], $r_s = 0.31$, $P = .0002$). However, correlation to estradiol was no longer significant if females on OE were excluded ($n = 100$, $P = .35$).

Impact of biological variables

SαKL exhibited a weak negative correlation with BMI and waist-hip-ratio ($r_s = -0.13$, and −0.12 (−0.20-−0.05), $P < .001$ for both, Figure 4A-B). Total cholesterol concentrations were only available in a subset of participants ($n = 161$), but also showed a weak negative correlation to sαKL ($r_s = -0.16$ (−0.29-−0.02), $P < .001$, Figure 4C. In contrast, a weak positive correlation was seen to estimated glomerular filtration rate (eGFR, $r_s = 0.25$ [0.18-0.31], $P < .0001$, Figure 4D). We also performed multivariate regression analysis with sαKL as the dependent variable, and age, sex, BMI, and eGFR as independent predictors. The effect of eGFR on sαKL concentrations remained statistically significant across all models. In contrast, the effect of BMI was no longer detectable once age is included. Age remained a significant predictor in all models.

There was no correlation between sαKL and fasting glucose, fasting insulin, hemoglobin A1c, homeostasis model

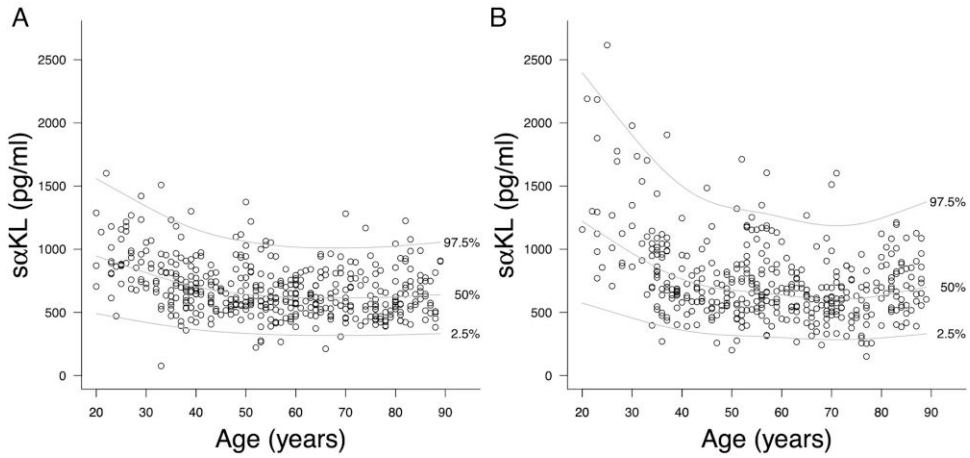

Figure 1. SαKL concentrations in males (A, $n = 435$) and females not taking estrogen containing OC/OE (B, $n = 389$) from the reference cohort. sαKL: soluble alpha klotho.

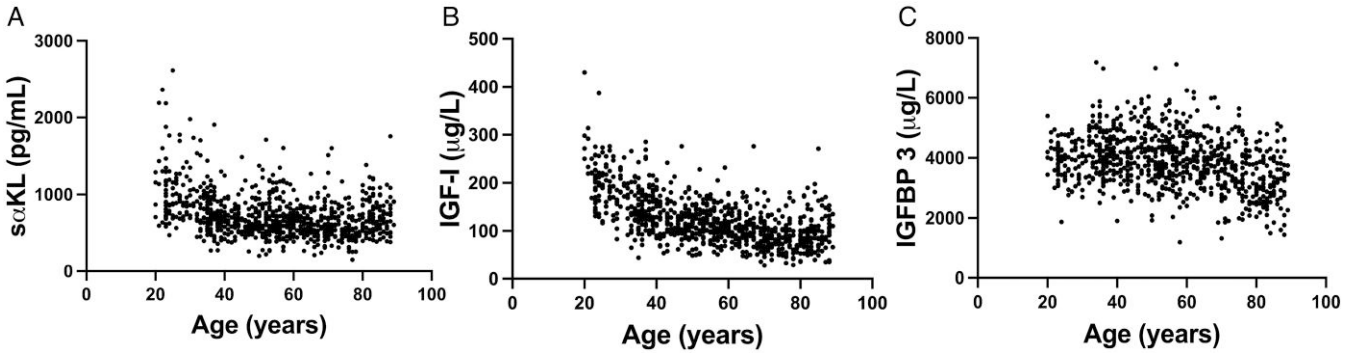

Figure 2. Correlation of age to sαKL concentrations (A), with IGF-I (B) and IGFBP 3 (C) in the reference cohort not taking OC/OE ($n = 824$). sαKL: soluble alpha klotho, IGF-I: insulin-like growth factor I, IGFBP 3: insulin-like growth factor binding protein 3. 2A: $r_s = -0.29$ (−0.36 to −0.24), $P < .0001$, 2B: $r_s = -0.58$ (−0.63 to −0.54), $P < .0001$, 2C: $r_s = -0.31$ (−0.35 to −0.22), $P < .0001$.

assessment-estimated insulin resistance, and insulin sensitivity index ($P = .35$, 0.07, 0.11, 0.10, and 0.37, respectively). SαKL concentrations were not significantly different between individuals fasting for different durations (<8 h [$n = 113$], 8-12 [$n = 27$] or >12 h [$n = 573$], $P = .06$, Figure 5).

There was no correlation of sαKL concentrations to gamma-glutamyl transpeptidase (GGT), alkaline phosphatase, glutamic oxaloacetic transaminase and albumin ($P = .13$, .53, .06, and .52, respectively), and negligible correlation to glutamic pyruvic transaminase ($r_s = 0.08$ [0.01-0.15], $P = .04$). SαKL did also not correlate with parameters of calcium metabolism (25-OH-vitamin D, calcium, phosphorous, and parathyroid hormone [PTH], $P = .82$, .78, .12, and .55, respectively).

IGF-I and IGFBP 3 correlated with more biological variables than sαKL. IGF-I concentrations negatively correlated with BMI ($r_s = -0.24$ [−0.30 to −0.18], $P < .0001$), waist-hip-ratio ($r_s = -0.12$ [−0.19-−0.04], $P = .002$), fasting glucose ($r_s = -0.15$-0.22 to −0.06), $P = .0006$), HbA1c ($r_s = -0.25$ [−0.32-−0.18], $P < .0001$), PTH ($r_s = -0.15$ [−0.22 to −0.07], $P < .0001$) and positively with eGFR ($r_s = 0.27$ [0.20 to 0.34], $P < .0001$), 25-OH-vitamin D ($r_s = 0.12$ [0.04-0.20], $P = .002$), calcium ($r_s = 0.15$ [0.06-0.23], $P < .0005$) and albumin ($r_s = 0.27$ [0.20-0.34], $P < .0001$). IGFBP 3 concentrations negatively correlated with age ($r_s = -0.31$ [−0.35 to −0.22], $P < .0001$), waist-hip-ratio ($r_s = -0.16$ [−0.24 to −0.09], $P < .0001$), HbA1c ($r_s = -0.12$ [−0.19 to −0.04], $P < .002$), PTH ($r_s = -0.21$ [−0.28 to −0.14], $P < .0001$), and positively with HOMA-IR ($r_s = 0.15$ [0.07-0.24], $P = .0004$), fasting insulin ($r_s = 0.13$ [0.06-0.21], $P = .002$), eGFR ($r_s = 0.29$ [0.22-0.36], $P < .0001$), GGT ($r_s = 0.18$ [0.10-0.25], $P < .0001$), 25-OH-vitamin D ($r_s = 0.13$ [0.06-0.21], $P = .0004$), calcium ($r_s = 0.15$ [0.06-0.23], $P = .0004$) and albumin ($r_s = 0.32$ [0.26-0.40], $P < .0001$).

Concentrations in patients with other pituitary diseases (NFPA and prolactinoma)

SαKL concentrations (pg/mL, median IQR) were not different between the population-based sample (A: 667 [537-836]) and patients with NFPA (B: 755 [676-906]; A vs. B, $P = .09$), but were significantly higher (though within the reference interval) in patients with prolactinoma (904 [759-1283]; A vs. C, $P < .0001$, Figure 6). Notably, in our cohorts, mean (IQR) of IGF-I and IGFBP 3 were within the respective reference intervals for age and sex in patients with both, NFPA and prolactinoma (NFPA: IGF-I: 115 [86.5-161.5], IGFBP 3: 3426 [2829-3536], prolactinoma: IGF-I: 144 [105-193], IGFBP 3: 3694 [3214-4199]).

Impact of estrogen status

To investigate a potential influence of estrogen status, we compared sαKL in the studies included in the above analyses

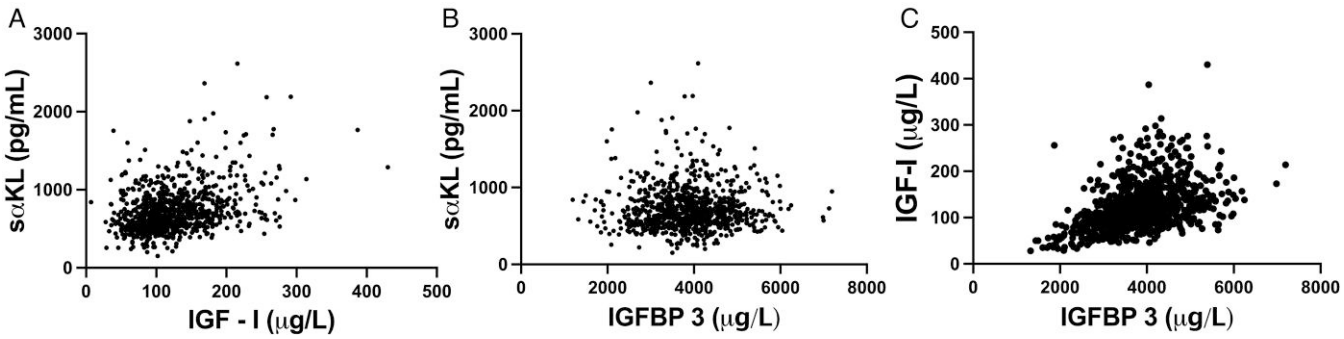

Figure 3. Correlation of sαKL with IGF-I (A) and IGFBP 3 concentrations (B) or IGF-I to IGFBP 3 concentrations (C) in subjects from reference sample not taking OC/OE ($n = 824$). sαKL, soluble alpha klotho; IGF-I, insulin-like growth factor I; IGFBP 3, insulin-like growth factor binding protein 3. 3A, $r_s = 0.31$ (0.26-0.38), $P < .0001$; 3B, $P = .56$; 3C, $r_s = 0.46$ (0.40-0.51), $P < .0001$.

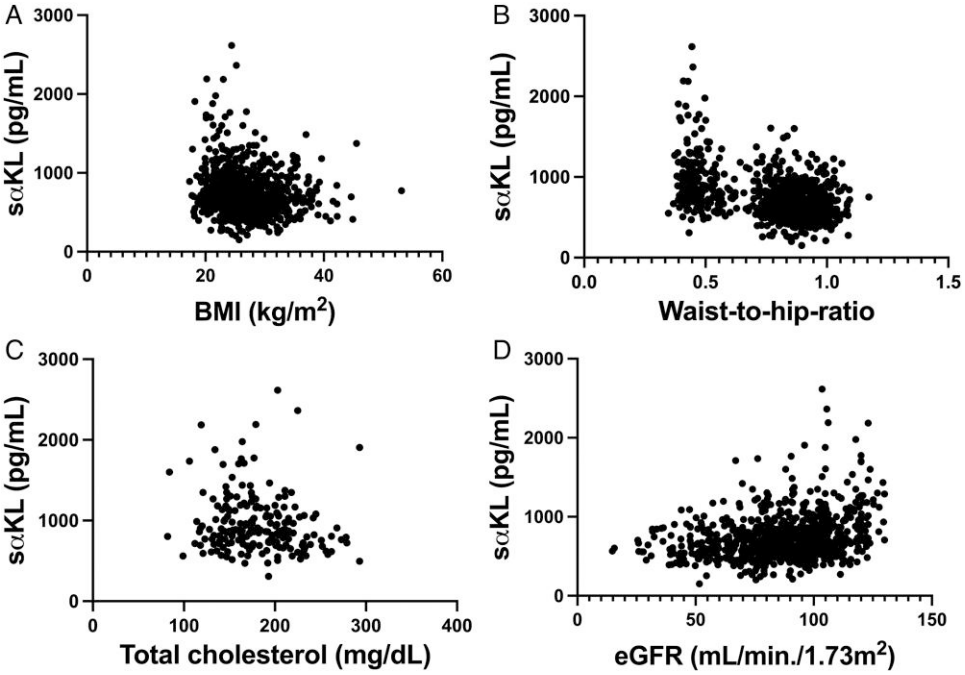

Figure 4. Correlation of sαKL concentrations to BMI (A), waist-hip-ratio (B), total cholesterol concentrations (C), and eGFR (D) in subjects from the reference sample not taking OC/OE ($n = 824$, data for cholesterol, $n = 161$). sαKL, soluble alpha klotho, BMI, body mass index (BMI), eGFR, estimated glomerular filtration rate. 4A, $r_s = -0.13$ (−0.20 to −0.06), $P < .001$; 4B, −0.12 (−0.20 to −0.05), $P < .001$; 4C, $r_s = -0.16$ (−0.29 to −0.02), $P < .001$, 4D, $r_s = 0.25$ (0.18-0.31), $P < .0001$.

(pre- [$n = 158$] and post-menopausal [$n = 231$] females without OE and males [$n = 435$]) to concentrations found in pre-menopausal females with estrogen containing OC ($n = 56$, Figure 7), and post-menopausal females on OE ($n = 10$). In pre-menopausal females, sαKL concentrations were not statistically different between individuals with or without OC (716 [639-939] vs. 687 [556-959]; $P = .72$), but the small group of post-menopausal females on OE (492 [316-703]) had lower sαKL compared with post-menopausal females without OE (668 [535-837], $P = .04$). As mentioned above, sαKL concentrations were slightly higher for females when analyzing the whole reference sample (without individuals on OC/OE). However, we found no difference in sαKL between the subgroups of pre-menopausal females (independent of estrogen status) and males <50 years (726 [582-891], $P = .80$), and between post-menopausal females with OE and males >50 years ((600 (506-748), $P = .42$). Only in the group of post-menopausal females without OE, sαKL concentrations were slightly but significantly higher when compared with males >50 years (600 [506-748], $P = .02$).

In female patients with prolactinoma, sαKL was higher in the patients without OC/OE ($n = 37$, 970 [798-1452]) compared with the few patients receiving OC/OE ($n = 6$, 690 [645-864], $P = .02$). However, sαKL was not different between any of the female patient groups (with or without OC/OE) compared with male patients ($P = .12$ and .49, respectively). In patients with NFPA (9 males/9 females) sαKL tended to be lower in males, but the difference did not reach statistical significance (males: 708 [285-891] vs. females: 890 [715-1092], $P = .06$).

Discussion

The primary goal of our study was to establish age- and sex-adjusted reference intervals for sαKL, a protein which we and others recently had proposed as a new biomarker in

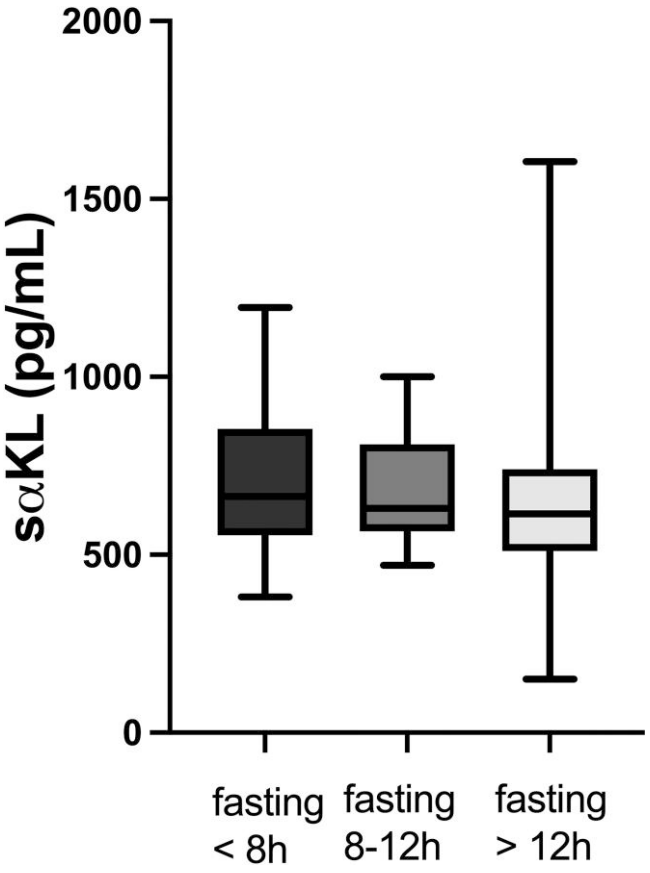

Figure 5. SαKL concentrations in individuals fasting < 8 h ($n = 113$), 8-12 h ($n = 27$) or > 12 h ($n = 573$). sαKL: soluble alpha klotho. $P > .05$.

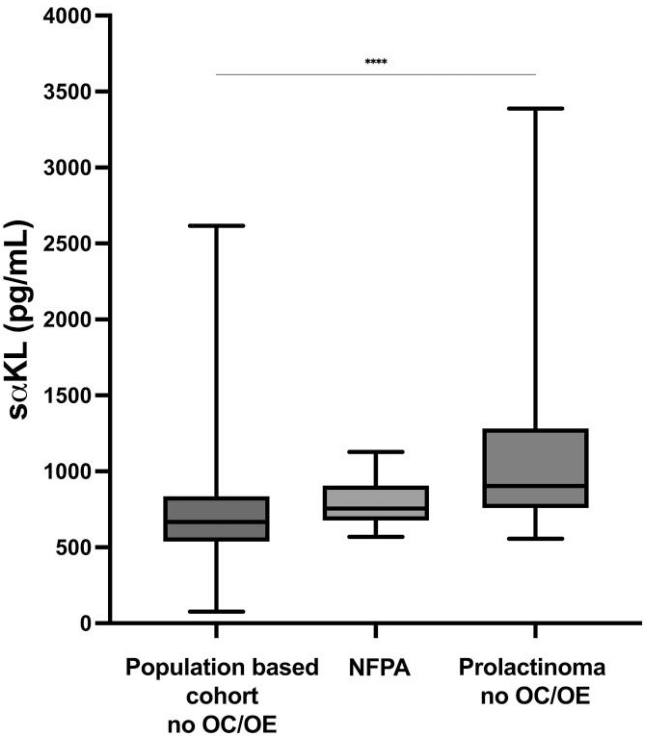

Figure 6. SαKL concentrations in individuals from the reference sample ($n = 824$), in patients with NFPA ($n = 18$) and in patients with prolactinoma ($n = 59$). The 3 groups only include individuals not taking OC/OE. sαKL, soluble alpha klotho, NFPA, non-functioning pituitary tumor. $****P < .0001$.

patients with acromegaly.[15-18,37-39] We first evaluated and optimized performance characteristics of a widely used ELISA, and studied pre-analytical stability of sαKL. We then measured sαKL concentrations in a large sample from the background-population, equally representing sexes and adult age groups. Detailed reference intervals were constructed, and the potential impact of different biological variables was analyzed. Finally, we compared sαKL concentrations obtained in our reference population to concentrations measured in patients with pituitary tumors other than somatotroph adenomas (namely NFPA and prolactinoma).

The sαKL assay used in our study (IBL ELISA) exhibited adequate analytical performance, but we found that 2 modifications to the manufacturers protocol were important: First, extending the initial incubation step from 1 h to overnight significantly improved reproducibility of the measurements within a plate, leading to better within- and between-assay coefficients of variation. Second, while the assay exhibits acceptable linearity across the concentration range seen in healthy subjects, we suggest diluting all samples at least 1:4. The reason is that the standard curve shows a plateau at concentrations exceeding 3000 pg/mL, potentially compromising accuracy at higher concentrations if samples are not adequately diluted. This is important particularly in patients with active acromegaly where sαKL can be very high.

We confirm sαKL concentrations do not differ between paired serum and EDTA plasma samples from the same individual,[40] and demonstrate pre-analytical robustness under different conditions for collection and storage. Parallel measurements with another commercially available klotho assay (IDK) revealed that results for the same sample disagree dramatically, confirming observations form previous studies using different assays.[21,40] We found less disagreement in patients with acromegaly compared with healthy controls, suggesting differences in sensitivity might play a role. Other possible explanations include the use of different standards for calibration, and the fact that different assays might recognize different isoforms of sαKL. Future studies are needed to better address this issue, but our observation emphasizes that only results from studies using the same sαKL assay can be compared.

We established reference intervals for the sαKL assay most widely used in the current literature, reflecting age- and sex-related changes in concentrations. While our study population was much larger than previously published studies using the same assay,[19,21,22,41] sαKL concentrations were in the same order of magnitude. Notably, the upper limits of the normal range for males and females in our study are higher than the upper limit initially reported by Yamazaki et al.[19] Moreover, in our study variation of sαKL concentrations within each age group was greater in females compared with males. Interestingly, this was primarily driven by an upward shift of the 97.5th percentile, while the 2.5th percentiles were comparable between sexes. Since our population mostly consists of individuals with Caucasian background, we cannot exclude an impact of ethnicity. More likely, the difference in cohort size might also play a role. It is intriguing that overall the upper limits of the normal range derived from our study are very close to the cutoff we previously found to best differentiate patients with active acromegaly from those with controlled disease, NFPA or healthy controls.[18] We did not find systematic

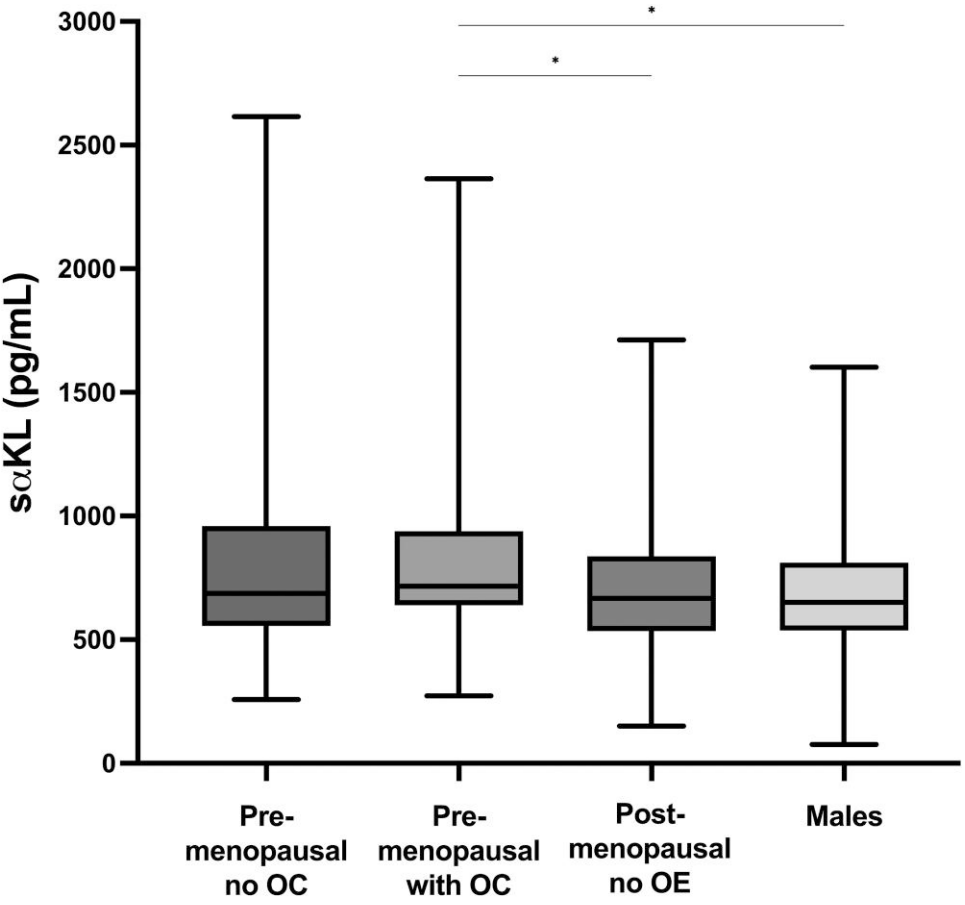

Figure 7. SαKL concentrations in pre-menopausal females without OC ($n = 158$), pre-menopausal females with OC ($n = 56$), post-menopausal females without OE ($n = 231$) and males ($n = 435$). sαKL: soluble alpha klotho. * $P < .05$.

differences in sαKL concentrations between males and females with acromegaly in our previous study,[18] but another study had reported this.[42] Our new data suggest it might be particularly important to use sex-specific upper limits of the normal range for interpretation of sαKL in patients with acromegaly.

Other than in patients with active acromegaly, in the background population sαKL showed only modest correlation with IGF-I, and not with IGFBP 3. Furthermore, the correlation with age for sαKL was weaker than that seen for IGF-I and IGFBP 3. Therefore, while associated with the GH-IGF-I axis to some degree, sαKL concentrations seem to be regulated differently. It has been shown that the enzymes ADAM 10 and 17 induce shedding of sαKL, and GH and IGF-I have been suggested to be involved in regulation of the expression of these enzymes.[7,10-12] We speculate that this indirect link, modified by other factors, explains the comparably weak association between sαKL and the GH-IGF-I axis under physiological conditions. At the same time, none of the factors analyzed in our study was associated with an increase of sαKL concentrations similar to that seen in active acromegaly.[16-18,39] This indicates a specific effect of excess GH concentrations on sαKL, supporting its use as a biomarker of disease activity in acromegaly. While the half-life of sαKL has only been described in animal studies, 1 study in patients with acromegaly describes a rapid decrease in sαKL concentrations within 2-6 days after successful pituitary surgery[17] which would be faster than the occurrence of normalization of IGF-I.[43]

SαKL exhibited a weak negative correlation with the variables age, BMI, waist-hip-ratio and total cholesterol and a positive correlation with eGFR when variables were considered individually. Since the variables might be correlated among each other, we also performed multivariate analysis. While the correlation of sαKL with eGFR remained significant, the association with BMI disappeared, indicating it was mainly an effect of age. The correlations of sαKL with age and eGFR were already shown in the literature.[19,44,45] Total cholesterol data were only available for a subset of our cohort, and further studies might be needed to understand a potential association with sαKL.

In our study, we did not observe correlations of sαKL with parameters of glucose metabolism. However, most individuals had normal glucose metabolism. Data in patients with diabetes are controversial, but some studies reported an impact of glucose metabolism on sαKL. Inclusion of patients with kidney diseases in some studies might have contributed to that observation.[46,47]

Concentrations of prolactin ($n = 199$) and estradiol ($n = 137$) were available only in a small subset of our cohort. SαKL correlated weakly to prolactin in all subjects, but to estradiol only in the all participants (including males and females on OE). The limited number of subjects is a limitation here, and further studies are necessary to clarify the potential impact of those hormones on sαKL concentrations.

We also did not find a correlation of sαKL with parameters of calcium metabolism, mirroring results from our previous study in patients with acromegaly.[18] Some studies had reported correlations between sαKL and parameters of calcium

metabolism in patients with kidney insufficiency.[48-51] However, in these patients, the transmembrane form of klotho is more relevant. In contrast, shedding and generation of sαKL is considered to be largely independent from calcium metabolism.

In patients with NFPA, sαKL was comparable to the background population. In contrast, concentrations tended to be slightly higher in patients with prolactinoma. We assume this is due to the younger age, and the higher proportion of females among patients with prolactinoma. We acknowledge that the NFPA group is small ($n = 18$), limiting the statistical power of a comparison. However, the absence of an obvious difference in sαKL concentrations in these patient groups to the background population seems to suggest that grossly elevated concentrations of sαKL as reported from patients with active acromegaly are not related to a pituitary tumor itself. Notably, in our study, patients with NFPA and prolactinoma all presented with IGF-I and IGFBP 3 concentrations within the reference interval for age and sex. We acknowledge that, although IGF-I concentrations were not different between patients with prolactinoma and NFPA, most of the patients with prolactinoma in our study were not operated. Therefore, we cannot exclude a co-expression of prolactin and GH, potentially explaining the slightly higher mean sαKL concentrations in this group.

In our study populations, estrogen status was not correlated with circulating sαKL concentrations. Generally, concentrations in all pre- and post-menopausal females without OC/OE were slightly higher compared with concentrations in all males. The small difference remained significant until advanced age. On the other hand, we found no difference in sαKL between pre-menopausal females with or without OC use. This is an important difference in the regulation compared with IGF-I, where several studies have shown that oral estrogens induce a state of hepatic GH resistance, leading to reduced IGF-I concentrations.[52-54] Only in the small groups of post-menopausal females with OE or female patients with prolactinoma with OE/OC, concentrations of sαKL were lower compared with the concentrations seen in the respective individuals without OE/OC. However, caution is needed in interpretation due to the limited size of the groups ($n = 10$ and $n = 6$, respectively). The absence of a difference in sαKL between the large groups of pre-menopausal females with and without OC suggests negligible influence of oral estrogens. A likely explanation is that hepatic expression—contributing to the majority of circulating IGF-I—is less relevant for sαKL. Recent reports suggested that anorexia, another condition associated with GH-resistance and low IGF-I, might also be associated with low sαKL concentrations.[55,56] Further studies are needed to understand the significance of and potential mechanisms behind this association.

In summary our findings support the concept that sαKL is a highly stable biomarker. The fact that it was specifically elevated in conditions of excess GH secretion, but less related to biological variables compared with IGF-I or IGFBP 3 suggest it might be an interesting new biomarker of disease activity in acromegaly. The reference intervals provided by our study might facilitate the use of sαKL as a biomarker in studies and clinical practice.

Supplementary material

Supplementary material is available at *European Journal of Endocrinology* online.

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Authors' contributions

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Data availability

The data underlying this article are presented in the article and in its online material, or are available from the authors upon reasonable request.

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