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Impact of preanalytical conditions and biological variables on urinary concentrations of soluble alpha klotho, a novel biomarker of growth hormone activity

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Background

There is a lack of practical screening parameters that are robust against influencing factors for diagnosing acromegaly. It has been shown that soluble alpha klotho (saKL), a peptide hormone, is elevated in serum samples of patients with growth hormone (GH) excess [1, 2] while biological factors including BMI, age and sex have less influence than compared to insulin-like growth factor (IGF)-I [3]. Urinary saKL may also function as a biomarker for growth hormone excess; however, the preanalytical stability as well as influencing biological parameters need to be assessed beforehand.

Methods

We collected spontaneous urine and serum samples from 20 healthy subjects along with anthropometric data including age, height and weight. A sandwich-ELISA (IBL, Hamburg, Germany) was used to measure serum and urine saKL. The stability of different storage temperatures was assessed by measuring fresh urine (baseline) and aliquots after four weeks of storage at 4° C, -20° C, -80° C. Centrifugation was performed for 10 minutes (10.000 rpm, 20° C) and cOmpleteTM Mini (Roche) was used as a protease inhibitor. Linearity was investigated by using different dilutions. Up to four freeze-thaw-cycles were performed, in which samples were frozen to -80° C and exposed to room temperature (RT) for 2 hours before refreezing. Samples were also exposed RT for 0, 2, 4, 24 and 48 hours before and after storage at -80° C. When the measured value was below that of the blank of the standard curve, we arbitrarily assigned a value of 1 pg/dL. Samples above blank but below the smallest standard point were arbitrarily set to 46.785 pg/dL (50% of the smallest standard point).

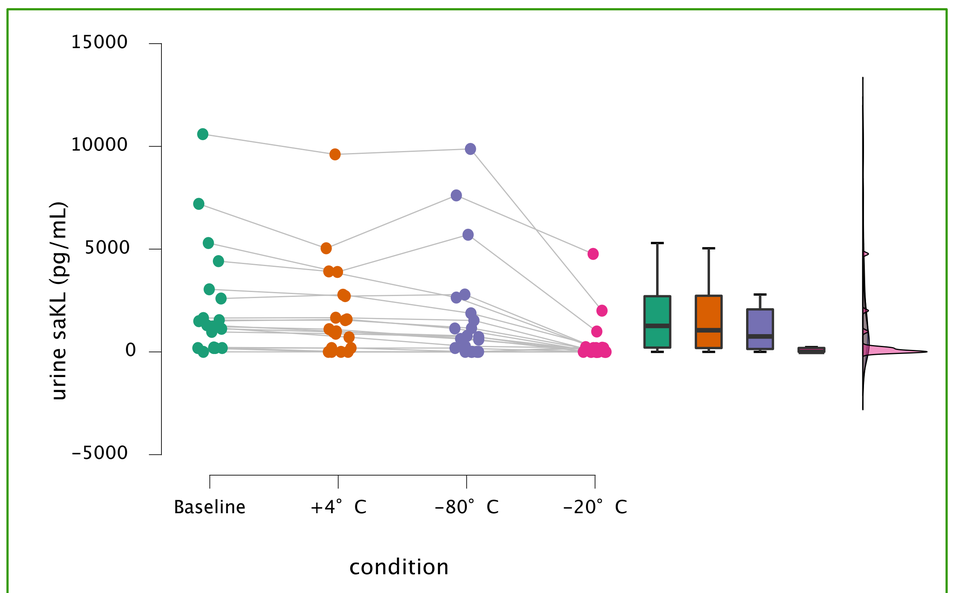
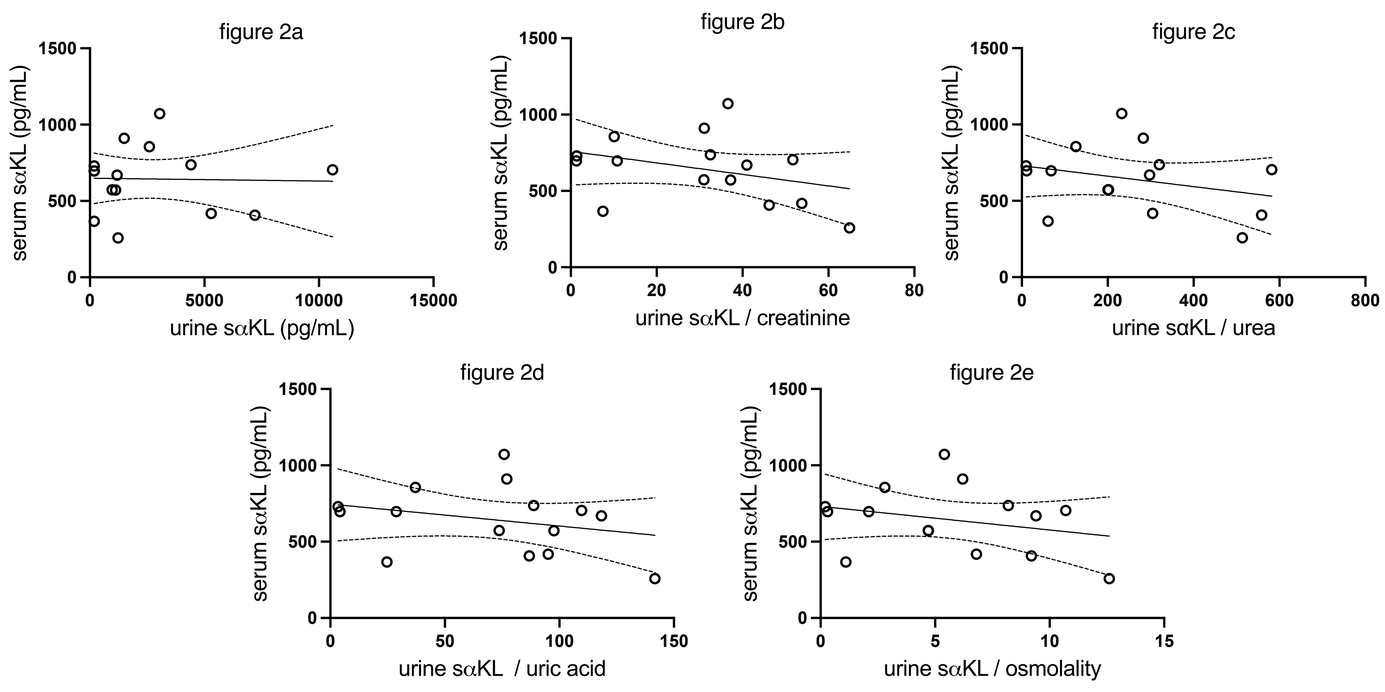


Figure 1: Descriptive image of the repeated measures ANOVA comparing baseline urinary saKL to after four weeks storage at various temperatures (4° C, -80° C and 20° C for four weeks) shows a significant concentration loss (p Bonferroni <.001) at -20° C and no significant differences at all other conditions. (n=20)

Results

Urine saKL is stable in different preanalytical conditions, however when stored at -20° C measured concentrations are significantly reduced compared the other conditions (baseline, +4° C, and -80° C; repeated measures ANOVA, post hoc analysis) (**Figure 1**). In the baseline measurement urine saKL was measurable in 19 of 20 healthy participants; in those 19, urine saKL fell below the Limit of Quantification in 47.4% when stored at -20° C. Neither protease inhibitor addition nor centrifugation prevented this decrease. The linearity of urinary saKL is robust. When exposed to up to four freeze-thaw-cycles the samples were stable (maximum percent coefficient of variation: 15.3%, maximum percentage change: 28.5%). Exposure to RT before and after freezing, did not lead to a significant loss in saKL concentration. Serum saKL and absolute urine saKL did not correlate, (Spearman's rho: 0.148; p-value: 0.599) (**Figure 2a**), after normalization of urine saKL for creatinine, urea, uric acid and osmolality this was still true (Spearman's rho: -0.350, -0.161, -0.282, and -0.196; p-values: 0.201, 0.567, 0.307, and 0.482 respectively) (**Figures 2b-e** respectively). Absolute urine saKL concentrations did not correlate with anthropometric data or traditional GH biomarkers (**Table 1**).



Figures 2a-e: A correlation was not shown between serum saKL and baseline urinary saKL (**Figure 2a**), urinary saKL corrected for creatinine (**Figure 2b**), Urea (**Figure 2c**) uric acid (**Figure 2d**) or osmolality (**Figure 2e**). (n=15)

Variable		Serum saKL	Absolute saKL in Urine	Urinary saKL/ creatinine	Urinary saKL/ urea	Urinary saKL/ uric acid	Urinary saKL/ osmolality
Age	Spearman's rho	-0.136	-0.074	-0.043	-0.057	-0.118	-0.072
	p-value	0.628	0.793	0.879	0.839	0.675	0.800
Weight	Spearman's rho	0.056	0.411	0.392	0.430	0.213	0.372
	p-value	0.844	0.128	0.148	0.110	0.446	0.172
Height	Spearman's rho	0.511	0.323	-0.013	0.075	-0.238	-0.038
	p-value	0.051	0.241	0.965	0.790	0.394	0.894
BMI	Spearman's rho	-0.232	0.362	0.446	0.464	0.311	0.407
	p-value	0.404	0.185	0.097	0.083	0.259	0.133
IGF-I x ULN	Spearman's rho	0.504	-0.261	-0.439	-0.479	-0.514	-0.479
	p-value	0.058	0.347	0.103	0.073	0.052	0.073
IGF BP 3 x ULN	Spearman's rho	0.289	-0.164	-0.239	-0.221	-0.311	-0.275
	p-value	0.295	0.559	0.389	0.427	0.259	0.320
hGH	Spearman's rho	0.082	-0.164	0.063	-0.018	0.061	0.061
	p-value	0.771	0.559	0.825	0.950	0.830	0.830

Table 1: Spearman's correlations did not show any significant association between anthropometric data or traditional growth hormone biomarkers and serum saKL, absolute urinary saKL (baseline) or normalized urinary saKL (baseline). (n=15)

Discussion

SaKL is measurable in spontaneous urine and is stable at room-temperature, 4° C and -80° C, and is resistant to repeated freezing and thawing. However, storage of urine for saKL measurement should be avoided at -20° C, as this is likely a eutectic temperature for urine, at which urine cannot fully freeze, making proteins in unfrozen areas susceptible to carbamoylation and alteration of the protein's tertiary structure as a result of urea breakdown; this process is likely as it has been shown for other protein hormones [4, 5]. As expected, and seen for serum saKL, urinary saKL was not influenced by the biological factors investigated here. Not unexpected, in the presented cohort of healthy individuals with normal IGF-I, we did not find correlations between urine saKL and GH-dependent parameters in serum [1]. It might be possible that correlations can only be detected with substantially higher saKL concentrations as seen in GH excess.

Sources

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Summary

Urinary saKL is stable in various preanalytical conditions, excluding storage at -20° C. This facilitates its investigation as a potential new biomarker for growth hormone action in situations of elevated growth hormone levels.

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