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Abstract

Background: α-Klotho is a transmembrane protein that can be cleaved and act as a circulating hormone (s-klotho). s-Klotho serum levels seem to reflect growth hormone (GH) secretory status. We investigated the role of s-klotho as a reliable marker of GH secretion in short children and the factors influencing its secretion.

Methods: We enrolled 40 short Egyptian children (20 GH deficiency [GHD] and 20 idiopathic short stature [ISS]). They underwent a pegvisomant-primed insulin tolerance test (ITT) and were accordingly reclassified as 16 GHD and 24 ISS. The samples obtained before and 3 days after pegvisomant administration, prior to the ITT, were used for assaying insulin-like growth factor (IGF)-I and s-klotho.

Results: IGF-I and s-klotho serum levels were not significantly different ($p = 0.059$ and $p = 0.212$, respectively) between GHD and ISS. After pegvisomant, a significant reduction in IGF-I and s-klotho levels was found in both groups. s-Klotho significantly correlated only with IGF-I levels in both groups.

Conclusions: s-Klotho mainly reflects the IGF-I status and cannot be considered a reliable biomarker for GH secretion in children.

Keywords: children; growth hormone (GH) deficiency diagnosis; IGF-I; klotho.

Introduction

The diagnosis of growth hormone deficiency (GHD), apart from the severe forms caused by genetic mutations, tumors, cancer treatment, etc. is not so straightforward. There is, in fact, a grey zone where the assessment of GH secretion in children is notoriously difficult because of the lack of accuracy of our investigational tools [1–3] and the variability of GH responses to stimulation tests [4–6]. The evaluation of spontaneous nocturnal GH secretion might be useful; however, it is too cumbersome, requiring multiple samplings and hospitalizations [7]. Pegvisomant-primed GH stimulation seems to be a promising technique, which, however, needs confirmation on a large number of subjects [8]. Nevertheless, even when supported by a detailed clinical evaluation and insulin-like growth factor (IGF)-I serum levels [9], some diagnoses remain doubtful, as shown by the "normalization" of GH secretion at retesting [10].

α-Klotho might be an additional tool to improve our accuracy in diagnosing GHD. α-Klotho, a transmembrane protein, serves as a co-receptor for fibroblast growth factor 23 (FGF-23), which inhibits the renal tubular absorption of phosphate and the synthesis of calcitriol [11, 12]. The ectodomain of α-klotho is enzymatically released in the circulation as s-klotho [13, 14], which exerts systemic effects on ion channels, attenuates insulin and IGF-I signaling and regulates calcium homeostasis [15, 16]. GH and s-klotho seem to be strictly correlated. In fact, α-klotho-deficient mice (*kl/kl* mice) are smaller compared with their wild-type counterparts, and their GH-producing cells show a reduced number of secretory granules [17]. Furthermore, treatment of these mice with α-klotho enhances GH

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secretion [18]. Children with organic GHD showed low levels of s-klotho [19], which significantly increased following a treatment with GH [20]. On the contrary, high levels of α-klotho were observed in acromegaly and they significantly decreased after surgery [21].

With this background, we wondered whether s-klotho might be helpful in the diagnosis of GHD and, in particular, whether a diagnostic cut-off limit might be identified. Furthermore, we investigated the mechanisms influencing its secretion.

Subjects and methods

We enrolled 20 short Egyptian children with reduced GH secretion (GH peak <10 ng/mL) after two classical pharmacological stimuli (clonidine and insulin tolerance test, ITT) (GHD) and 20 subjects with normal GH secretion (idiopathic short stature, ISS) (GH peak >10 ng/mL). All children underwent a pegvisomant-primed GH stimulation test, with pegvisomant acting as an enhancer of GH secretion, in order to avoid false positive results [8]. Pegvisomant (1 mg/kg of body weight) was injected subcutaneously and, 3 days after, an ITT was performed [8]. The samples obtained before and 3 days after pegvisomant administration, prior to the ITT, were used for measuring IGF-I and s-klotho. The area under the curve (AUC) during the ITT test was calculated according to the trapezoidal rule. After the study was completed, the patients who showed a normal GH response were regularly followed up as outpatients, in order to clinically confirm the diagnosis of ISS. The study was approved by the Local Committee in Cairo and the parents of the children gave their consent to the study after a full explanation of it was given.

According to the results of the pegvisomant-primed ITT and the follow-up evaluation, the patients were reclassified as 16 GHD and 24 ISS. All the patients with GHD showed idiopathic isolated GHD. The chronological age was 9.48 years (standard deviation [SD] = 2.84) and 11.49 years (SD = 1.98) (p = 0.003), body mass index (BMI) −0.96 standard deviation score (SDS) (SD = 0.90) and −1.26 SDS (SD = 1.33) and height −3.49 SDS (SD = 0.63) and −3.25 SDS (SD = 0.58) for GHD and ISS children, respectively. The measures of height and BMI were converted to SDS according to Tanner et al. [22] and Cole et al. [23]. Basal IGF-I levels were −2.10 SDS (SD = 2.67) and −1.14 SDS (SD = 1.37) for the GHD and ISS groups, respectively. Seven subjects had IGF-I concentrations below −2.0 SDS in the GHD group. The values of IGF-I were converted to SDS according to Elmlinger et al. [24].

Magnetic resonance imaging (MRI) of the hypothalamus and the pituitary region was performed in all patients, with normal results.

Serum GH levels were measured with a commercially available chemiluminescent method (IDS-iSYS, ImmunoDiagnostic Systems Ltd, Newcastle upon Tyne, UK), which has no cross-reaction with pegvisomant. Serum IGF-I was measured with a fully automated immunochemistry analyzer, Immulite 2000 (Siemens Diagnostics, Milan, Italy). Growth hormone and IGF-I methods are based on solid-phase two-site immunometric sandwich assays with a chemiluminescence signal.

Serum s-klotho levels were measured by commercially available enzyme-linked immunosorbent assays (Immuno-Biological Laboratories Co, Gunma, Japan); the sensitivity of the assay was 6.15 pg/mL. Intra-assay coefficients of variation (CVs) were 3.1% and 3.5% at concentrations of 2968.78 pg/mL and 186.64 pg/mL, respectively; inter-assay CVs were 2.9% and 11.4% at concentrations of 2903.01 pg/mL and 165.47 pg/mL, respectively.

Statistical analysis

Quantitative variables were expressed as mean values and SDs as they were normally distributed (the Shapiro-Wilk test). Qualitative variables were summarized as counts and percentages. The comparisons between the diagnostic groups were evaluated with a chi-squared test for categorical variables and a Student's t-test (for independent data or paired data) for continuous variables. Pearson's r coefficient was used to analyze correlation. Multiple regression models for repeated data were used to explore associations among s-klotho, IGF-I and the diagnostic groups (in all models we included sex, age, height, BMI, GH peak and AUC to obtain estimated coefficients adjusted for these covariates). All tests were two-sided, and $p < 0.05$ was considered statistically significant. Data analysis was performed with the statistical package Stata (release 14.0, 2015, Stata Corporation, College Station, TX, USA).

Results

Basal IGF-I (125 [SD = 110] vs. 188 [SD = 91] ng/mL; p = 0.059) and s-klotho (1397 [SD = 697] vs. 1760 [SD = 975] pg/mL; p = 0.212) levels were not significantly different between GHD and ISS, respectively (Figures 1 and 2).

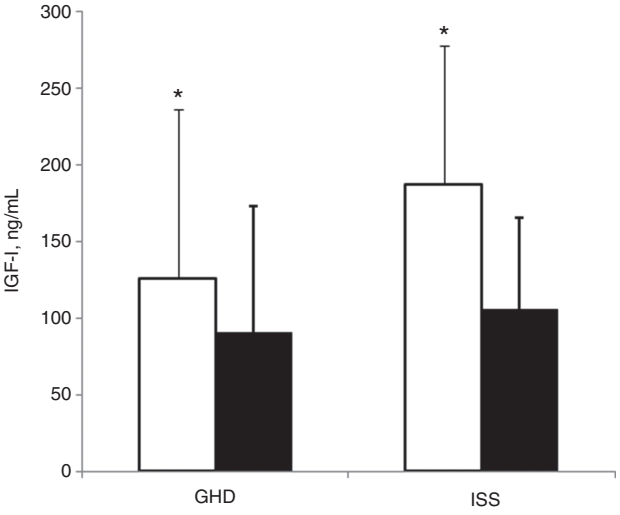

Figure 1: Serum levels of IGF-I before (white boxes) and after pegvisomant priming (black boxes) in GHD and ISS children. Data are expressed as mean and standard deviation.
*p < 0.05 before pegvisomant vs. after pegvisomant priming in the respective groups (t-test).

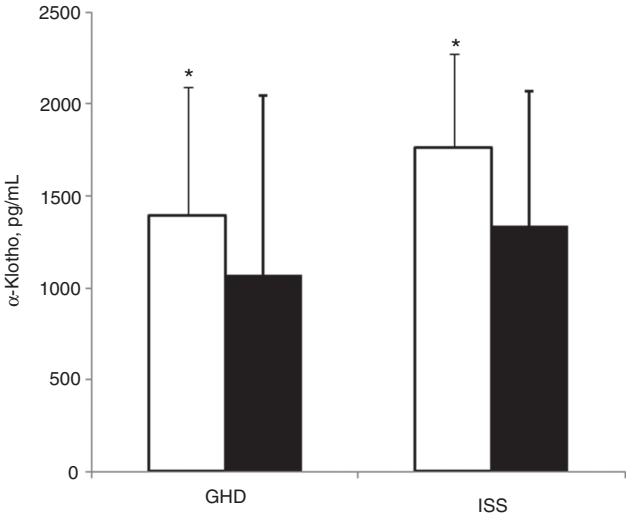

Figure 2: Serum levels of s-klotho before (white boxes) and after pegvisomant priming (black boxes) in GHD and ISS children. Data are expressed as mean and standard deviation.
*$p < 0.05$ before pegvisomant vs. after pegvisomant priming in the respective groups (t-test).

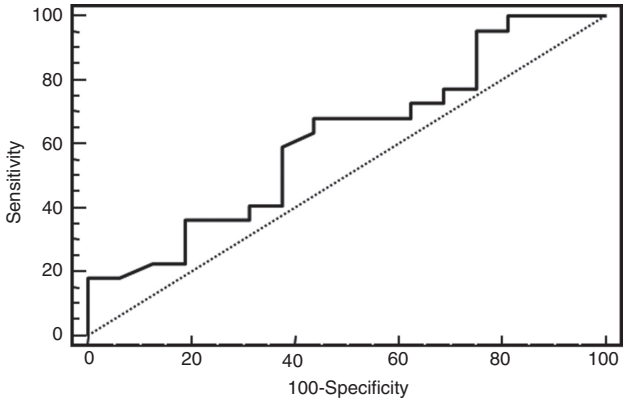

Figure 3: Receiver operating characteristic (ROC) analysis for s-klotho.
Sensitivity: 68.2%; specificity: 56.2%; criterion: >1240 pg/mL.

After pegvisomant priming, a significant reduction of IGF-I was observed in the GHD group (125 [SD = 110] vs. 90 [SD = 83] ng/mL; $p < 0.002$) as well as in the ISS group (188 [SD = 91] vs. 107 [SD = 59] ng/mL; $p < 0.001$) (Figure 1). The delta of IGF-I was significantly greater in the ISS than in the GHD group (84.3 [SD = 59.9] vs. 35.7 [SD = 41.8] ng/mL; $p = 0.008$). s-Klotho also significantly decreased both in the GHD group (1397 [SD = 697] vs. 1069 [SD = 516] pg/mL; $p = 0.002$) and in the ISS subjects (1760 [SD = 975] vs. 1339 [SD = 728] pg/mL; $p < 0.001$) (Figure 2), but the delta of s-klotho was not different between the two groups (GHD: 395 [SD = 422], ISS: 570 [SD = 331]; $p = 0.1$) and the post-pegvisomant values were also not different (GHD: 1069 [SD = 516] pg/mL; ISS: 1339 [SD = 728] pg/mL; $p = 0.1$).

As shown in Figure 3, receiver operating characteristic (ROC) analysis could not identify a threshold to differentiate GHD from non-GHD children.

s-Klotho basal levels significantly correlated with IGF-I levels both before and after pegvisomant priming (GHD: before priming $r = 0.4173$, $p = 0.040$; after priming $r = 0.5604$, $p = 0.0298$; and ISS: before priming $r = 0.7098$, $p = 0.0002$; after priming $r = 0.5428$, $p = 0.009$), but not with the parameters of GH secretion such as AUC or GH peak.

In a multiple regression model with s-klotho as a dependent variable (and IGF-I, time, diagnostic group and basal s-klotho as independent variables), its level was correlated with IGF-I ($p = 0.002$).

Discussion

The aim of this study was to verify whether s-klotho might be useful in the screening for and/or as an adjunctive tool in the diagnosis of GHD. Unfortunately, the results of this study contradict our initial hypothesis. We found, in fact, that GHD and ISS children showed superimposable s-klotho values and, consequently, no cut-off limit by the ROC analysis was found. Furthermore, s-klotho was neither associated with the parameters of GH secretion such as AUC or GH peak nor with auxological parameters such as height and/or BMI. The only correlation we found was with serum IGF-I, which is secreted by the liver under GH stimulation and, therefore, reflects the GH status [25], but it also greatly depends on the nutritional status [26]. Altogether, it might be concluded that s-klotho is a good marker of IGF-I status but not directly of GH secretion.

The IGF-I association with s-klotho is very well demonstrated by the behavior of the two hormones during the pegvisomant priming. Following pegvisomant administration, there is, in fact, a clear drop in both IGF-I and s-klotho levels, while GH levels, on the contrary, increase. This suggests a positive role for IGF-I, and not for GH, in s-klotho secretion. Obviously, if there is a significant decrease in GH levels, as seen in the postoperative stage of patients operated for GH-secreting tumors [21], a significant decrease in IGF-I levels will also be observed and, consequently, a drop in s-klotho levels. This evidence explains the results reported in the paper by Neidert and confirms his suggestion that s-klotho might be useful in the follow-up for these patients.

Furthermore, we looked for factors modulating the response of s-klotho to the GH receptor (GHR) blockade.

In the multiple regression analysis, the only factor influencing α-klotho was IGF-I, which would support the hypothesis of reduced s-klotho secretion following a drop in IGF-I levels. However, association does not mean causality and, therefore, we cannot exclude that reduced secretion of that hormone depends on other mechanisms. For example, it could be possible that GHR blockade results in a reduction of proteolysis of α-klotho from the cell membrane and decreased s-klotho levels, as GH is able to induce proteolytic activity [27]. The only possibility of verifying the dependence of s-klotho on IGF-I would be to maintain high IGF-I serum levels by continuous IGF-I administration. This might be the aim of future studies.

However, our results are consistent with those previously reported in children by other authors [18–20] and, furthermore, they explain the mechanism for their results.

A further outcome of this study is the confirmation of the accuracy of pegvisomant priming in the differential diagnosis of GHD from ISS, as four out of 20 children previously classified as GHD were in fact ISS.

A limitation of the study might be the low number of subjects evaluated, which could have hampered the statistical analysis, thus explaining the lack of statistical difference in α-klotho between the GHD and ISS groups. A further study with a larger number of patients is therefore needed in order to clarify this point.

In conclusion, this study increases our knowledge about the mechanism regulating s-klotho secretion; however, it also clearly shows that s-klotho cannot be employed in the diagnosis of GHD, while confirming its efficacy in the follow-up of patients after surgery for GH-secreting tumors.

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