Serum alpha-klotho concentrations in girls with anorexia nervosa

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ORIGINAL ARTICLE

Abstract

BACKGROUND: Soluble α-klotho may influence energy homeostasis. It also plays a role in calcium-phosphate and vitamin D_3 metabolism regulation. Two so far published studies have demonstrated that serum α-klotho levels in patients with AN are decreased, but their relationships with BMI and metabolic disturbances in these patients remain unclear.
OBJECTIVES: The aim of the study was to assess the association between serum soluble α-klotho levels and glucose, calcium-phosphorus and vitamin D_3 metabolism in girls with acute AN.
METHODS: Serum soluble α-klotho concentrations were evaluated using commercially available ELISA kit in 31 Polish girls with restrictive AN and 29 healthy controls (C). Moreover, anthropometric measurements (weight, height, BMI) and laboratory assays (serum fasting glucose, insulin, HOMA-IR, total calcium, phosphorus as well as 25-hydroxy vitamin D_3 and calcitriol) were performed.
RESULTS: The mean serum α-klotho concentrations in the AN group were significantly lower than in the C group even after adjustment for BMI. Significant correlations between serum α-klotho and body mass (r=0.54; *p*=0.009), BMI (r=0.48; *p*=0.02), serum calcitriol (r=0.48; *p*=0.03), insulin (r=0.49; *p*=0.008) and HOMA-IR (r=0.54; *p*=0.006) were observed in the AN, but not in healthy controls.
CONCLUSIONS: Serum α-klotho concentrations in female adolescents with AN are decreased in comparison with normal weight girls and strongly associated with their nutritional status, insulin sensitivity and active vitamin D_3 levels.

INTRODUCTION

Anorexia nervosa (AN) is an eating disorder characterized by self-induced starvation and excessive weight loss (Oświęcimska 2003). The essence of AN is the person striving to obtain a slim silhouette by deliberately limiting the amount of food eaten, undergoing strenuous physical exercise and inducing vomiting or using laxatives and/or diuretics to lose weight. It is the third common chronic illness among adolescents that leads to a significant emaciation or even severe wasting (Herpertz-Dahlmann 2015). Anorexia nervosa is considered a brain disease with severe metabolic effects on the entire body. Therefore it may serve as a biological model of fat tissue depletion, energy imbalance and/or deficit in humans (Maïmoun *et al.* 2015).

Alpha-klotho, named after the Greek Goddess of Fate, Klotho, who spins the thread of life, was originally characterized as an aging suppressor gene, also known as the messenger of healthy bodies (Fantuzzi 2014).

The human klotho gene – *Kl* is located on chromosome 13. It encodes a type I transmembrane protein composed of the extracellular, transmembrane and cytoplasmatic domains (Kuro-o *et al.* 1997, Nabeshima 2009). Klotho is expressed primarily in the kidney, parathyroid glands, choroid plexus of the brain and adipose tissue (Kuro-o *et al.* 1997). It has been described an essential cofactor for the binding of fibroblast growth factor (FGF 23) to its cognate receptor and thus playing an important role in regulation of phosphate homeostasis (Urakawa *et al.* 2006).

Soluble α-klotho generated either by alternative splicing or shedding the extracellular domain of transmembrane protein affects numerous biological functions as: adipogenesis, angiogenesis, calcium and phosphate metabolism (Kuro-o *et al.* 1997; Chihara *et al.* 2006; Shimada *et al.* 2009; Imura *et al.* 2007). It is involved in the maintenance of vascular health, regulation of insulin signalling and exerts antioxidant, anti-inflammatory and antineoplastic effects (Kurosu *et al.* 2005; Yamamoto *et al.* 2005; Mori *et al.* 2000; Nakatani *et al.* 2009).

Recent reports demonstrated the relationship between α-klotho and BMI and suggest that this protein may influence systemic glucose metabolism and energy homeostasis (Razzaque 2012; Amitani *et al.* 2013).

Two so far published studies reported that serum α-klotho levels in patients with AN are decreased, but their relationships with BMI and metabolic disturbances in these patients remain unclear (Amitani *et al.* 2013; Wolf *et al.* 2015). Thus, we aimed to study the association between serum soluble α-klotho levels and metabolism of glucose, calcium-phosphorus and vitamin D_3 in girls with acute AN.

MATERIALS AND METHODS

Subjects

We recruited 31 girls (mean age: 14.6±1.3 years, range: 11.8–16.6 years) who, following pediatric examination, psychological evaluation, and psychiatric consultation, were diagnosed with a restricting type of AN in accordance with the classification in the *American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders V* (American Psychiatric Association 2013).

These criteria comprise persistent restriction of energy intake leading to significantly low body weight (in context of what is minimally expected); either an intense fear of gaining weight or of becoming fat and disturbance in the way one's body weight or shape is experienced (American Psychiatric Association 2013). Expected body weight was established using normal ranges for a Polish population of females (Palczewska & Niedźwiedzka 2001).

We also assessed the patients using a semi-structured interview (*The Eating Disorder Examination*) (Cooper & Fairburn 1987).

Participants in the AN were examined during the first 2 days of hospitalization at the *Department of Pediatric Endocrinology, University Hospital No 1 in Zabrze, Medical University of Silesia in Katowice, Poland* prior the refeeding therapy. Eligibility criteria consisted of a stable general medical condition, the absence of concomitant diseases (gastrointestinal bleeding, dehydration, peptic ulcer disease, liver and kidney dysfunction) and normal results of additional laboratory tests (serum electrolytes, aspartate and alanine aminotransferases, and creatinine). Girls with any organic or psychiatric disorders, other than AN that could cause cachexia, were excluded from the study. The mean duration of the disease prior to hospitalization was 13.0±8.9 months. Two patients with AN had primary amenorrhea. In 29 girls with anorexia nervosa secondary amenorrhea had been present for a mean duration of 9.9±7.4 months. During hospitalization, patients were placed on bed rest and they received psychotherapy.

The control group consisted of 29 age-matched (mean age: 14.0±1.5 years; range: 12.1–17.9 years), normal weight and regularly menstruating female volunteers who were recruited from secondary schools.

All of the females that we examined were in Tanner IV–V pubertal stages.

None of the participants took any medications, including hormonal drugs within the past 3 months or had infections within the last month before the study. All examined girls were non-smokers.

BMI (body weight [kg]/height [m²]) and standard deviation score (SDS) for BMI were calculated for all participants according to current Polish populational normal ranges (Palczewska & Niedźwiedzka 2001). In the AN group we also calculated the mean rate of body weight loss prior to hospitalization (weight loss

[kg]/duration of the disease [months]), which was 1.3±1.2 kg/month.

Clinical characteristics of the examined groups of girls are given in Table 1.

The study was conducted according to the Declaration of Helsinki and approved by the Bioethics Committee of the Medical University of Silesia in Katowice (registry number KNW/0022/KB1/70/13). Informed written consent for participation in the study was obtained from all study subjects and their parents or legal guardians.

Laboratory analyses

To determine hormone concentrations, blood was drawn between 7:00 AM and 8:30 AM after at least a 12-hour fast. Serum was frozen at –70 °C until the time of assay. Serum soluble α-klotho concentrations were determined using the immunoenzymatic method with the application of the ELISA kit (IBL International, Hamburg, Germany) according to the manufacturer's protocol. The sensitivity of the kit was 6.15 pg/ml. The intra- and inter-assay coefficient of variations were 3.5% and 11.1%, respectively.

Serum calcitriol levels with the application of the ELISA kit (IBL International, Hamburg, Germany). The sensitivity of the kit was 4.8 pg/ml. The intra- and inter-assay coefficient of variations were 6.7% and 9.0%, respectively.

Serum total calcium, phosphate, alkaline phosphatase and fasting glucose levels were evaluated by enzymatic colorimetric assay and serum insulin, "intact" parathormone (iPTH) and 25-hydroxy vitamin D_3 (25-OHD$_3$) concentrations were assayed using ECLIA (*electro-chemiluminescence immunoassay*) using Cobas 600 analyzer (Roche Diagnostics, Basel, Switzerland).

HOMA-IR index was calculated in accordance with the model described elsewhere (Hermsdorff *et al.* 2009).

Statistical analysis

We prepared the database using Excel 2000 (Microsoft Corporation, Albuquerque, New Mexico, USA). We carried out the statistical analysis using Statistica 6.0 software (StatSoft Inc., Tulsa, Oklahoma, USA). A normal data distribution was assessed using the Shapiro-Wilk test; homogeneity of variance was computed using Levene's test. We present the results as means ± standard deviation (SD). We employed the Student's t-test or the Mann-Whitney U test (if the distribution of data differed from normal) for intergroup comparisons. Pearson`s or Spearman's (if the distribution of data differed from normal) correlation coefficients were used to estimate linear relationships between variables. We used analysis of covariance (ANCOVA) to remove the effects of parameters that significantly differed between the groups. The *p*-values <0.05 were considered to be statistically significant.

RESULTS

Anthropometric measurements

Mean body weight, BMI and BMI-SDS were significantly lower In the AN group (*p*<0.0001) (Table 1).

Laboratory assays

Mean serum total calcium, phosphates, alkaline phosphatase, iPTH, fasting glucose, insulin, HOMA-IR and calcitriol levels were significantly lower in AN girls in comparison with healthy controls. There were no statistically significant differences in serum 25-OHD$_3$ between the examined groups of adolescents (Table 2).

Mean serum soluble α-klotho in anorectic girls (743.0±37.2 pg/ml) was significantly higher in comparison to the control group (*p*<0.0001), even after adjusting for BMI (*p*<0.0001) (Figure 1).

Significant positive linear correlations between serum α-klotho and body weight (r=0.54; *p*=0.009), BMI (r=0.48; *p*=0.02), calcitriol (r=0.45; *p*=0.03), insulin (r=0.49; *p*=0.008) and HOMA-IR were noted in the AN group. There were no such correlations in healthy controls (Table 3).

In the AN group, no statistically significant relationships between serum α-klotho and the following were observed: illness duration, maximum body mass before

Tab. 1. Clinical characteristics of the examined groups of girls.

Parameter	AN (n=31)	Control (n=29)	*p*-value
	mean ± SD (range)		
age [years]	14.6±1.3 (11.8–16.6)	14.0±1.5 (12.1–17.9)	0.06
body weight [kg]	40.5±6.6 (18.7–51.0)	52.6±6.2 (40.6–63.0)	<0.0001*
height [m]	1.63±0.06 (1.44–1.60)	1.62±0.04 (1.52–1.71)	0.28
BMI [kg/m²]	15.09±1.99 (9.11–18.20)	19.94±1.95 (15.93–24.03)	<0.0001*
BMI-SDS	−1.79±0.72 (−3.38 to −0.54)	0.20±0.62 (−1.4–1.3)	<0.0001*
maximal body weight prior to the disease onset [kg]	53.77±12.25 (28.00–90.00)		
duration of the disease [months]	13.0±8.9 (4.0–36.0)		
body weight loss [kg]	13.1±9.5 (1.00–46.00)		
rate of body weight loss [kg/month]	1.34±1.22 (0.08–4.76)		
duration of amenorrhea [months]	9.9±7.4 (3.0–30.0)		

AN – anorexia nervosa; BMI – body mass index; SDS – standard deviation score; * statistically significant

the onset of the disease, loss of body mass, the rate of body weight loss and duration of amoenorrhea.

DISCUSSION

Our results confirmed the previous findings that serum α-klotho levels are decreased in AN (Amitani *et al.* 2013; Wolf *et al.* 2015), but a novel finding of this study
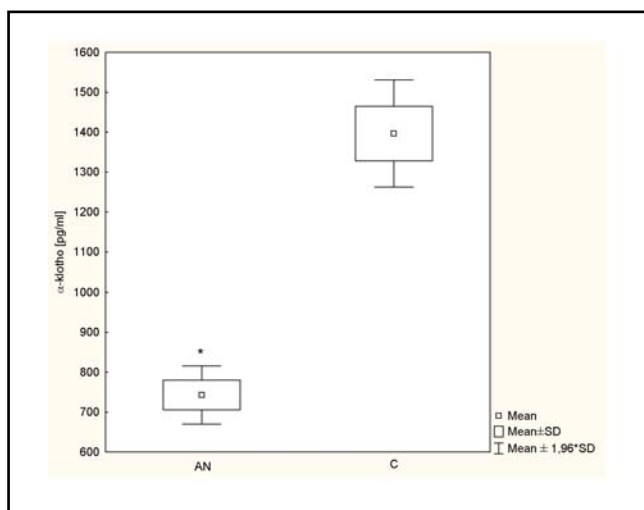

Fig. 1. Serum soluble α-klotho concentrations in the examined groups of girls. AN – anorexia nervosa; C – control; *p<0.0001; SE – standard error

is the observation that AN patients exhibit a significant positive correlation between serum soluble α-klotho and body weight as well as BMI. There was no such relationship in the control group.

Amitani *et al.* (2013) examined serum α-klotho levels in the adult female patients with AN and demonstrated that low plasma klotho levels in the acute phase of the disease increased significantly with the recovery of BMI. This observation was confirmed by others in adolescents, however there was not control group in this study (Wolf *et al.* 2015). Despite apparent relationship between serum α-klotho levels and increase of BMI during refeeding demonstrated in the aforementioned studies there was no linear correlation between these parameters. The plausible explanation of this fact may be the small sample of examined patients (12 and 19, respectively), whereas our study involved 31 girls with AN.

Low klotho levels in AN may contribute to the disease complications, as abolishing klotho function by

Tab. 2. Results of laboratory assays in the examined groups of girls.

Parameter	AN (n=31)	Control (n=29)	p-value
	mean ± SD (range)		
total calcium [mmol/l]	2.44±0.15 (2.03–2.77)	2.52±0.07 (2.41–2.72)	0.006*
phosphate [mmol/l]	1.22±0.16 (0.35–1.48)	1.33±0.16 (0.92–1.63)	0.04*
alkaline phosphatase [U/l]	78.7±96.2 (26.0–580.0)	147.4±81.3 (50.0–358.0)	<0.0001*
iPTH [pg/ml]	23.1±10,3 (8.5–49.8)	43.7±25.1 (17.3–147.0)	0.0002*
25-OHD$_3$ [ng/ml]	21.6±8.7 (5.3–37.6)	19.4±7.8 (7.9–38.6)	0.31
calcitriol [ng/ml]	99.8±10.7 (80,5–118,8)	133.9±12.0 (99.8–148.7)	<0.0001*
fasting glucose [mg/dl]	81.2±9.64 (55.0–100.0)	86.4±5.1 (77.0–94.0)	0.02*
insulin [μIU/ml]	5.89±3.86 (0.39–14,67)	11.36±3.76 (2.84–22.6)	<0.0001*
HOMA-IR	1.12±0.96 (0.07–3.11)	2.32±0.96 (0.06–5.25)	<0.0001*

AN – anorexia nervosa; iPTH – intact parathormone; 25-OHD$_3$ – 25-hydroxy vitamin D$_3$; HOMA-IR – Homeostatic Model Assessment of Insulin Resistance; * statistically significant

Tab. 3. Analysis of correlations among serum soluble α-klotho concentrations and results of anthropometric and laboratory measurements in examined groups of girls.

Parameter	α-klotho [pg/ml]	
	AN (n=31)	Control (n=29)
body weight [kg]	r=0.54* p=0.009	r=0.12 p=0.53
BMI [kg/m²]	r=0.48* p=0.02	r=0.15 p=0.43
BMI-SDS	r=0.39 p=0.07	r=0.13 p=0.49
total calcium [mmol/l]	r=-0.09 p=0.25	r=-0.13 p=0.52
phosphate [mmol/l]	r=-0.11 p=0.24	r=0.05 p=0.81
alkaline phosphatase [U/l]	r=0.01 p=0.97	r=0.01 p=0.94
25-OHD$_3$ [ng/ml]	r=-0.12 p=0.567	r=-0.09 p=0.65
calcitriol [ng/ml]	r=0.45* p=0.03	r=0.13 p=0.50
iPTH [pg/ml]	r=-0.20 p=0.36	r=0.12 p=0.95
fasting glucose [mg/dl]	r=0.23 p=0.30	r=-0.08 p=0.66
insulin [mIU/ml]	r=0.49* p=0.008	r=-0.06 p=0.77
HOMA-IR	r=0.54* p=0.006	r=0.09 p=0.61

AN – anorexia nervosa; BMI – body mass index; SDS – standard deviation score; iPTH – intact parathormone; 25-OHD$_3$ – 25-hydroxy vitamin D$_3$; HOMA-IR – Homeostatic Model Assessment of Insulin Resistance; * statistically significant

knockout or knockdown of *Kl* results in the generation of lean mice with decreased white adipose tissue accumulation, hypogonadism, muscle atrophy, osteopenia, abnormalities in glucose metabolism and shortened lifespan (Kuro-o *et al.* 1997). $Kl^{-/-}$ mice are also resistant to obesity induced by high-fat diet. Interestingly, the disturbances observed in the animal model of klotho deficiency are very similar to these found in AN (Westmoreland *et al.* 2015).

Recent studies suggest that klotho is involved in adipogenesis by promoting differentiation of preadipocytes into adipocytes. Chihara *et al.* (2005) proved that klotho mRNA expression increases during the first 3 days of adipocyte differentiation and markers of this process such as C/EBPα, C/EBPβ, C/EBPδ, PPAR-γ and aP2 decrease with the suppression of *Kl* gene (Chihara *et al.* 2005). Moreover, elimination of klotho function reduces intracellular lipid accumulation in the liver (Ohnishi *et al.* 2011).

In healthy subjects serum klotho does not correlate with body weight or BMI and our findings are consistent with the results of previous studies (Pedersen *et al.* 2013; Gkentzi *et al.* 2014). Thus, our findings support the hypothesis by Wolf *et al.* (2015) that reduced klotho levels may directly contribute to the reduction of fat mass and BMI observed in AN.

Another novel finding of our study is the positive correlation between serum α-klotho levels and insulin as well as HOMA-IR in the AN group.

Serum insulin levels and HOMA-IR are reduced in AN, protecting these subjects against hypoglycemia (Prioletta *et al.* 2011, Dostálova *et al.* 2009). Similarly, mice lacking klotho function have reduced pancreatic insulin content, but still develop hypoglycemia owing to increased insulin sensitivity (Utsugi *et al.* 2000). On the other hand, overexpression of klotho leads to biochemical features of insulin resistance (Kurosu *et al.* 2005).

Mechanisms underlying klotho effects on glucose metabolism are unclear. Klotho may influence insulin signaling either via its expression in tissues involved in carbohydrate metabolism or as a hormone, after release of the klotho extracellular domain from renal tubular cells (Chen *et al.* 2007). Kurosu *et al.* (2005) demonstrated that klotho does not bind to the insulin receptor itself, but supresses its ligand-stimulated autophosphorylation in a dose-dependent manner. Conversly, insulin is reported to stimulate cleavage and release of the extracellular domain of klotho by ADAM10 and ADAM17 (Chen *et al.* 2007). This protein may also be linked to the glucose and energy metabolism indirectly, through stimulation of FGF23 production in bone as elevated FGF23 concentrations in human may be considered a biomarker of dyslipidemia, insulin resistance and metabolic syndrome. (Smith *et al.* 2012; Hanks *et al.* 2015; Wójcik *et al.* 2012).

Serum calcitriol levels in our study were significantly lower in patients with AN in comparison with the healthy controls. On the other hand, serum 25-OHD$_3$ concentrations did not differ between the groups. This suggests altered vitamin D$_3$ metabolism in chronic, severe malnutrition and has been reported elsewhere (Aarskog *et al.* 1986; Veronese *et al.* 2015).

Lower serum active vitamin D$_3$ in undernourished girls may result in decreased calcium and phosphates and elevated intact parathormone due to compensatory mechanisms of calcium-phosphate balance, which was confirmed by our results. Another reason of hypophosphatemia in AN is decreased insulin concentration. Insulin promotes phosphate and glucose uptake in peripheral tissues and to ensure an adequate supply of phosphate to support energy production it enhances renal phosphate retention at the same time (Pi & Quarles 2013).

Klotho plays an important role in regulation of vitamin D$_3$ metabolism and inappropriate expression of this protein is constantly associated with abnormal serum 1,25-dihydroxy vitamin D$_3$ concentrations (Wang TT *et al.* 2005). In turn, it has been demonstrated that calcitriol may directly stimulate klotho mRNA expression in the kidney, through a putative vitamin D–responsive element (VDRE) that has been identified in the promoter of the human klotho gene (Tsujikawa *et al.* 2003).

In our study we observed significant positive correlations between α-klotho and calcitriol levels in AN, but not in healthy controls. Since α-klotho restores to the normal values during refeeding (Amitani *et al.* 2013; Wolf *et al.* 2015) we suggest that this protein may be potentially involved in the disturbances of vitamin D$_3$ metabolism observed in AN.

In conclusion, we have confirmed that serum α-klotho concentrations in female adolescents with AN are decreased in comparison with normal weight girls. Positive associations between α-klotho and nutritional status, insulin sensitivity and active vitamin D$_3$ levels may suggest that reduced klotho may contribute to the metabolic disturbances occurring in malnutrition. Our findings support the hypothesis that klotho as a multiple function regulatory protein may be a common link between carbohydrate and calcium-phosphate metabolism.

ACKNOWLEDGEMENTS

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