Vaspin and selected indices of bone status in girls with anorexia nervosa

Waspina a wybrane wykładniki stanu kośćca u dziewcząt z jadłowstrętem psychicznym

Zofia Ostrowska[1], Katarzyna Ziora[2], Joanna Oświęcimska[2], Elżbieta Świętochowska[1], Bogdan Marek[3], Dariusz Kajdaniuk[3], Joanna Strzelczyk[1], Karolina Gołąbek[1], Małgorzata Morawiecka-Pietrzak[1], Kinga Wołkowska-Pokrywa[1], Beata Kos-Kudła[3]

[1]*Department of Medical and Molecular Biology, School of Medicine with the Division of Dentistry in Zabrze, Medical University of Silesia, Katowice, Poland*

[2]*Department of Paediatrics, School of Medicine with the Division of Dentistry in Zabrze, Medical University of Silesia, Katowice, Poland*

[3]*Department of Pathophysiology and Endocrinology, School of Medicine with the Division of Dentistry in Zabrze, Medical University of Silesia, Katowice, Poland*

Abstract

Introduction: In vitro studies indicate that vaspin may act as a regulator of bone metabolism. The aim of the study was to evaluate the relationship between vaspin and bone metabolism in girls with anorexia nervosa (AN), as well as the potential involvement of OPG and RANKL in this relationship.

Material and methods: Serum vaspin, OC, CTx, OPG, and sRANKL were determined by ELISA in 50 girls with AN and in 30 healthy controls aged 13 to 17 years.

Results: Girls with AN exhibited significant reduction in body weight, BMI, and Cole index as well as a significant increase in serum level of vaspin compared to healthy participants. These changes were associated with a significant decrease in serum OC and CTx levels and a significant increase in OPG and sRANKL, while the OPG/sRANKL ratio was significantly decreased. BMI and Cole index correlated negatively and significantly with CTx levels in the control group (C), girls with AN, and all study participants (C+AN). Girls with AN showed a significant negative correlation between BMI, the Cole index, and OPG levels. The combination group (C+AN) showed a significant positive correlation between BMI, Cole index, and the OPG/sRANKL ratio. In this group of girls vaspin levels correlated positively and significantly with sRANKL and negatively with body weight, BMI, Cole index, and OPG/sRANKL ratio. Girls with AN showed a significant negative correlation between vaspin levels and the OPG/sRANKL ratio.

Conclusions: Undernourishment and associated deficit of adipose tissue may result in inadequate vaspin production and bone metabolism disorders in girls with AN. Vaspin acts as a coordinator of the dynamic balance between bone formation and resorption processes; its action is affected by the cytokines of the RANKL/RANK/OPG system. Changes in the relationships between vaspin, bone markers, OPG, and RANKL might contribute to the development of osteoporosis in girls with AN. **(Endokrynol Pol 2016; 67 (6): 599–606)**

Key words: *anorexia nervosa; girls; vaspin; bone metabolism; OPG; sRANKL; OPG/sRANKL ratio*

Streszczenie

Wstęp: Badania *in vitro* sugerują, że waspina może mieć znaczenie w regulacji metabolizmu kostnego. Celem pracy była ocena związku między waspiną a metabolizmem kostnym u dziewcząt z jadłowstrętem psychicznym (AN) z uwzględnieniem ewentualnego udziału OPG i RANKL w mechanizmie powiązań między nimi.

Materiał i metody: U 50 dziewcząt z AN i 30 zdrowych w wieku 13–17 lat oceniono stężenia waspiny, OC, CTx, OPG i sRANKL w surowicy metodą ELISA.

Wyniki: U dziewcząt z AN wykazano istotne zmniejszenie masy ciała, BMI i wskaźnika Cole'a oraz znamienny wzrost stężenia waspiny w surowicy w porównaniu z grupą dziewcząt zdrowych. Zmianom tym towarzyszyła znamienna supresja stężeń OC, CTx oraz wzrost stężeń OPG i sRANKL w surowicy przy istotnie obniżonych wartościach wskaźnika OPG/sRANKL. Wartości wskaźników BMI i Cole'a korelowały istotnie i ujemnie ze stężeniami CTx w grupie kontrolnej (C), u dziewcząt z AN i u wszystkich dziewcząt łącznie (grupa C+AN) i OPG (u dziewcząt z AN). Istotną i dodatnią korelację wykazano między wartościami wskaźników BMI i Cole'a a wartościami wskaźnika OPG/sRANKL w grupie C+AN. W grupie tej stężenia waspiny korelowały istotnie i dodatnio ze stężeniami sRANKL, a ujemnie z wartościami masy ciała, BMI, wskaźnika Cole'a i wskaźnika OPG/sRANKL. Ujemną i znamienną korelację między stężeniami waspiny a wartościami wskaźnika OPG/sRANKL zanotowano u dziewcząt z AN.

Wnioski: Niedożywienie i związany z nim deficyt tkanki tłuszczowej mogą indukować nieprawidłowości w produkcji waspiny i metabolizmie kostnym u dziewcząt z AN. Waspina wpływa na dynamiczną równowagę między procesami tworzenia i resorpcji tkanki kostnej, a ważną rolę w mechanizmie tego oddziaływania odgrywają cytokiny systemu RANKL/RANK/OPG. Zaburzenia w powiązaniach między waspiną, markerami kostnymi oraz OPG i RANKL mogą wpływać na rozwój osteoporozy u dziewcząt z jadłowstrętem psychicznym. **(Endokrynol Pol 2016; 67 (6): 599–606)**

Słowa kluczowe: *jadłowstręt psychiczny; dziewczęta; waspina; metabolizm kostny; OPG; sRANKL; wskaźnik OPG/sRANKL*

✉ Zofia Ostrowska M.D., Department of Medical and Molecular Biology, School of Medicine with the Division of Dentistry in Zabrze, Silesian Medical University, Jordana St. 19, 41–808 Zabrze, Poland, phone: +48 32 272 21 71, e-mail: ozdrasiek@wp.pl

Introduction

Vaspin, a visceral adipose tissue-derived serine protein-ase inhibitor, was isolated in 2005 from visceral adipose tissues of Otsuka Long-Evans Tokushima Fatty (OLETF) rat, an animal model of abdominal obesity and type 2 diabetes mellitus [1, 2]. Animal studies revealed that vaspin improved glucose tolerance, increased insulin sensitivity, reduced food intake, lowered blood glucose, and exhibited anti-inflammatory action [2–4]. Vaspin expression was almost undetectable in the diabetes-resistant lean rats (LETO) in comparison to OLETF rats. Experimental studies of Gonzales et al. [5] showed that vaspin gene expression in rat white adipose tissue was the lowest after fasting; leptin administration and chronic treatment with metformin increased its expression. Decreased levels of growth hormone (GH) and thyroid hormones also suppressed vaspin expression [5].

In humans, vaspin expression was demonstrated in subcutaneous and visceral adipose tissue of obese individuals with normal glucose tolerance [6]. Vaspin expression in visceral adipose tissue increased along with an increase in body mass index (BMI), body fat percentage and glucose concentration in hour 2 of an oral glucose tolerance test [6,7]. Subcutaneous vaspin expression was positively correlated with waist-to-hip ratio (WHR) and fasting serum insulin concentration [8]. In the normal glucose-tolerant group, vaspin serum concentration was higher in women compared to men, and it correlated positively with body fat mass and BMI [9]. Elevated vaspin serum concentrations were associated with obesity and impaired glucose tolerance; however, study participants with diabetes did not exhibit such correlations [10, 11]. Some researchers believe that elevation of serum vaspin may arise as a compensatory mechanism for obesity and insulin resistance [8, 12–14]. El-Mesalamy et al. [15] demonstrated vaspin elevation in non-obese and obese type 2 diabetes patients compared with the control subjects. Women with poor glycaemic control (HbA$_{1c}$ > 7%) had higher vaspin levels than those with HbA$_{1c}$ < 7% [14].

Serum vaspin concentrations are related to physical activity; the concentrations are lower in normal-weight and regularly exercising individuals but tend to increase in obese people on weight loss regimens combined with physical exercise programs [10]. Handisuraya et al. [16] observed that bariatric surgery-induced weight loss resulted in a reduction of serum vaspin that correlated significantly with the reduction of circulating leptin, insulin, and C-peptide levels and with the amelioration of insulin sensitivity. In obese children, vaspin levels were significantly higher compared to their normal-weight peers [17, 18]. Vaspin levels were positively correlated

with triglycerides, HOMA-IR, and BMI, while their correlation with adiponectin levels was negative. The body fat percentage was found to be the strongest predictor of visceral vaspin, and insulin sensitivity seems to be the strongest determinant of subcutaneous vaspin expression [17]. On the other hand, Körner et al. [19] revealed that vaspin concentration was lower in obese girls compared to lean controls; also, no correlation was found between serum vaspin and BMI. It is generally believed that differences in vaspin levels between obese individuals (children, adolescents, adults) are related to gender, age, physical activity, and hormone metabolism.

Few researchers have studied vaspin levels in individuals with body mass deficits [20, 21]. Bergmann and Sypniewska [20] found an approximately 50% decrease in vaspin levels in their study participants with BMI < 25 kg/m² compared to overweight subjects (BMI 25–29.9 kg/m²). Vehapoglu et al. [21] observed that vaspin concentrations were significantly lower in underweight prepubertal children (youngest age = 2 years) compared to the control group. Our preliminary investigations revealed vaspin elevation in girls with AN aged 13 to 17 years as compared to normal-weight, age-matched controls (C) [22]. The combination group (C+AN) exhibited a negative correlation between vaspin levels and body weight and BMI, suggesting that undernourishment might cause abnormal levels of vaspin.

The most recent in vitro studies seem to indicate that, along with leptin, adiponectin, resistin, visfatin, apelin and omentin-1 [23–27], vaspin may regulate bone metabolism. Kamio et al. [28] concluded that vaspin inhibited RANKL-induced osteoclastogenesis in RAW264.7 cells and bone marrow cells. It also attenuated the apoptosis of human osteoblasts [29]. Hence the hypothesis that changes in vaspin levels observed in girls with AN [22] might play a role in inducing changes in bone metabolism found in these patients, the effect of which might be mediated by the RANKL/RANK/OPG system. Therefore, we designed a study to examine the relationship between vaspin and the well-established markers of bone metabolism, i.e. osteocalcin (OC) — a marker of bone formation, and C-terminal telopeptide of type I collagen α 1 chain (CTx) — a marker of bone resorption in girls with AN. We also considered the role of the cytokines of the RANKL/RANK/OPG system, i.e. OPG and sRANKL in this relationship. The obtained results might help to gain deeper insight into the pathomechanisms of osteoporosis development in girls with AN.

The aim of the study was to evaluate the relationship between vaspin and bone metabolism in girls with AN, taking into consideration a potential role of OPG and RANKL in this relationship.

Material and methods

The study group consisted of 50 girls aged 13 to 17 years, hospitalised at the Paediatric Endocrinology Division of the Paediatric Department in Zabrze, who, following an examination by paediatricians and a psychiatrist, were diagnosed with AN based on the American Psychiatric Association's classification and diagnostic tool, i.e. a DSM-IV of 1994. The girls with AN underwent all tests during the first three days of hospital stay, i.e. prior to the launch of therapy. All other somatic or mental disorders that might lead to cachexia were ruled out. The mean age of the AN patients was 15.02 ± 1.52 years (Table I). All had secondary amenorrhoea. The duration of the disease was 3-60 months. The control group consisted of 30 healthy, regularly menstruating girls (mean age 15.99 ± 2.19 years) with no endocrine or other disorders that could affect bone tissue metabolism; they were all schoolgirls from the city of Zabrze, who volunteered to participate in the study.

Objective assessment included anthropometric evaluation such as weight and height measurements. Body mass index (BMI; weight/height^2 [kg/m^2]) and the Cole index (CI; weight × height^2 × 100 [%]) were calculated [23]. The Cole index reflects the nutritional status of an individual and encompasses the following categories: wasting — < 75%; undernourished — 75–85%; mildly undernourished — 85–90%; adequately nourished — 90–100%; and overnourished — > 110% [acc. to 24]. The mean body weight of our AN patients was: 39.89 ± 5.401 kg, BMI: 15.61 ± 2.08 kg/m^2, and the Cole index: 79.88 ± 0.19%. The mean body weight of the control participants was: 55.93 ± 7.21 kg, BMI: 20.54 ± ± 2.12 kg/m^2, and the Cole index: 100.18 ± 0.89% (Table I).

On the day of the examination the girls did not report any complaints; none of them suffered from an acute infection during the preceding month. Blood samples for the determination of vaspin, OC, CTx, as well as OPG and its soluble ligand sRANKL were collected between 08.00 and 09.00 hours after a 12-hour fast. Serum obtained by centrifugation was frozen and stored at –75°C until assay. Determinations of vaspin, OC, CTx, OPG, and sRANKL concentrations were performed using High-Sensitivity Human ELISA kits: vaspin (BioVendor Laboratorni medicina a.s., Czech Republic), OC (DSL Inc., USA), CTx (Nornic Bioscience Diagnostics A/S, Denmark), OPG, and sRANKL (Biomedica, Austria). The respective sensitivity and intra- and interassay errors were: 0.01 ng/mL, 7.6% and 7.7% for vaspin; 0.05 ng/mL, 5.8 and 7.3% for OC; 0.08 nmol/L, 5.2 and 6.7% for CTx; 0.14 pmol/L, 7 and 7.5% for OPG; 0.04 pmol/L, 5 and 7% for sRANKL.

The database was prepared using Excel 2000 (Microsoft Corporation). Statistical analysis was carried out

Table I. *Mean values of age, height, body weight, body mass index (BMI), the Cole index (CI), mean serum levels of vaspin, osteocalcin (OC), C-terminal telopeptide of type I collagen α 1 chain (CTx), osteoprotegerin (OPG), soluble receptor activator of nuclear factor-κB ligand (sRANKL), and mean values of the OPG/sRANKL ratio in girls with anorexia nervosa and in the control group*

Tabela I. *Średni wiek, wzrost, masa ciała, wskaźnik masy ciała (BMI), wskaźnik Cole'a (CI), średnie stężenia waspiny, osteokalcyny (OC), C-terminalnego usieciowanego telopeptydu łańcucha α1 kolagenu typu I (CTx), osteoprotegeryny (OPG), rozpuszczalnego ligandu receptora aktywatora czynnika jądrowego-κB (sRANKL) oraz wartości wskaźnika OPG/ /sRANKL w grupie kontrolnej i u dziewcząt z jadłowstrętem psychicznym*

Variables	Groups	
	Control group (n = 30)	Anorexia nervosa (n = 50)
Age (years)	15.99 ± 2.19	15.02 ± 1.57
Height [m]	1.64 ± 0.09	1.61 ± 0.08
Body weight [kg]	55.93 ± 7.21	**39.89 ± 5.41***
BMI [kg/m²]	20.54 ± 2.12	**15.61 ± 2.08***
CI (%)	100.18 ± 0.89	**79.88 ± 0.19***
Vaspin [ng/mL]	0.42 ± 0.08	**0.58 ± 0.08***
OC [μmol/L]	4.34 ± 1.05	**3.52 ± 1.59***
CTx [nmol/L]	5.92 ± 1.72	**3.55 ± 2.50***
OPG [pmol/L]	3.40 ± 0.64	**4.69 ± 0.80***
sRANKL [pmol/L]	0.51 ± 0.17	**0.62 ± 0.28***
OPG/sRANKL ratio	12.38 ± 4.65	**8.86 ± 2.86***

*p ≤ 0.05 *vs.* control group

with Statistica 10 for Windows (StatSoft Inc., USA). All data were tested for normality of distribution with the Shapiro-Wilk algorithm. The Student t-test was used to determine the significance of intergroup differences (normal distribution of variables). In the case of non-normal distribution, the significance was tested using the Mann-Whitney U test. The relationships between the BMI, Cole index and OC, CTx, OPG, sRANKL, and the the OPG/sRANKL ratio as well as between vaspin and age, height, body weight, BMI, Cole index, OC, CTx, OPG, sRANKL and the OPG/sRANKL ratio in control participants (C), girls with AN, and the combination group of C+AN were analysed using the Spearman's ranked correlation test. Statistical significance was set at p ≤ 0.05.

The study was approved by the Bioethics Committee of the Silesian Medical University in Katowice (KNW/0022/KB1/105/09). Informed consent to participate in the study was obtained from the patients, their parents, or guardians.

Table II. *Correlation between body mass index (BMI), Cole index (CI) and osteocalcin (OC), C-terminal telopeptide of type I collagen α1 chain (CTx), osteoprotegerin (OPG), soluble receptor activator of nuclear factor-κB ligand (sRANKL) levels, and the OPG/sRANKL ratio in the control group (C), in girls with anorexia nervosa (AN), and in all girls (C+AN)*

Tabela II. *Tabela II. Korelacja między wartościami wskaźnika masy ciała (BMI) i wskaźnika Cole'a (CI) a osteokalcyną (OC), C-terminalnym usieciowanym telopeptydem łańcucha α1 kloagenu typu I (CTx), osteoprotegeryną (OPG), rozpuszczalnym ligandem receptora aktywatora czynnika jądrowego-κB (sRANKL) i wartościami wskaźnika OPG/sRANKL w grupie kontrolnej (C), u dziewcząt z jadłowstrętem psychicznyn (AN), i u wszystkich dziewcząt łącznie (C + AN)*

Variables		Values of correlation coefficients		
		C (n = 30)	AN (n = 50)	C+AN (n = 80)
BMI [kg/m²]	OC [μmol/L]	−0.322	0.211	−0.202
	CTx [nmol/L]	**−0.476***	**−0.380***	**−0.345***
	OPG [pmol/L]	−0.079	**−0.270***	−0.163
	sRANKL [pmol/L]	0.058	0.150	−0.066
	OPG/sRANKL ratio	0.060	0.146	**0.250***
CI (%)	OC [μmol/L]	−0.299	0.199	−0.150
	CTx [nmol/L]	**−0.488***	**−0.419***	**−0.334***
	OPG [pmol/L]	−0.190	**−0.209***	−0.098
	sRANKL [pmol/L]	−0.051	0.169	−0.070
	OPG/sRANKL ratio	0.082	0.160	**0.257***

*p ≤ 0.05 — statistically significant values of correlation coefficients

Results

The mean body weight, BMI, and the Cole index were significantly lower in girls with AN compared to healthy controls, while the mean serum vaspin was significantly higher. All these changes were associated with marked suppression of the mean serum OC and CTx and an increase in the mean serum OPG and sRANKL, while the mean value of the OPG/sRANKL ratio was decreased compared to normal-weight controls (Table I).

In all groups, i.e. the control participants, girls with AN, and the combination C+AN group, the BMI and Cole indexes correlated negatively and significantly with CTx. Girls with AN also exhibited a negative and significant correlation between the BMI and Cole indexes and OPG. The C+AN group showed a positive and significant correlation between the BMI and Cole indexes and the OPG/sRANKL ratio (Table II).

In the combination group (C+AN), vaspin levels were positively and significantly correlated with sRANKL, while their correlation with body weight, the BMI, and Cole indexes and the OPG/sRANKL ratio was negative. Girls with AN had a negative and significant correlation between serum vaspin levels and the OPG/ /sRANKL ratio values (Table III).

Discussion

In our previous [22] and present studies, we found an increase in serum vaspin in girls with AN compared to normal-weight healthy controls with normal BMI range (group C). We also observed a negative and significant correlation between body weight, BMI, and vaspin levels in all study participants (group C+AN), i.e. in the group that exhibited considerable body weight differences. Additionally, the results of the present study revealed a negative and significant correlation between the Cole index and serum vaspin in this group. These findings indicate that undernourishment and associated adipose tissue deficit may result in inadequate vaspin production in girls with AN.

Few researchers studied circulating vaspin levels in underweight persons, but some observed a significant decrease in serum vaspin in individuals with BMI < 25 kg/ /m² compared to overweight participants [20]. Mean vaspin levels were significantly lower in underweight prepubertal children with thinness grades of 1, 2, and 3 and body weights < 90% of ideal body weight due to loss of appetite and less frequent hunger episodes compared to the control group [21]. The authors concluded that in underweight children, decreased vaspin levels should be considered in the aetiology of anorexia. It was also shown that although circulating vaspin was lower in slim and regularly exercising study subjects than in untrained individuals [10, 32], its concentrations increased in obese people on weight loss regimens combined with physical exercise programs [10]. This paradox has been accounted for by the fact that the serum vaspin level is differentially regulated in the non-active resting state and after exercise. Dietary

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Table III. *Correlation between vaspin levels and age, height, body weight, body mass index (BMI), the Cole index (CI), as well as osteocalcin (OC), C-terminal telopeptide of type I collagen α 1 chain (CTx), osteoprotegerin (OPG), soluble receptor activator of nuclear factor-κB ligand (sRANKL) levels, and the OPG/sRANKL ratio in the control group (C), in girls with anorexia nervosa (AN), and in all girls (C+AN)*

Tabela III. *Korelacja między stężeniami waspiny a wiekiem, wzrostem, masą ciała, wskaźnikiem masy ciała (BMI), wskaźnikiem Cole'a (CI) oraz stężeniami osteokalcyny (OC), C-terminalnego usieciowanego telopeptydu łańcucha α 1 kolagenu typu I (CTx), osteoprotegeryną (OPG), rozpuszczalnym ligandem receptora aktywatora czynnika jądrowego-κB (sRANKL) oraz wartościami wskaźnika OPG/sRANKL w grupie kontrolnej (C), u dziewcząt z jadłowstrętem psychicznym (AN), i u wszystkich dziewcząt łącznie (C+AN)*

Variables		Values of correlation coefficients		
		C (n = 30)	AN (n = 50)	C+AN (n = 80)
Vaspin [ng/mL]	Age (years)	−0.084	0.185	0.054
	Height [m]	0.236	0.073	0.207
	Body weight [kg]	−0.077	−0.153	**−0.204***
	BMI [kg/m²]	−0.264	−0.136	**−0.298***
	CI (%)	−0.021	−0.160	**−0.268***
	OC [μmol/L]	0.018	−0.049	−0.120
	CTx [nmol/L]	0.138	0.299	0.123
	OPG [pmol/L]	−0.138	−0.046	−0.085
	sRANKL [pmol/L]	0.142	0.133	**0.250***
	OPG/sRANKL ratio	0.301	**−0.298***	**−0.231***

*p ≤ 0.05 — statistically significant values of correlation coefficients

interventions [33–35] and bariatric surgery [16, 36] used in obese people usually resulted in weight loss. It has been speculated that differences in vaspin concentrations seen in underweight individuals are associated with gender, age, physical activity, and interference of several endogenous factors, mainly hormones.

Studies carried out by Gonzales et al. [5] revealed that white adipose tissue (WAT) vaspin mRNA levels were down-regulated in hyperthyroid rats and significantly increased in hypothyroid animals in comparison to euthyroid ones. Hence the final conclusion that thyroid status plays an important role in the regulation of vaspin in rats. Abnormal levels of vaspin in hypo- and/or hyperthyroid patients have been reported with controversial results. Cinar et al. [37, 38] showed that vaspin levels were not correlated with TSH levels in hypothyroid patients before treatment and after establishment of euthyroidism. On this basis the authors concluded that vaspin might be differently regulated in humans and rodents. Although it was not meant to investigate the relationship between vaspin and thyroid function in humans, one study examined the relation between TSH and vaspin levels before and after weight loss by bariatric surgery (RYGB) [16]. Handisuraya et al. [16] showed that RYGB induced a significant reduction in circulating TSH levels, which correlated positively with changes in vaspin levels. The Gonzales et al. [5] studies also revealed that WAT vaspin

mRNA levels were down-regulated in GH-deficient rats compared to control animals, suggesting that vaspin may be particularly sensitive to the influence of GH. The above-mentioned experimental and clinical data suggested that somatotroph cell function can play a role in vaspin regulation. Thus, it could be hypothesised that, in girls with AN, circulating vaspin levels might be influenced by changes in GH and thyroid hormone concentrations. Persistent fasting results in disturbances in somatoliberin and somatostatin secretion in AN patients. Despite elevated GH levels, insulin-like growth factor I (IGF-I) is reduced, which leads to an acquired GH-resistant state [39–42]. Patients with AN exhibit high GH concentrations both at baseline and after somatoliberin or thyreoliberin stimulation, while somatostatin levels remain low. On the other hand, a tendency towards suppression of the hypothalamic-pituitary-thyroid axis was also found [24, 41, 43, 44]. A decrease of 5′-deiodinase activity compromises conversion of thyroxine (T_4) to 3,5,3′-triiodothyronine (T_3), giving rise to the hormonally inactive 3,3′,5′-triiodothyronine, i.e. reverse T_3 (rT_3) [24, 42, 44]. Experimental studies in rats also demonstrated that vaspin concentrations decreased after fasting and were partially recovered after leptin treatment [5], results which were then confirmed by human studies [7]. On the other hand, vaspin levels were negatively correlated with adiponectin [7]. Hence, changes in leptin and adiponec-

tin concentrations observed in girls with AN [26, 35, 45] might possibly affect serum vaspin concentrations.

The most recent studies, especially *in vitro* studies, indicated that, along with leptin, adiponectin, resistin, visfatin, apelin and omentin-1 [24–27], vaspin may also act as a bone metabolism regulator. Kamio et al. [28] found that vaspin inhibited RANKL-induced osteoclastogenesis in RAW264.7 cells and bone marrow cells (BMCs) as well as the RANKL-induced expression of nuclear factor of activated T cells c1 (NFATc1) in RAW264.7 cells and BMCs. Vaspin also inhibited the RANKL-induced upregulation of matrix metalloproteinase-9 and cathepsin K in RAW264.7 cells. Hence the final conclusion that vaspin downregulates osteoclastogenesis in part by inhibiting expression of the transcription factor NFATc1 [28]. Vaspin also attenuated the apoptosis of human osteoblasts [29]. Western blot analysis indicates that, depending on the dose, vaspin upregulates the expression of anti-apoptotic gene Bcl-2 and downregulates the expression of BAX, which plays a crucial role in apoptosis induction. Vaspin stimulates the phosphorylation of ERK; hence, pretreatment of human osteoblasts with ERK inhibitor PD98059 blocks the ERK pathway. However, vaspin does not stimulate the phosphorylation of p38, JNK, or Akt. It attenuates the apoptosis of human osteoblasts by activating the MAPK/ /ERK signalling; this suggests that vaspin might be involved in bone metabolism regulation (via its protective effect on osteoblasts). Based on the *in vitro* results, it is likely that changes in vaspin concentrations observed in girls with AN might, along with changes in other adipose tissue hormones [26, 27], play a role in bone metabolism disturbances with the RANKL/RANK/OPG system being involved also. To our knowledge there have been no studies on the relationship between circulating vaspin levels and bone metabolism in girls with AN with the consideration of the role of the RANKL/ /RANK/OPG system's cytokines in this relationship.

Our investigations have shown that undernourishment and extreme weight loss may not only lead to abnormal levels of vaspin but also to bone metabolism disturbances. Reduction in body weight, BMI, the Cole index, and serum vaspin elevation in girls with AN were associated with significant suppression of OC and CTx and an increase in serum OPG and sRANKL, while the OPG/sRANKL ratio was significantly decreased. In all study groups (C, AN, and C+A) the BMI and Cole indexes were negatively and significantly correlated with CTx concentrations. In girls with AN, the BMI and Cole indexes also correlated negatively and significantly with OPG concentrations. In the combination group, i.e. C+AN, there was a positive and significant correlation between the BMI and Cole indexes and the OPG/ /sRANKL ratio as well as between serum vaspin and sRANKL. Vaspin concentrations were negatively and significantly correlated with the OPG/sRANKL ratio in girls with AN and the combination group (C+AN). Differences in results from in vivo and in vitro studies on the relationships between bone metabolism, RANKL/RANK/OPG system cytokines, and vaspin may be associated with the interference of several endogenous factors, mainly hormones (i.e. oestrogens, glucocorticoids, parathyroid hormone, and vitamin D), adipose tissue hormones other than vaspin, and cytokines, whose concentrations are altered in girls with AN [25–27, 41, 43, 44, 46–52]. These factors may have a direct or RANKL/RANK/OPG system-mediated effect on bone metabolism. However, an indirect influence of vaspin on bone metabolism should also be taken into consideration, e.g. via its effect on the secretion of several osteotropic factors including leptin, adiponectin, or proinflammatory cytokines.

Vaspin was shown to inhibit adiponectin and stimulate leptin secretion in the adipose tissue [7]. Hence, it might be hypothesised that the effect of vaspin on bone metabolism could be partly mediated via these adipokines. It is well known that osteoclastogenesis is increased by adiponectin via the RANKL/OPG mechanism [53, 54]. In vitro studies revealed that adiponectin stimulated RANKL and inhibited OPG expression in human osteoblasts through the MAPK signalling pathway [53]. Leptin, on the other hand, is involved in the control of bone mass by a complex mechanism that has an important role in the central nervous system (CNS) and osteoblastic β2-adrenergic receptors [55, 56]. Our studies in girls with AN revealed a positive and significant correlation between changes in adiponectin concentrations and OPG, and a negative correlation between changes in adiponectin concentrations and the OPG/sRANKL ratio. On the other hand, the OPG/ /sRANKL ratio was positively and significantly correlated with changes in leptin levels. Adiponectin was found to be an independent predictor of OPG, while adiponectin and leptin turned out to be independent predictors of the OPG/sRANKL ratio [26]. It has been suggested that vaspin might also modify the actions of pro-inflammatory cytokines, including TNF-α, IL-6, or IL-1 [32, 57, 58]. Vaspin attenuates inflammatory cytokine-induced nuclear factor-κB (NF-κB) activation via the activation of AMP-activated protein kinase (AMPK) in vascular endothelial cells [57]. Liu et al.'s [58] studies showed that vaspin inhibited TNF-α and IL-1-mediated activation of NF-κB and its downstream molecules in a concentration-dependent manner. Hence it could be speculated that vaspin might also modify the effects of pro-inflammatory cytokine actions in bone tissue. In girls with AN, we found positive and significant correlations between changes in IL-1β, IL-6

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concentrations, and CTx as well as between changes in TNF-α levels and sRANKL, while the correlation between changes in TNF-*a* concentrations and the OPG/ /sRANKL ratio was negative. IL-6 and IL-1β were shown to be independent predictors of CTx; TNF-α and IL-6 turned out to be independent predictors of sRANKL, while TNF-α, IL-6, and IL-1β were independent predictors of the OPG/sRANKL ratio [59].

As already mentioned, GH and thyroid hormones might affect vaspin secretion and act as regulators of bone metabolism. In patients with AN, a significant age-related correlation was found between GH, free T_4, and free T_3 and markers of bone formation and resorption [39, 40, 43]. IGF-I and free T_3 were shown to be independent predictors of CTx in girls with AN while GH was an independent predictor of OC in both girls and women with AN [43]. Our studies on girls with AN revealed that free T_3 was an independent predictor of sRANKL and the OPG/sRANKL ratio [52]. It has been suggested that GH resistance and changes in the hypothalamic-pituitary-thyroid axis may play a role in the osteopaenia and decreased peak bone mass frequently associated with AN [39–41]. On this basis it could be speculated that changes in GH and thyroid hormone concentrations observed in patients with AN might affect vaspin concentrations and hence modulate bone remodelling.

To sum up: undernourishment and associated deficit of adipose tissue may result in inadequate serum vaspin concentrations and bone metabolism disturbances in girls with AN. A relationship found between vaspin and soluble ligand sRANKL, and the OPG/sRANKL ratio, indicates that vaspin might regulate the dynamic balance between bone formation and resorption processes through the cytokines of the RANKL/RANK/ /OPG system. However, it cannot be ruled out that the relationship might involve some osteotropic agents, mainly hormones and cytokines (whose concentrations are abnormal in AN and which modulate or are modulated by vaspin). Changes in the relationships between vaspin, the above-mentioned osteotropic agents, bone markers, and cytokines of the RANKL/ /RANK/OPG system might underlie the development of osteoporosis in girls with AN.

Conclusions

Undernourishment and associated deficit of adipose tissue may cause inadequate vaspin production and bone metabolism disturbances in girls with AN.

Vaspin acts as a coordinator of the dynamic balance between bone formation and resorption processes; its action is affected by the cytokines of the RANKL/RANK/ /OPG system.

Changes in the relationships between vaspin, bone markers, OPG, and RANKL might be involved in the development of osteoporosis in girls with AN.

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