



Selected pro-inflammatory cytokines, bone metabolism, osteoprotegerin, and receptor activator of nuclear factor- κ B ligand in girls with anorexia nervosa

Wybrane cytokiny prozapalne, metabolizm kostny, osteoprotegeryna i ligand receptora aktywatora czynnika jądrowego- κ B u dziewcząt z jądłowstrętem psychicznym

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Abstract

Introduction: It has been indicated that disturbances in the production of certain pro-inflammatory cytokines might contribute to the development of osteoporosis in girls with anorexia nervosa (AN). The aim of the study was to determine whether girls with AN exhibited a relationship between IL-1 β , IL-6, TNF- α , bone turnover markers (OC and CTx), OPG, sRANKL, and the OPG/sRANKL ratio.

Material and methods: Serum IL-1 β , IL-6, TNF- α , OC, CTx, OPG, and sRANKL were determined by ELISA in 59 girls with AN and in 17 healthy counterparts, aged 13 to 17 years.

Results: Girls with AN showed significant reduction in body weight, BMI, BMI-SDS, and Cole index compared to the controls. These changes were associated with a significant increase in IL-1 β , IL-6, TNF- α , OPG, and sRANKL concentrations and a decrease in bone markers and the OPG/sRANKL ratio. Significant negative correlations were found between BMI, the Cole index and CTx, OPG (girls with AN); between BMI and OC, CTx as well as the Cole index and CTx (the control group — C); between BMI, the Cole index and IL-1 β , IL-6, TNF- α , CTx in all study participants (group AN+C). The combined group AN+C also exhibited positive correlation between BMI, the Cole index, and the OPG/sRANKL ratio. Girls with AN showed positive correlations between IL-1 β , IL-6, and CTx as well as between TNF- α and sRANKL whereas the correlation between TNF- α and the OPG/sRANKL ratio was negative (IL-6 and IL-1 β were identified to be independent predictors of CTx, TNF- α and IL-6 independently predicted sRANKL while TNF- α , IL-6, and IL-1 β were independent predictors of the OPG/sRANKL ratio). The control participants exhibited negative correlations between IL-1 β and OPG and positive correlations between IL-1 β and sRANKL (IL-1 β was found to be an independent predictor of OPG and sRANKL). In the AN+C group, IL-1 β correlated negatively with OC and OPG and positively with sRANKL, while IL-6 and TNF- α positively correlated with CTx (IL-6 and TNF- α turned out to be independent predictors of CTx, IL-1 β of OPG while IL-6, TNF- α , and IL-1 β were independent predictors of sRANKL and the OPG/sRANKL ratio).

Conclusions: The relationship between the nutritional status and IL-1 β , IL-6, and TNF- α concentrations as well as bone status indicators seems to indicate that abnormalities observed regarding the concentrations of pro-inflammatory cytokines and bone remodelling in girls with AN might result from malnutrition. Correlations between IL-1 β , IL-6, TNF- α , bone markers, OPG, its ligand sRANKL, and/or the OPG/sRANKL ratio suggest potential involvement of these cytokines in the mechanism underlying the lack of the expected bone mineral density increase in adolescent girls. (*Endokrynol Pol* 2015; 66 (4): 313–321)

Key words: anorexia nervosa; girls; pro-inflammatory cytokines; bone metabolism; OPG; sRANKL

Streszczenie

Wstęp: Istnieją sugestie, że zaburzenia w wytwarzaniu niektórych cytokin prozapalnych mogą współuczestniczyć w mechanizmie prowadzącym do rozwoju osteoporozy u dziewcząt z jądłowstrętem psychicznym (AN, *anorexia nervosa*). Celem pracy było wykazanie, czy u dziewcząt z AN istnieje związek między IL-1 β , IL-6, TNF- α , markerami obrotu kostnego (OC i CTx), OPG, sRANKL i wskaźnikiem OPG/sRANKL.

Materiał i metody: U 59 dziewcząt z AN i 17 zdrowych w wieku 13–17 lat oceniono stężenia IL-1 β , IL-6, TNF- α , OC, CTx, OPG i sRANKL w surowicy metodą ELISA.

Wyniki: U dziewcząt z AN wykazano istotne obniżenie masy ciała, BMI, BMI-SDS oraz wskaźnika Cole'a w porównaniu z grupą kontrolną. Zmianom tym towarzyszył istotny wzrost stężeń IL-1 β , IL-6, TNF- α , OPG, sRANKL przy zmniejszonym stężeniu markerów kostnych i obniżonym wskaźniku OPG/sRANKL. Wykazano ujemną korelację między wskaźnikami BMI i Cole'a a CTx i OPG (dziewczęta z AN); między BMI a OC i CTx oraz między wskaźnikiem Cole'a a CTx (grupa kontrolna — C); między wskaźnikami BMI i Cole'a a IL-1 β , IL-6, TNF- α , CTx (u wszystkich badanych łącznie: grupa AN+C). W grupie AN+C wskaźniki BMI i Cole'a korelowały ponadto dodatnio z wskaźnikiem OPG/sRANKL. U dziewcząt z AN stwierdzono dodatnią korelację między IL-1 β i IL-6 a CTx, a także między TNF- α a sRANKL oraz ujemną między TNF- α a wskaźnikiem OPG/sRANKL (udokumentowano, że IL-6 i IL-1 β są niezależnymi predyktorami dla CTx, TNF- α i IL-6 dla sRANKL a TNF- α , IL-6 i IL-1 β dla wskaźnika OPG/sRANKL). W grupie kontrolnej IL-1 β korelowała ujemnie z OPG a dodatnio z sRANKL (stwierdzono, że IL-1 β jest niezależnym predyktorem dla OPG i sRANKL). W grupie AN+C zanotowano ujemną korelację między IL-1 β a OC i OPG, a dodatnią między IL-1 β a sRANKL oraz między IL-6 i TNF- α a CTx (wykazano, że IL-6 i TNF- α są niezależnymi predyktorami dla CTx, IL-1 β dla OPG a IL-6, TNF- α i IL-1 β dla sRANKL i wskaźnika OPG/sRANKL).

Wnioski: Wykazana zależność między stopniem odżywienia a stężeniami IL-1 β , IL-6, TNF- α oraz wskaźnikami stanu kości może świadczyć o tym, że przyczyną obserwowanych nieprawidłowości w stężeniach badanych cytokin prozapalnych i przebudowie tkanki kostnej u dziewcząt z AN może być niedożywienie. Istnienie powiązań między stężeniami IL-1 β , IL-6, TNF- α a markerami kostnymi, OPG, jej ligandem sRANKL i/lub wskaźnikiem OPG/sRANKL wskazuje na możliwy współdziałanie wymienionych cytokin w mechanizmie prowadzącym do braku oczekiwanego wzrostu gęstości mineralnej kości u dziewcząt w okresie dorostania. (Endokrynol Pol 2015; 66 (4): 313–321)

Słowa kluczowe: jadłowstręt psychiczny; dziewczęta; cytokiny prozapalne; metabolizm kostny; OPG; sRANKL

Introduction

It has been widely acknowledged that osteoporosis is a common complication of AN [1, 2]. Its aetiology is multifactorial. Well-documented disturbances in the production, release, and action of several osteotropic agents, mainly hormones and cytokines (including some pro-inflammatory cytokines), such as: interleukin (IL)-1 β , IL-6, and tumour necrosis factor α (TNF- α) [3–8], might play a role in the mechanism leading to either a decrease or lack of the expected increase in bone mineral density (BMD) during adolescence [1].

Studies estimating IL-1 β , IL-6, and TNF- α production in patients with AN can be divided into two categories [3, 8]. The first category concentrates on *in vitro* studies evaluating spontaneous or mitogen-induced secretion of the above mentioned cytokines from peripheral blood mononuclear cells, the other concerns studies involving measuring their concentrations in body fluids of the AN patients and/or adipose tissue cytokine expression [3, 8]. However, the obtained results have not always been clear, which makes it difficult to form any final conclusions. The most significant differences were reported regarding the levels of circulating pro-inflammatory cytokines. Some researchers found an increase in circulating IL-1 β , IL-6, or TNF- α in patients with AN compared to healthy individuals [9–14]. Others did not find significant differences in the concentrations of these cytokines between the study and control groups [15–19]. Nevertheless, others noted that serum TNF- α levels were undetectable in patients and controls [9, 20–25], while IL-6 concentrations were subnormal [25]. The majority of the investigations were carried out in adults, especially women [3, 8]; only a few studies comprised girls with AN [14, 19, 24–26]. After refeeding, the concentrations of IL-1 β , IL-6, and TNF- α usually returned to levels characteristic of normal-

weight controls [9, 10, 20, 21, 24]. However, this finding was not confirmed by other investigators [3, 16].

In vitro studies revealed that pro-inflammatory cytokines such as IL-1, IL-6, and TNF- α are important regulatory determinants of bone remodelling [27–30]. It has been found that osteoblasts express IL-1 receptors. Interleukin-1 stimulates the proliferation of osteoblast precursors and, depending on local concentration, may stimulate or inhibit collagen and alkaline phosphatase synthesis. IL-1 also inhibits the synthesis of osteocalcin (OC), stimulates bone tissue resorption, and promotes the synthesis and secretion of collagenase by an osteoblastic cell line. It activates mature osteoclasts and stimulates the recruitment and differentiation of precursor cells [27]. IL-1-induced stimulation of osteoclastogenesis occurs indirectly via enhanced expression of receptor activator of nuclear factor- κ B ligand (RANKL) in osteoblasts and marrow stromal cells [31–33]. Interleukin-6 is a protein synthesised by lymphocytes and other cell types including osteoblasts and osteoclasts. Small doses of this cytokine stimulate the recruitment of osteoclast precursors while large doses increase osteoclast activity. Interleukin-6 stimulates osteoclastogenesis and enhances the action of parathyroid hormone-related protein (PTHrP) [27]. Liu et al. [34] indicated that an interaction between prostaglandin E₂ (PE₂) and IL-6 promotes osteoclast differentiation via the RANKL/RANK/osteoprotegerin (OPG) system. Sanchez et al. [35] found that IL-6 inhibited OPG expression but did not modify RANKL mRNA level, ultimately resulting in a decrease of the OPG/RANKL ratio and hence increased bone resorption. TNF- α stimulates osteoblast precursor proliferation but inhibits their maturation through a decrease in the synthesis of type 1 collagen and alkaline phosphatase activity [27]. Through synergistic interaction with IL-1, TNF- α stimulates the fusion of osteoclast precursors into multinucleated osteoclasts

in bone marrow cells *in vitro*; it also activates mature osteoclasts [27]. TNF- α enhances RANKL expression and bone resorption by osteoclasts [36–39]. The above data suggest that cytokines may regulate osteoclastogenesis not only directly, but also indirectly via the RANKL/RANK/OPG system [27–39]. Through an increase in the expression of RANKL and/or a decrease in OPG expression, IL-1 β , IL-6, or TNF- α may suppress the OPG/RANKL ratio resulting in enhanced bone resorption [27–39].

The results of *in vitro* investigations also indicate that some adipocyte-secreted factors, including the above-mentioned pro-inflammatory cytokines, might modify the balance of OPG/RANKL [40–42]. It was shown that human adipocytes regulated the expression of OPG and RANKL in human osteoblastic cell lines *in vitro*. They inhibited and stimulated the expressions of RANKL and OPG, respectively [40, 42]. The OPG/RANKL ratio in primary human preosteoblasts increased (mRNA and protein) when stimulated with adipocyte-secreted factors. Osteoblasts that were pre-stimulated with adipocyte-secreted factors inhibited the formation of osteoclasts [42].

Only a few researchers have studied the relationship between pro-inflammatory cytokines and bone status in patients with AN [25, 43]. Increased concentrations of OC and the C-terminal telopeptide of type I collagen α 1 chain (CTx) and reduced OPG levels observed in young women with AN and depressive disorder were associated with a significant increase in TNF- α level [43]. TNF- α concentrations were positively correlated with CTx and negatively with OPG [43]. However, in adolescent girls with AN, IL-1 β and TNF- α levels were undetectable, IL-6 concentrations markedly lowered, bone formation markers suppressed (bone-specific alkaline phosphatase — BSAP and OC), and resorption marker (N-terminal telopeptide of type I collagen α 1 chain — NTx) increased [25]. These inconsistencies regarding a potential relationship between bone markers in patients with AN caused us to undertake a research project in this area.

The aim of the study was to determine whether girls with AN showed any relationships between IL-1 β , IL-6, TNF- α , bone turnover markers (OC and CTx), OPG, sRANKL, and the OPG/sRANKL ratio.

Material and methods

The study comprised 59 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. the

Table I. Mean values of age, body weight, height, body mass index (BMI), standard deviation score for BMI (BMI-SDS), the Cole index, mean serum levels of selected pro-inflammatory cytokines, osteocalcin (OC), C-terminal telopeptide of type I collagen α 1 chain (CTx), osteoprotegerin (OPG), soluble receptor activator of nuclear factor- κ B ligand (sRANKL), and mean value of the OPG/sRANKL ratio in girls with anorexia nervosa and in the control group

Tabela I. Średni wiek, masa ciała, wzrost, wskaźnik masy ciała (BMI), odchylenie standardowe dla BMI (BMI-SDS), wskaźnik Cole'a, średnie stężenia wybranych cytokin prozapalnych, osteokalcyny (OC), karboksyterminalnego usieciowanego telopeptydu łańcucha α 1 kolagenu typu I (CTx), osteoprotegeryny (OPG), rozpuszczalnego ligandu receptora aktywatora czynnika jądrowego- κ B (sRANKL) oraz wartość wskaźnika OPG/sRANKL u dziewcząt z jadłowstrętem psychicznym i w grupie kontrolnej

Variables	Groups	
	Anorexia nervosa (n = 59)	Control (n = 17)
Age (years)	15.27 \pm 1.62	15.71 \pm 1.68
Height [m]	1.62 \pm 0.06	1.66 \pm 0.04
Body weight [kg]	39.17 \pm 3.59*	55.27 \pm 6.80
BMI [kg/m ²]	15.27 \pm 1.84*	20.38 \pm 2.19
BMI-SDS	-2.52 \pm 1.10*	0.05 \pm 1.01
Cole index (%)	78.31 \pm 0.99*	100.17 \pm 0.86
IL-1 β [pg/mL]	9.77 \pm 1.31*	4.43 \pm 1.69
IL-6 [pg/mL]	3.52 \pm 0.69*	2.12 \pm 0.94
TNF- α [pg/mL]	5.55 \pm 1.23*	2.51 \pm 0.70
OC [μ mol/L]	0.92 \pm 0.73*	3.65 \pm 2.31
CTx [nmol/L]	7.21 \pm 0.91*	8.06 \pm 0.87
OPG [pmol/L]	5.40 \pm 0.89*	3.58 \pm 1.30
sRANKL [pmol/L]	0.39 \pm 0.03*	0.22 \pm 0.01
OPG/sRANKL ratio	12.85 \pm 1.43*	16.27 \pm 1.08

*p \leq 0.05 vs. control group

DSM-IV of 1994. 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 15.27 \pm 1.62 years (Table I). All had primary or secondary amenorrhoea. The duration of the disease was 3 to 60 months. The control group consisted of 17 healthy regularly menstruating girls (mean age 15.71 \pm 1.68 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.

The height and body weight of all participants were measured, and their body mass index (BMI; weight/height²) and BMI standard deviation score (BMI-SDS) calculated. BMI-SDS was assessed using gender- and

age-specific BMI percentiles developed for the Polish population by Palczewska et al. [44]. The mean body weight of girls with AN was 39.17 ± 3.59 kg, mean BMI was 15.27 ± 1.84 kg/m², and the mean BMI-SDS was -2.52 ± 1.10 . The Cole index was also calculated, which reflects the nutritional status of an individual and encompasses the following categories: < 75%: wasting; 75–85%: undernourished; 85–90% mildly undernourished; 90–100% adequately nourished; and > 110% overnourished [acc. to 45]. The mean value of the Cole index in our AN patients was $78.31 \pm 0.90\%$. The mean body weight of the control participants was 55.27 ± 6.80 kg, BMI was 20.38 ± 2.19 kg/m², BMI-SDS was -0.05 ± 1.01 , and the Cole index was $100.17 \pm 0.86\%$ (Table I).

On the day of the examination the girls did not report any complaints; none of them suffered from acute infection during the preceding month. Blood samples (8 mL) for the determination of IL-1 β , IL-6, TNF- α , and bone markers, i.e. OC (bone formation marker) and the CTx (bone resorption marker) as well as OPG and its soluble ligand sRANKL, were collected between 08.00 and 09.00 after a 12-hour fast. Centrifuged serum was frozen and stored at -75°C until assay. Determinations of the concentrations of selected pro-inflammatory cytokines, OC, CTx, OPG, and sRANKL were performed by High Sensitivity Human ELISA kits: IL-1 β (Quantikine R&D System, Inc., USA), IL-6 (BioVendor — Laboratorni medicina a.s., Czech Republic), TNF- α (eBioscience, Austria), OC (DSL Inc., USA), CTx (Nornic Bioscience Diagnostics A/S, Denmark), OPG, and sRANKL (Biomedica, Austria). The respective sensitivity intra- and inter-assay errors were: 1 pg/mL, 4.8 and 5.6% for IL-1 β ; 0.81 pg/mL, 4.4 and 9.1% for IL-6; 0.13 pg/mL, 8.5 and 9.8% for TNF- α ; 0.05 $\mu\text{mol/L}$, 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 with Statistica 10 for Windows (StatSoft Inc., USA). The 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, the Cole index, IL-1 β , IL-6, TNF- α , OC, CTx, OPG, sRANKL, and the OPG/sRANKL ratio in girls with AN, control participants (C), and the combination group of AN+C were analysed using Spearman's correlation. The level of significance was set at $p \leq 0.05$.

Stepwise regression was used to determine whether and which of the investigated cytokines were independent predictors of bone markers, cytokines of the RANKL/RANK/OPG system, and the OPG/sRANKL

ratio (model entry was set at $p = 0.05$, and model exit at $p = 0.05$).

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

Results

Mean body weight, BMI, BMI-SDS, and the Cole index were significantly lower and mean serum IL-1 β , IL-6, and TNF- α significantly higher in girls with AN compared to healthy controls. The changes in the concentrations of the pro-inflammatory cytokines were associated with considerable suppression of the mean serum levels of bone markers (OC and CTx) and elevation of the mean OPG and sRANKL levels compared to healthy participants with normal body weight. The mean value of the OPG/sRANKL ratio was significantly lower in girls with AN than in the control group (Table I).

Girls with AN exhibited a negative and significant correlation between BMI, the Cole index, and CTx as well as OPG. In the control group, a negative and significant correlation was revealed between BMI and OC, CTx as well as between the Cole index and CTx (Table II). In the combined AN+C group, BMI and the Cole index correlated negatively and significantly with serum IL-1 β , IL-6, TNF- α , and CTx and positively with the OPG/sRANKL ratio (Table II).

In girls with AN, IL-1 β and IL-6 correlated positively and significantly with CTx. TNF- α was positively and significantly correlated with sRANKL and negatively with the OPG/sRANKL ratio. IL-6 and IL-1 β were shown to be independent predictors of CTx ($R^2 = 0.1105$, $p = 0.048$); TNF- α and IL-6 turned out to be independent predictors of sRANKL ($R^2 = 0.1684$, $p = 0.013$) while TNF- α , IL-6, and IL-1 β were independent predictors of the OPG/sRANKL ratio ($R^2 = 0.1706$, $p = 0.034$) (Table III).

In the control group IL-1 β was negatively and significantly correlated with OPG and positively with sRANKL. IL-1 β turned out to be an independent predictor of OPG ($R^2 = 0.2552$, $p = 0.044$) and sRANKL ($R^2 = 0.1998$, $p = 0.049$) (Table III).

In the combined AN+C group, IL-1 β correlated negatively with OC and OPG and positively with sRANKL while IL-6 and TNF- α were positively correlated with CTx. IL-6 and TNF- α were independent predictors of CTx ($R^2 = 0.0407$, $p = 0.049$). IL-1 β turned out to be an independent predictor of OPG ($R^2 = 0.0598$, $p = 0.047$) while IL-6, TNF- α , and IL-1 β were shown to be independent predictors of sRANKL ($R^2 = 0.1331$, $p = 0.032$) and the OPG/sRANKL ratio ($R^2 = 0.1079$, $p = 0.041$) (Table III).

Table II. Correlation between body mass index (BMI), the Cole index and the selected pro-inflammatory cytokines, osteocalcin (OC), C-terminal telopeptide of type I collagen α 1 chain (CTx), osteoprotegerin (OPG), soluble receptor activator of nuclear factor- κ B ligand (sRANKL), and the OPG/sRANKL ratio in girls with anorexia nervosa (AN), in the control group (C), and in all girls (AN + C)

Tabela II. Korelacja między wskaźnikiem masy ciała (BMI), wskaźnikiem Cole'a a wybranymi cytokinami prozapalnymi, osteokalcyną (OC), karboksyterminalnym usieciowanym telopeptydem łańcucha α 1 kolagenu typu I (CTx), osteoprotegeryną (OPG), rozpuszczalnym ligandem receptora aktywatora czynnika jądrowego- κ B (sRANKL) i wartościami wskaźnika OPG/sRANKL u dziewcząt z jadłowstrętem psychicznym (AN), w grupie kontrolnej (C) i u wszystkich dziewcząt łącznie (AN+C)

Variables		Values of correlation coefficients		
		AN (n = 59)	C (n = 17)	AN+C (n = 76)
BMI [kg/m ²]	IL- β 1 [pg/mL]	0.098	0.144	-0.576*
	IL-6 [pg/mL]	-0.174	-0.227	-0.552*
	TNF- α [pg/mL]	0.191	0.144	-0.478*
	OC [μ mol/L]	0.206	-0.533*	-0.186
	CTx [nmol/L]	-0.379*	-0.483*	-0.329*
	OPG [pmol/L]	-0.266*	-0.067	-0.069
	sRANKL [pmol/L]	0.147	0.028	-0.040
	OPG/sRANKL ratio	0.145	-0.015	0.248*
Cole index (%)	IL- β 1 [pg/mL]	0.025	-0.046	-0.565*
	IL-6 [pg/mL]	-0.127	-0.222	-0.449*
	TNF- α [pg/mL]	0.094	-0.046	-0.459*
	OC [μ mol/L]	0.207	-0.380	-0.123
	CTx [nmol/L]	-0.326*	-0.517*	-0.321*
	OPG [pmol/L]	-0.300*	0.189	-0.072
	sRANKL [pmol/L]	0.171	-0.019	-0.007
	OPG/sRANKL ratio	0.114	0.020	0.235*

* $p \leq 0.05$ — statistically significant values of correlation coefficients

Discussion

Girls with AN usually exhibit suppression of bone formation and resorption markers, which correlates with BMI and/or BMD, and indicates a decrease in bone turnover [46–63]. It was also observed that an increase in serum OPG correlated negatively with BMI and/or BMD [51, 58–63]. However, several authors reported a slight or significant increase in bone resorption markers in patients with AN, which was associated with a significant decrease in bone formation markers [1]. The suppression of bone resorption markers in girls with AN observed by the majority of researchers might indicate an inhibiting effect of high OPG concentrations on osteoclast activity [51]. Consistent with our previ-

ous results [58–63], the present findings also show a decrease in serum bone markers (OC and CTx) as well as a significant increase in OPG and sRANKL, associated with a noticeable decrease in the OPG/sRANKL ratio. The high correlation between CTx and/or OC and the OPG/sRANKL ratio unassociated with or associated with a moderate correlation between bone markers and OPG and/or sRANKL seems to suggest that it is the OPG/RANKL ratio rather than OPG and/or RANKL alone that determines osteoclast differentiation and activation as well as apoptosis thereof [58–63]. The low values of the OPG/sRANKL ratio paralleled by high OPG and sRANKL concentrations confirmed in the present study along with some degree desynchronisation of the relationships between these cytokines and bone formation and resorption markers [58–63] indicate that girls with AN might suffer from a compromise in the mechanism which controls bone remodelling and/or mechanism compensating for enhanced bone turnover. Adolescent females with AN, examined by Munoz-Calvo et al. [64], exhibited a significant decrease in the OPG/RANKL ratio correlated with an increase in RANKL as well as a positive and significant correlation between the OPG/RANKL ratio and BMD. However, the researchers did not observe a significant OPG increase in the sera of girls with AN or any relationships between OPG, RANKL, and BMD. Hence, they concluded that the decrease in the OPG/RANKL ratio only partly explained the increased bone loss observed in their study population with AN. 17β -oestradiol is known to be a major regulator of bone metabolism and BMD in women and girls suffering from AN. However, both OPG and RANKL, and OPG in particular, are regulated not only by 17β -estradiol, but also by other hormones and cytokines (maybe also pro-inflammatory cytokines), whose effect might be similar or opposite to that of 17β -estradiol [27]. It has been demonstrated that pro-inflammatory cytokines influence the bone tissue directly and/or indirectly, via the RANKL/RANK/OPG system [27–39]. Since it has been documented that IL-1 β , IL-6, and TNF- α stimulate bone resorption both *in vitro* and *in vivo* [27–30], it seems reasonable to presume that any potential disturbances in the production of these cytokines in patients with AN might play a role in the development of osteoporosis.

Numerous researchers investigated the concentrations of pro-inflammatory cytokines in patients with AN. However, the obtained results were not always consistent. Several authors reported normal [11, 17, 18], decreased [25], or increased IL-1 β levels [10, 14]; normal [11, 15, 17–19] or increased IL-6 concentrations [1, 9, 12] as well as normal [11, 17–19] or increased TNF- α levels [12, 13, 16], compared to healthy weight controls. Our female adolescents with AN exhibited a significant in-

Table III. Correlation^a between the selected pro-inflammatory cytokines, osteocalcin (OC), C-terminal telopeptide of type I collagen α 1 chain (CTx), osteoprotegerin (OPG), soluble receptor activator of nuclear factor- κ B ligand (sRANKL), and the OPG/sRANKL ratio in girls with anorexia nervosa (AN), in the control group (C) and in all girls studied (AN+C)

Tabela III. Korelacja^a między wybranymi hormonami tkanki tłuszczowej, osteokalcyną (OC), karboksyterminalnym usięciowanym telopeptydem łańcucha α 1 kolagenu typu I (CTx), osteoprotegeryną (OPG), rozpuszczalnym ligandem receptora aktywatora czynnika jądrowego- κ B (sRANKL) i wartościami wskaźnika OPG/sRANKL u dziewcząt z jadłowstrętem psychicznym (AN), w grupie kontrolnej (C), i u wszystkich dziewcząt łącznie (AN + C)

Variables	Bone markers		Cytokines of RANKL/RANK/OPG system		OPG/sRANKL ratio	Groups
	OC [μ mol/L]	CTx [nmol/L]	OPG [pmol/L]	sRANKL [pmol/L]		
IL-1 β [pg/mL]	-0.040	0.262*	-0.092	0.107	-0.106	AN (n = 59)
IL-6 [pg/mL]	0.081	0.271*	0.072	-0.216	-0.109	
TNF- α [pg/mL]	0.138	-0.019	0.104	0.355*	-0.274*	
Stepwise regression model ^b	-	IL-6	-	TNF- α	TNF- α	
		IL-1 β		IL-6	IL-6	
R ²		0.1105		0.1684	0.1706	
p		0.048		0.013	0.034	
IL-1 β [pg/mL]	0.217	0.097	-0.553*	0.487*	-0.189	C (n = 17)
IL-6 [pg/mL]	-0.161	-0.133	0.183	0.213	-0.053	
TNF- α [pg/mL]	-0.202	0.041	0.159	0.061	-0.111	
Stepwise regression model ^b	-	-	IL-1 β	IL-1 β	-	
R ²			0.2552	0.1998		
p			0.044	0.049		
IL-1 β [pg/mL]	-0.234*	0.181	-0.289	0.290*	-0.212	AN+C (n = 76)
IL-6 [pg/mL]	0.178	0.219*	0.049	0.152	-0.201	
TNF- α [pg/mL]	0.156	0.269*	0.121	0.127	0.193	
Stepwise regression model ^b	-	IL-6	IL-1 β	IL-6	IL-6	
		TNF- α		TNF- α	TNF- α	
R ²		0.0407	0.0598	0.1331	0.1079	
p		0.049	0.047	0.032	0.041	

^ap \leq 0.05 — statistically significant values of correlation coefficients; ^bDisplayed value represents the model variance accounted for by these parameters; ^cParameters entering the stepwise regression model

crease in serum IL-1 β (similar to the study participants of Allende et al. [10] and Ziora et al. [14]), IL-6 (similar to the study participants of Pomeroy et al. [9], Kahl et al. [12], and Misra & Klibansky [1]), and TNF- α (similar to study participant of Nakai et al. [11], Kahl et al. [12], and Angello et al. [13]). The above-mentioned inter-study discrepancies in IL-1 β , IL-6, or TNF- α levels in patients with AN might be due to differences in group size; the number of participants was often quite small [3], which clearly affected the results of statistical analyses. Also, determinations were made using different biological materials and methods of unequal sensitivity and specificity. Some authors determined pro-inflammatory cytokine levels using ELISA [10, 20, 65] whereas others chose RIA [16, 18, 66] or IRMA [16]. These methods, as

explained in their respective instruction manuals, allow the measurement of serum immunoradioactivity and detection of biologically inactive cytokines or their fragments. Others tested the biological activity of cytokines using bioassays [3, 8, 9, 23]. It cannot be ruled out that discrepancies in IL-1 β , IL-6, or TNF- α levels might also be associated with different times of sample collection. Due to circadian patterns in certain cytokines secretion, e.g. TNF- α [67, 68], it is important that material sampling should be scheduled at the same time each day. In our study, cytokine determinations were made with ultrasensitive, latest-generation ELISA, which made it possible to measure biologically active cytokines, especially in reference to IL-1 β . Blood samples for the determination of pro-inflammatory cytokines and bone

markers were always collected between 08.00 and 09.00 after a 12-hour fast.

Our findings, similar to previously reported results of other researchers [14, 19], indicate a significant correlation between the investigated cytokines and BMI and the Cole index in girls with AN and in control participants, when analysed collectively (AN+C), most probably due to considerable differences in body weight. Separate analyses of AN and control groups did not reveal such relationships. On the other hand, BMI and/or the Cole index correlated significantly with OC, CTx, and/or OPG in all study groups, i.e. groups with AN and the control group, when analysed separately, and AN+C analysed collectively. All girls also exhibited a significant correlation between BMI, the Cole index, and the OPG/sRANKL ratio. The relationship between the nutritional status and IL-1 β , IL-6, and TNF- α levels as well as the above-mentioned bone status indices might suggest that malnutrition could be the underlying cause of abnormalities observed both in pro-inflammatory cytokines and bone remodeling.

Our results also seem to demonstrate relationships between selected pro-inflammatory cytokines and certain bone status indices. IL-1 β was negatively correlated with OPG and positively with sRANKL in healthy girls with normal body weight. It also turned out to be an independent predictor of OPG and sRANKL. Our patients with AN showed a positive correlation between IL-1 β and CTx. IL-1 β was an independent predictor of CTx and the OPG/sRANKL ratio. However, in the combined group (AN+C), IL-1 β was negatively correlated with OC and OPG, and positively with sRANKL. Interleukin-1 β turned out to be an independent predictor of OPG, sRANKL, and the OPG/sRANKL ratio. Previous *in vitro* investigations revealed that, depending on its local concentration, IL-1 β may stimulate or inhibit osteoblasts through specific receptors [27]. It also stimulates bone resorption via the RANKL/RANK/OPG system [31–33]. *In vitro* investigations demonstrated that IL-1 β enhanced the expression of the RANKL gene in osteoblasts and marrow stromal cells leading to a shift in the OPG/sRANKL ratio toward a functional excess of sRANKL, and, consequently, increased bone resorption [31–33]. The negative and positive correlation between IL-1 β and OPG and sRANKL, respectively, revealed in our study, seems to indicate that IL-1 β might be capable of changing not only sRANKL but also OPG expression. However, some direct or indirect influence of other, for example hormone-related factors (such as oestrogen deficiency), on OPG expression cannot be excluded [27].

Similar to our findings, Misra & Klibansky [1] also observed increased IL-6 concentrations in AN; however, a stepwise regression analysis revealed that this cytokine was not an independent predictor of BMD or

bone resorption markers in their patients. Our results showed a positive correlation only between IL-6 and CTx in the combination (AN+C) and AN groups, but not in the control. However, a stepwise regression analysis demonstrated that IL-6 was an independent predictor of CTx, sRANKL, and the OPG/sRANKL ratio. Numerous researchers reported that IL-6 stimulated osteoclastogenesis *in vitro* [69]. The cytokine was also demonstrated to act as a regulator of bone resorption via the RANKL/RANK/OPG system [34, 35]. Sanchez et al. [35] suggested that although IL-6 did not influence the expression of RANKL, it inhibited OPG secretion, thus exerting a suppressive effect on the OPG/RANKL ratio.

There is evidence that TNF- α enhances RANKL expression *in vitro* with resultant suppression of the OPG/RANKL ratio and stimulation of osteoclastic bone resorption [27, 30, 36–39]. In young women with AN and a depressive disorder, an increase in serum OC and CTx and low OPG levels were associated with TNF- α elevation [43]. There was also a positive correlation between TNF- α and OPG. Regarding our study participants we found relationships between TNF- α and CTx and/or sRANKL and the OPG/sRANKL ratio in the AN and AN+C groups. In the AN+C group TNF- α correlated positively with CTx while in AN patients TNF- α was positively correlated with sRANKL and negatively with the OPG/sRANKL ratio. Moreover, in the AN group TNF- α turned out to be an independent predictor of sRANKL and the OPG/sRANKL ratio while in the AN+C group TNF- α turned out to be an independent predictor of CTx, sRANKL, and the OPG/sRANKL ratio. These findings seem to confirm the hypothesis on the potential role of this cytokine in the development of osteoporosis in patients with AN.

When considering the possible contribution of pro-inflammatory cytokines to osteoporosis in patients with AN, it should be kept in mind that the endocrine changes seen in these individuals including sex steroid suppression as well as enhanced cortisol and β -endorphin secretion might, on one hand, affect cellular immunity [3], and, on the other hand, modify bone remodelling via the RANKL/RANK/OPG system [27, 62]. Based on the results of *in vitro* investigations and our findings, this effect might be presumed to occur directly or indirectly via cytokines, also IL-1 β , IL-6, or TNF- α . Oestrogens have been demonstrated to stimulate osteoblasts to express the OPG gene while suppression is caused by 1,25(OH) $_2$ D $_3$, glucocorticosteroids and PTH [27, 28]. It is believed that the inhibition of the OPG gene by glucocorticosteroids and an increase in RANKL expression by these hormones might be among the important mechanisms that contribute to the development of osteoporosis — also in patients with AN.

Conclusions

The relationship between the nutritional status and IL-1 β , IL-6, TNF- α as well as bone status indicators seems to indicate that abnormalities in the concentrations of pro-inflammatory cytokines and bone remodelling observed in AN patients might result from undernutrition.

Correlations between IL-1 β , IL-6, TNF- α , bone markers, OPG, its ligand sRANKL, and/or the OPG/sRANKL ratio suggest potential involvement of these cytokines in the mechanism underlying the lack of the expected bone mineral density increase in adolescent girls.

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