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TGF-β1, bone metabolism, osteoprotegerin, and soluble receptor activator of nuclear factor-κB ligand in girls with anorexia nervosa

TGF-β1, metabolizm kostny, osteoprotegeryna i rozpuszczalny ligand receptora aktywatora czynnika jądrowego-κB u dziewcząt z jadłowstrętem psychicznym

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Abstract

Introduction: Numerous investigations, and especially *in vitro* studies, indicate that TGF-β1 may act as an important regulator of bone remodelling. Thus, it could be expected that disturbances of this cytokine production observed by several researchers might play a role in the mechanism leading to the development of osteoporosis in girls with anorexia nervosa (AN). The aim of the study was to determine whether 1) girls with AN exhibited a relationship between TGF-β1 and bone metabolism (as assessed based on serum OC and CTx concentrations) and 2) whether OPG and sRANKL might modify the possible relationship between TGF-β1 and bone metabolism.

Material and methods: Serum concentrations of TGF-β, OC, CTx, OPG, and its soluble ligand sRANKL were determined by ELISA in 60 girls with AN and in 20 healthy controls (C). All study participants were aged 13 to 17 years.

Results: Body weight, BMI, BMI-SDS and the Cole index, serum TGF-β1, OC, CTx, and the OPG/sRANKL ratio were significantly reduced, while OPG and sRANKL levels were significantly increased, in girls with AN compared to healthy participants. BMI and the Cole index correlated negatively and significantly with serum CTx and OPG (AN group) or CTx only (groups C and C + AN). Girls with AN showed a positive and significant correlation between the Cole index and serum TGF-β1. The combination group (C + AN) showed a positive and significant correlation between BMI, the Cole index, and the OPG/sRANKL ratio and TGF-β1 concentration, while TGF-β1 correlated positively and significantly with OC concentrations and the OPG/sRANKL ratio. The Cole index and BMI were identified to be significant and independent predictors of CTx (C, AN, and C+AN groups) and OPG (AN group); the Cole index, BMI, and TGF-β1 independently predicted the OPG/sRANKL ratio (C, AN, and C + AN groups); TGF-β1 was found to be an independent predictor of OC (C + AN group).

Conclusions: Changes in bone markers, OPG, and/or OPG/sRANKL ratio observed in girls with AN are associated with changes in serum TGF-β1 concentrations. TGF-β1 suppression in girls with AN might lead to disturbances in the relationship between bone metabolism and the OPG/sRANKL system, which, in turn, might compromise the mechanism compensating for bone remodelling disturbances. **(Endokrynol Pol 2016; 67 (5): 493–500)**

Key words: *anorexia nervosa; girls; TGF-β1; bone metabolism; OPG; sRANKL*

Streszczenie

Wstęp: Liczne badania, zwłaszcza *in vitro* wskazują, że TGF-β1 odgrywa istotną rolę w regulacji remodelingu kostnego. Na tej podstawie można przypuszczać, że obserwowane przez niektórych badaczy zaburzenia w produkcji tej cytokiny mogłyby współuczestniczyć w mechanizmie prowadzącym do rozwoju osteoporozy u dziewcząt z jadłowstrętem psychicznym (AN). Celem badań było: 1) wykazanie, czy u dziewcząt z AN istnieje związek między TGF-β1 a metabolizmem kostnym (ocenianym na podstawie stężeń w surowicy OC i CTx), 2) ustalenie, czy OPG i sRANKL mogą mieć znaczenie w mechanizmie ewentualnej zależności między TGF-β1 a metabolizmem kostnym.

Materiał i metody: U 60 dziewcząt z AN i 20 zdrowych w wieku 13–17 lat oznaczono stężenia TGF-β1, OC, CTx, OPG i jej rozpuszczalnego ligandu sRANKL w surowicy metodą ELISA.

Wyniki: U dziewcząt z AN wykazano istotne obniżenie masy ciała, wskaźników BMI i Cole'a, BMI-SDS oraz stężeń TGF-β1, OC i CTx w surowicy, jak również wartości wskaźnika OPG/sRANKL przy istotnym wzroście stężeń OPG i sRANKL w porównaniu do grupy kontrolnej. Wartości wskaźników BMI i Cole'a korelowały znamiennie i ujemnie ze stężeniami CTx i OPG (grupa AN) lub stężeniami CTx (grupy C i C + AN). W grupie AN wartości wskaźnika Cole'a korelowały istotnie i dodatnio ze stężeniami TGF-β1, a w grupie C + AN znamienną i dodatnią korelację wykazano między wartościami wskaźników BMI i Cole'a a wartościami wskaźnika OPG/sRANKL i stężeniami TGF-β1 oraz między stężeniami TGF-β1 a stężeniami OC i wartościami wskaźnika OPG/sRANKL. Wykazano, że: wskaźnik

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Cole'a i BMI są niezależnymi predykatorami dla CTx (grupy C, AN, C + AN) oraz OPG (grupa AN); wskaźnik Cole'a, BMI i TGF-β1 są niezależnymi predyktorami dla OPG/sRANKL (grupy C, AN, C + AN); TGF-β1 jest niezależnym predyktorem dla OC (grupa C + AN).
Wnoski: Obserwowanym u dziewcząt z AN zmianom w stężeniach markerów kostnych, OPG, sRANKL i/lub wskaźniku OPG/sRANKL towarzyszą zmiany w stężeniach TGF-β1. Supresja stężeń TGF-β1 u dziewcząt z AN może prowadzić do naruszenia powiązań między metabolizmem kostnym a układem OPG/sRANKL, co może skutkować upośledzeniem mechanizmu kompensującego zaburzenia w przebudowie kośćca. **(Endokrynol Pol 2016; 67 (5): 493–500)**

Słowa kluczowe: *jadłowstręt psychiczny; dziewczęta; TGF-β1; metabolizm kostny; OPG; sRANKL*

Introduction

Transforming growth factor β (TGF-β) belongs to the family of multifunctional regulatory polypeptides. The majority of cells synthesising these polypeptides express receptors that are specific to them, which shows the cells' autocrine action. The polypeptides may also act via a paracrine pathway. TGF-β is a multifactorial cytokine; it takes part in angiogenesis, stimulates extracellular matrix protein synthesis and degradation, regulates apoptosis, promotes the epithelial to mesenchymal transition, and inhibits the growth of hematopoietic, endothelial, and lymphatic cells. TGF-β has various isoforms; three of them are found in humans, i.e. TGF-β1, TGF-β2, and TGF-β3; however, TGF-β1 constitutes the largest source of TGF-β in bone [1-5]. Numerous investigations, and especially *in vitro* studies, indicate that TGF-β1 may act as an important regulator of bone remodelling by influencing both bone formation and bone resorption as well as the production of certain pro-inflammatory cytokines [2, 5–8]. A role of TGF-β1 in coupling bone resorption with formation was also suggested [4].

The role of TGF-β1 in the regulation of bone formation has been well established [2, 3, 7]. Autocrine stimulation by TGF-β1 enhances osteoblast proliferation, blocks apoptosis of osteoblasts, and recruits osteoblastic precursors or matrix-producing osteoblasts to the site through chemotactic attraction. TGF-β1 also enhances the production of extracellular bone matrix protein in the early stages of osteoblast differentiation. On the other hand, it inhibits the later phase of osteoblast proliferation and mineralisation. These later stages are positively regulated by other growth factors such as bone matrix proteins (BMP) [9]. Thus, TGF-β1 cooperates with BMP to regulate osteoblast differentiation. Several reports also indicate that Runt-related transcription factor 2 (Runx2), the master transcription factor in bone formation, is regulated by TGF-β1 and BMP-2 [10].

The role of TGF-β1 in osteoclastogenesis and bone resorption is very complex [2, 3, 7]. It is clear that TGF-β1 mediates osteoclast functions, including their maturation, apoptosis, and recruitment of osteoclast precursors from spleen or bone marrow. TGF-β1 has a biphasic effect on the osteoclast maturation. When TGF-β1 is added into the culture along with receptor activator of nuclear factor-κB ligand (RANKL) and macrophage colony stimulating factor (M-CSF), it induces osteoclastogenesis of haematopoietic and other osteoclast precursors. TGF-β1 triggers the expression of nuclear factor-κB (NF-κB) and receptor activator of nuclear factor-κB (RANK) in osteoclast precursors, and RANKL/RANK interaction is important for prolonged survival and augmented differentiation of osteoclast precursors into osteoclasts. When osteoclast precursors were cultured with osteoblasts, their activation was attenuated when they were stimulated with high levels of TGF-β1 (0.1–10 ng/mL). However, low levels of TGF-β1 (1-100 pg/mL) promoted osteoclast maturation. High levels of TGF-β1 upregulated the expression of osteoprotegerin (OPG) and downregulated the expression of RANKL by osteoblasts [2, 3, 7, 11].

Osteopaenia, or low bone mass, and osteoporosis are among the most severe and common complications of anorexia nervosa (AN) [12–16]. Several studies have reported a positive relationship between body mass, body mass index (BMI), and/or bone mineral density (BMD) in adolescents with AN [15, 17–22]. Adolescents with AN also have lower levels of biochemical markers of bone formation and resorption than normal-weight controls, indicating a decrease in bone turnover [15, 23–31]. This is in contrast to normal-weight adolescents, who have increased levels of bone metabolism markers, particularly in earlier puberty, consistent with increased bone modelling [15]. On the other hand, serum sRANKL [25–31] and/or OPG levels [23–31] are significantly increased in girls with AN while the OPG/sRANKL ratio is significantly decreased [32, 25–31]. Munoz-Calvo et al. [32] were the only researchers who did not find significant changes in OPG levels in girls with AN who exhibited a significantly increased sRANKL level and OPG/sRANKL suppression. Our previous studies [26–31] indicate some desynchronisation between the RANKL/RANK/OPG system and bone remodelling (especially bone resorption) in girls with AN.

Disturbances in bone remodelling observed in girls with AN are frequently associated with abnormal concentrations of numerous osteotropic factors, not only

hormones [15, 33–35], but also cytokines including anti- and proinflammatory cytokines [5, 30, 36]. Considering the fact that TGF-β1, a ubiquitous growth factor, is not a pro- but an anti-inflammatory cytokine that controls both osteoblast and osteoclast differentiation, and therefore balances between bone formation and resorption [2, 7, 8], it might be expected that possible disturbances in its production could have some role in the mechanism leading to the development of osteoporosis in adolescents with AN. Raymond et al. [37] investigated spontaneous or mitogen-induced secretion of TGF-β1 from peripheral blood mononuclear cells in patients with AN. There were also clinical studies aimed at determining serum TGF-β1 concentrations [38, 39]. However, these investigations were only carried out in young women, the patient groups were small, and the obtained results ambiguous. Raymond et al. [37] did not observe significant changes in spontaneous and concanavalin A-induced *in vitro* TGF-β1 production in peripheral blood mononuclear cells of patients with AN. Pomeroy et al. [38] found significant elevation of TGF-β, while Corcos et al. [39] found a significant decrease of TGF-β2, in young women with AN compared to the control participants. All these data [2, 7, 8, 36], along with our previous results [25–31], indicate that the abnormal TGF-β concentrations in women with AN demonstrated by several authors [38, 39] might promote the development of osteoporosis in these patients, possibly through OPG and/or RANKL. To the best of our knowledge, the relationship between potential changes in TGF-β1 concentrations and bone markers in girls with AN has not been studied so far. Therefore, we decided to undertake these investigations.

The aim of the study was to determine whether 1) girls with AN exhibited a relationship between TGF-β1 and bone metabolism (as assessed based on serum osteocalcin — OC and collagen type I crosslinked carboxyterminal telopeptide — CTx) and 2) whether OPG and sRANKL might modify the possible relationship between TGF-β1 and bone metabolism.

Material and methods

The study involved 60 girls aged 13 to 17 years with restrictive form of AN according to Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) classification published by the American Psychiatric Association in 1994. The average disease duration was 12.1 months (range 3–36 months). All examined girls were at the pubertal stage of Tanner IV-V. All had primary or secondary amenorrhoea. They had normal liver and kidney functions; no severe somatic complications or psychiatric disorders were observed. On recruitment, no patients were taking medications known to affect the nutritional and bone status including calcium or vitamin D supplements. During hospitalisation, patients were placed at bed rest, which is the standard care. The control group comprised 20 age-matched, healthy, regularly menstruating adolescent females with no endocrine or other disorders that might possibly influence adipose tissue and bone metabolism. During the three-month period before the study, the control participants did not take calcium or vitamin D supplements.

The height and body weight of all participants were measured, and their body mass index (BMI), standard deviation score (BMI-SDS), and Cole index were calculated. The Cole index reflects the nutritional status and encompasses the following categories: wasting < 75%; undernourished 75–85%; mildly undernourished 85–90%; adequately nourished 90–100%; and overnourished — > 110% [40]. On the day of the examination the girls did not report any complaints, and none of them suffered from acute infection during the preceding month. Blood samples for determination of TGF-β1, OC, CTx, OPG, and sRANKL were collected between 08.00 and 09.00 hours following a 12-hour fast. Centrifuged serum was frozen and stored at –75°C until assayed for the TGF-β1, bone markers, OPG, and sRANKL.

Determinations of TGF-β1, OC, CTx, OPG, and sRANKL levels were performed by ELISA using the following kits: TGF-β1 (eBioscience, Austria), OC (DSL Inc., USA), CTx (Nordic Bioscience Diagnostics A/S, Denmark), OPG and sRANKL (Biomedica, Austria). The respective sensitivity, intra-, and inter-assay coefficient of variations were: 8.6 pg/mL, 3.2 and 4.9% for TGF-β1; 0.05 μmol/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 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 BMI, Cole index, TGF-β1, OC, CTx, OPG, sRANKL, and the OPG/sRANKL ratio were analysed by Spearman's correlation. The level of significance was set at $p \leq 0.05$.

Stepwise regression was used to determine whether BMI, Cole index, and TGF-β1 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 $p = 0.05$).

This study was approved by the Bioethics Committee at the Medical University of Silesia in Katowice (No. L. dz. KNW/0022/KB1/105/09), and written informed

Table I. *Mean values of age, body mass, height, body mass index (BMI), standard deviation score for BMI (BMI-SDS), Cole index, mean serum levels of transforming growth factor β1 (TGF-β1), osteocalcin (OC), collagen type I crosslinked carboxyterminal telopeptide (CTx), osteoprotegerin (OPG), soluble receptor activator of nuclear factor-kB ligand (sRANKL), and mean value of OPG/sRANKL ratio in girls with anorexia nervosa and 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 transformującego czynnika wzrostu β1 (TGF-β1), osteokalcyny (OC), karboksyterminalnego usieciowanego telopeptydu łańcucha α 1 kolagenu typu I (CTx), osteoprotegeryny (OPG), rozpuszczalnego ligandu receptora aktywatora czynnika jądrowego-kB (sRANKL) oraz średnia wartość wskaźnika OPG/sRANKL u dziewcząt z jadłowstrętem psychicznym i w grupie kontrolnej*

Variables	Groups	
	Anorexia nervosa (n = 60)	Control group (n = 20)
Age (years)	15.27 ± 1.62	15.71 ± 1.68
Height [m]	1.62 ± 0.06	1.66 ± 0.04
Body mass [kg]	**39.17 ± 3.59***	55.28 ± 6.80
BMI [kg/m^2]	**15.27 ± 1.84***	20.38 ± 2.19
BMI-SDS	**–2.52 ± 1.10***	0.05 ± 1.01
Cole index (%)	**78.31 ± 0.99***	100.17 ± 0.86
TGF-β1 [ng/mL]	**2.85 ± 0.50***	4.28 ± 0.94
OC [μmol/L]	**1.98 ± 2.31***	3.64 ± 2.69
CTx [nmol/L]	**6.13 ± 0.62***	8.76 ± 0.77
OPG [pmol/L]	**5.41 ± 0.88***	3.57 ± 1.31
sRANKL [pmol/L]	**0.40 ± 0.03***	0.23 ± 0.01
OPG/sRANKL ratio	**10.30 ± 1.42***	15.87 ± 1.09

*$p \leq 0.05$ *vs.* control group

consent was obtained from all examined participants and their parents or legal guardians before participation.

Results

Mean body weight, BMI, BMI-SDS, the Cole index, and mean serum TGF-β1 concentration were significantly lower in girls with AN compared to healthy controls (group C). The changes in the mean concentration of TGF-β1 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).

In girls with AN, the Cole index and BMI correlated negatively and significantly with CTx and OPG concentrations. A negative and significant correlation was also revealed between BMI, the Cole index, and CTx (groups C and C + AN). Girls with AN exhibited a positive and significant correlation between the Cole index and serum TGF-β1. In the C + AN group, BMI and the Cole index correlated positively and significantly with the OPG/sRANKL ratio and serum TGF-β1 while TGF-β1 correlated positively and significantly with OC and the OPG/sRANKL ratio (Table II).

In the control group, the Cole index and BMI turned out to be independent predictors of CTx ($R^2 = 0.1394$, $p < 0.05$) while the Cole index, BMI, and TGF-β1 were independent predictors of the OPG/sRANKL ratio ($R^2 = 0.2658$, $p < 0.01$). In girls with AN, the Cole index

Table II. *Correlation between body mass index (BMI), Cole index, transforming growth factor β1 (TGF- β1), osteocalcin (OC), collagen type I crosslinked carboxyterminal telopeptide (CTx), osteoprotegerin (OPG), soluble receptor activator of nuclear factor-kB ligand (sRANKL), and OPG/sRANKL ratio in the control group (C), in girls with anorexia nervosa (AN), and in all girls (C + AN)*

Tabela II. *Korelacja między wskaźnikiem masy ciała (BMI), wskaźnikiem Cole'a, transformującym czynnikiem wzrostu β1 (TGF-β1), osteokalcyną (OC), karboksyterminalnym usieciowanym telopeptydem łańcucha α 1 kolagenu typu I (CTx), osteoprotegeryną (OPG), rozpuszczalnym ligandem receptora aktywatora czynnika jądrowego-kB (sRANKL) i wartościami wskaźnika OPG/sRANKL w grupie kontrolnej (C), u dziewcząt z jadłowstrętem psychicznym (AN) i w obydwu grupach łącznie (C + AN)*

Variables		C (n = 20)	AN (n = 60)	All girls C + AN (n = 80)
BMI [kg/m^2]	TGF-β1 [ng/mL]	0.258	0.187	**0.647***
	OC [μmol/L]	–0.318	0.207	0.140
	CTx [nmol/L]	**–0.485***	**–0.392***	**–0.329***
	OPG [pmol/L]	–0.243	**–0.267***	–0.129
	sRANKL [pmol/L]	0.031	0.143	–0.050
	OPG/sRANKL ratio	0.206	0.181	**0.248***
Cole index [%]	TGF-β1 [ng/mL]	0.243	**0.327***	**0.643***
	OC [μmol/L]	–0.346	0.247	–0.083
	CTx [nmol/L]	**–0.591***	**–0.339***	**–0.327***
	OPG [pmol/L]	–0.015	**–0.378***	–0.154
	sRANKL [pmol/L]	–0.016	0.179	0.041
	OPG/sRANKL ratio	0.257	–0.209	**0.235***
TGF-β1 [ng/mL]	OC [μmol/L]	0.197	0.301	**0.296***
	CTx [nmol/L]	0.117	–0.373	0.104
	OPG [pmol/L]	–0.287	–0.335	–0.082
	sRANKL [pmol/L]	–0.191	0.158	0.023
	OPG/sRANKL ratio	0.299	0.290	**0.319***

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

Table III. *Stepwise regression modelling for predictors of changes in indices of bone status: osteocalcin (OC), collagen type I crosslinked carboxyterminal telopeptide (CTx), osteoprotegerin (OPG), soluble receptor activator of nuclear factor-κB ligand (sRANKL) and OPG/sRANKL ratio in the control group (C; n = 20), in girls with anorexia nervosa (AN; n = 60), and in all girls (C + AN; n = 80). The covariates entered into the model were body mass index (BMI), Cole index and transforming growth factor β1 (TGF-β1)*

Tabela III. *Model regresji krokowej dla predyktorów zmian w wykładnikach stanu kośćca: osteokalcyny (OC), karboksyterminalnego usieciowanego telopeptydu łańcucha α1 kolagenu typu I (CTx), osteoprotegeryny (OPG), rozpuszczalnego ligandu receptora aktywatora czynnika jądrowego-κB (sRANKL) oraz wskaźnika OPG/sRANKL w grupie kontrolnej (C; n = 20), u dziewcząt z jadłowstrętem psychicznym (AN; n = 60) i w obydwu grupach łącznie (C + AN; n = 80). Współzmiennymi wchodzącymi do modelu są wskaźnik masy ciała (BMI), Cole'a indeks oraz transformujący czynnik wzrostu β1 (TGF-β1)*

	F ratio	P values	Variability contributed by specific variable (%)	Cumulative variability explained by model (%)	Groups
CTx [nmol/L]					
Cole index (%)	2.24	0.009	4.46	4.46	
BMI [kg/m²]	3.81	0.027	9.48	13.94	
OPG/sRANKL ratio					C
Cole index (%)	8.34	0.006	15.35	15.35	(n = 20)
BMI [kg/m²]	6.84	0.003	7.96	23.31	
TGF-β1 [ng/mL]	5.31	0.003	3.27	26.58	
CTx [μmol/L]					
Cole index (%)	3.32	0.047	6.35	6.35	
BMI [kg/m²]	3.42	0.041	6.13	12.48	
OPG [pmol/L]					
Cole index (%)	8.38	0.006	14.60	14.60	AN
BMI [kg/m²]	5.81	0.005	4.90	19.50	(n = 60)
OPG/sRANKL ratio					
Cole index (%)	12.57	< 0.001	31.77	31.77	
BMI [kg/m²]	19.74	< 0.001	28.53	60.30	
TGF-β1 [ng/mL]	15.98	0.047	5.42	65.72	
OC [μmol/l]					
TGF-β1 [ng/mL]	8.39	0.008	24.40	24.40	
CTx [nmol/L]					
Cole index (%)	6.24	0.015	9.57	9.57	All girls
BMI [kg/m²]	3.84	0.027	2.13	11.70	C + AN
OPG/sRANKL ratio					(n = 80)
TGF-β1 [ng/mL]	6.27	0.015	8.92	8.92	
Cole index (%)	3.92	0.024	2.15	11.07	
BMI [kg/m²]	3.27	0.027	2.59	13.66	

and BMI were shown to be independent predictors of CTx ($R^2 = 0.1248$, $p < 0.05$) and OPG ($R^2 = 0.1950$, $p < 0.01$) while the Cole index, BMI, and TGF-β1 independently predicted the OPG/sRANKL ratio ($R^2 = 0.6572$, $p < 0.001$). In the combination group (C + AN), TGF-β1 was identified to be an independent predictor of OC ($R^2 = 0.2440$, $p = 0.008$); the Cole index and BMI independently predicted CTx ($R^2 = 0.1170$, $p < 0.05$) while TGF-β1, the Cole index, and BMI were independent predictors of the OPG/sRANKL ratio ($R^2 = 0.1366$, $p = 0.027$) (Table III).

Discussion

There are only two reports on serum TGF-β concentrations in patients with AN; however, the results obtained by the two research teams are inconsistent [38, 39]. Pomeroy et al. [38] observed significant elevation of serum TGF-β in young women with AN (n = 16, mean age 23.3 ± 0.5 years) compared to healthy women with normal body weight (n = 11, mean age 27.7 ± 2.0 years). After a 10-day treatment, TGF-β concentrations decreased significantly in women with AN and returned to normal following the restoration of normal body weight. The authors suggest that elevated serum TGF-β may contribute to the immunosuppression observed in untreated AN patients. On the other hand, Corcos et al. [39] found that serum TGF-β2 was significantly decreased in patients with AN (n = 29, mean age 20.1 ± 1.9 years) compared to healthy participants (n = 20, mean age 23.7 ± 2.7 years). They concluded that changes in serum TGF-β2 of women with AN might result from undernourishment and/or specific deregulation of the anti/pro-inflammatory balance. Solmi et al. [5] performed a systemic PubMed literature search up to 31 December 2013 and meta-analysed cross-sectional or longitudinal studies comparing circulating TGF-β between patients with AN and healthy controls. Pooling the data from one cross-sectional [39] and one longitudinal study [38] revealed that TGF-β was not significantly different between AN and control women (standardised mean differences – SMD = 0.24, 95% CI = –1.63–2.10, p = 0.8). This might have been due to small study samples (45 women with AN and 31 healthy controls, altogether) and result inconsistency (TGF-β elevation [38] and TGF-β2 decrease [39] compared to the control). Differences in serum TGF-β concentrations might have also resulted from differences in the specificity, sensitivity, and precision of the assay methods used. Pomeroy et al. [38] measured TGF-β concentrations using bioassay (BIO) [41] whereas Corcos et al. [39] determined TGF-β2 levels by commercial ELISA — sandwich type kit. In our study, serum TGF-β1 was determined with an ultrasensitive ELISA. The method's sensitivity was 8.6 pg/mL, and intra- and inter-assay coef-

ficient of variations were: 3.2% and 4.9%, respectively. It should also be emphasised that the above-mentioned investigations were carried out in young women while we examined adolescent girls with AN.

Serum TGF-β1 concentrations in our adolescent study subjects with AN were comparable to those determined in young women studied by Corcos et al. [39]. Our patients with AN had significantly lower serum TGF-β1 compared to healthy controls with normal body weight. The changes in serum TGF-β1 were associated with significant weight loss and a decrease in BMI, BMI-SDS, and the Cole index. A significant suppression of OC and CTx concentrations and the OPG/sRANKL ratio was also found, while serum OPG and sRANKL were significantly increased. In girls with AN, the Cole index and BMI correlated negatively and significantly with CTx and OPG concentrations. A negative and significant correlation was also revealed between BMI, the Cole index, and CTx (groups C and C + AN). In the C + AN group, BMI and the Cole index correlated positively and significantly with the OPG/sRANKL ratio, while the Cole index correlated positively and significantly with serum TGF-β1. The obtained results, and especially correlation analyses, seem to indicate that abnormalities in TGF-β1 and several bone markers observed in girls with AN may result from chronic starvation and resultant malnutrition. In our regression model, the Cole index contributed to 4.46% and 15.35% (control group — C), 6.35% and 31.77% (AN group), and 9.57% and 2.15% (C + AN group) of CTx and OPG/sRANKL ratio variability, respectively. BMI contributed to 9.48% and 7.96 (C group), 6.13% and 28.53% (AN group), and 2.13% and 2.59% (C + AN group) of CTx and OPG/sRANKL ratio variability, respectively. Moreover, in girls with AN, the Cole index contributed to 14.60% and BMI contributed to 4.90% of OPG variability. Adipose tissue depletion caused by starvation and malnutrition may affect serum OPG and/or sRANKL and thereby also the OPG/sRANKL ratio. The latest *in vitro* studies show that human adipocytes regulate the expression of OPG and RANKL in human osteoblastic cells with osteoblastic cells not only producing less RANKL but also more OPG. The OPG/RANKL ratio markedly increased in primary human preosteoblasts (mRNA and proteins) when stimulated with adipocyte-secreted factors [42–44].

Differences in results from *in vivo* and *in vitro* studies regarding the relationship between TGF-β1 and bone status indices might be related to interferences from those endogenous factors, the concentrations of which are severely altered in patients with AN including oestrogens, glucocorticoids (GC), parathyroid hormone (PTH), vitamin D, and cytokines other than TGF-β1 (i.e. IL-1, IL-6, TNF-α, INF-γ, and PGE_2) [15, 25–31, 33–36], which modulate or are modulated by TGF-β1 signalling in a number of ways. The above-mentioned osteotropic factors might modify bone remodelling either directly (via specific receptors) or/and indirectly (via the RANKL/RANK/OPG system), and with or without TGF-β1 as a mediator [1, 15, 27–32, 34, 45–47].

Oestrogens stimulate TGF-β1 production in osteoblasts, and promotes osteoblast proliferation and differentiation. Oestrogens may also inhibit osteoblast apoptosis and prevent bone loss by promoting osteoclast apoptosis through a TGF-β-dependent mechanism. Oestrogen receptor α (ER-α) was identified as a corepressor for Smad activity, and Smad3 was identified as an enhancer of ER-α-mediated transcriptional activity, indicating that oestrogen and TGF-β1 coordinate actions during bone formation [7]. Therefore, oestrogen is a potent anabolic agent in the bone. Profound oestrogen deficiency observed in girls with AN [12, 15, 28, 33, 34] may adversely influence bone remodelling. GC are known to promote osteoblast apoptosis and inhibit osteoblast proliferation and differentiation while promoting the differentiation of osteoclasts. GC up-regulate TGF-β1 expression in osteoblasts; however, unlike oestrogens, they synergise with TGF-β-enhanced osteoclast formation by stimulating the priming of osteoclast progenitors for differentiation into osteoclasts [7]. Thus, hyperactivity of the hypothalamic-pituitary-adrenal axis seen in girls with AN may negatively affect bone remodelling. PTH enhances bone formation by increasing TGF-β-mediated type I collagen production in osteoblasts, and it promotes bone resorption by binding to PTH receptor on osteoblasts and stimulating osteoblasts to increase their expression of OPG. Thus, PTH acts as a double-edged sword for bone remodelling. Vitamin D is an important regulator of calcium homeostasis in the bone. The active metabolite of vitamin D (1,25-OH_2D_3) stimulates bone formation by promoting osteoblast differentiation and extracellular matrix mineralisation. Moreover, 1,25-OH_2D_3 promotes TGF-β1 secretion and binding by human osteoblasts. At physiological doses, it also inhibits PTH-mediated bone resorption [7]. However, TGF-β1 stimulates vitamin D receptor expression in mature osteoblast cell line. Fonseca et al. [48] reported prevalent vitamin D insufficiency in young women with AN. Calcium and vitamin D intake among patients with AN is presumably low due to dietary restriction. Other authors [49] studied overall school-aged population and revealed that vitamin D intake was only 23% of the reference daily intake while calcium intake was approximately 62.1% of the daily recommendations. Our previous study [50] revealed elevated serum PTH levels in almost 25% of females with AN. Like other hormonal aberrations observed in malnutrition, low oestrogen levels can also contribute to higher PTH. As females

with AN do better than controls regarding vitamin D intake, vitamin D deficiency should be more evident in the normal weight controls, with PTH elevations in both groups.

Changes in the concentrations of several pro-inflammatory cytokines observed in females with AN [36] may modify bone remodelling [30]. These cytokines can modulate or are modulated by TGF-β1. IL-1 stimulates TGF-β1 activity in the bones of the calvaria, but it inhibits TGF-β1 secretion by osteoblast-like cells. On the other hand, TGF-β1 inhibits IL-1 receptor expression on osteoclast precursors [2]. TGF-β1 has a stimulating effect on IL-6 mRNA expression in primary rat osteoblasts, IL-6 mRNA, and protein expression in murine bone marrow stromal cells as well as IL-6 expression by human osteoblasts [2]. However, IL-6 also proved to mediate PGE_2-induced suppression of OPG production by osteoblasts [51]. PGE_2 stimulates TGF-β1 expression in osteoblasts. TGF-β1 stimulates PGE_2 production by osteoblasts as well as osteoblast proliferation through a PGE_2-dependent mechanism [52]. TGF-β1 and TNF-α synergise and antagonise each other's function in bone tissue. TGF-β potentiates TNF-α-stimulated osteoclast formation [53]. On the other hand, it attenuates TNF-α-induced osteoblast apoptosis and bone resorption [54, 55]. TNF-α opposes the inhibitory effects of TGF-β1 on proliferation of bone marrow haematopoietic precursors and TGF-β1-induced up-regulation of collagen [56]. There is also cross-talk between TGF-β1 and INF-γ in bone. TGF-β1 antagonises INF-γ-induced suppression of osteoclast formation [53]. The results of our previous investigations in girls with AN indicate that significant suppression of bone marker concentrations and increased OPG and sRANKL levels associated with a significant reduction of the OPG/sRANKL ratio are concomitant with a significant increase in osteoclastogenesis modulators including serum IL-1β, IL-6, and TNF-α. IL-6 and IL-1β were identified to be independent predictors of CTx; TNF-α and IL-6 independently predicted sRANKL, while TNF-α, IL-6, IL-1β were independent predictors of the OPG/sRANKL ratio [30]. No significant changes were revealed regarding INF-γ concentrations in females with AN [36, 39]. However, the production of INF-γ by stimulated peripheral blood mononuclear cells was decreased [57] or elevated in subjects with AN compared to controls [36,37]. To our knowledge, so far there have been no reports on PGE_2 concentrations and the relationship between PGE_2 and bone metabolism in girls with AN.

Changes in the concentrations of the above-mentioned hormones and several cytokines including TGF-β1 suppression may result in desynchronisation between bone formation and/or resorption markers and the OPG/sRANKL system. We observed such desynchronisation in our previous study [26, 30, 31] and the present investigation concerning girls with AN. The positive and significant correlation between serum TGF-β1 and OC concentrations as well as between TGF-β1 and the OPG/sRANKL ratio demonstrated in the total study population (C + AN) presenting with substantial differences in body weight indicates a possible regulatory role of this cytokine in bone remodelling. In our regression model, TGF-β1 contributed to 24.40% (C + AN group) of OC variability. It also contributed to 3.27% (C group), 5.42% (AN group), and 8.92% (C + AN group) of OPG/sRANKL ratio variability. These results are suggestive of a relationship between TGF-β1 and OC, OPG/sRANKL ratio in our study subjects and seem to confirm the hypothesis generated from *in vitro* studies, namely that TGF-β1 might play a role in the regulation of bone remodelling in girls with AN with concurrent involvement of the RANKL/RANK/OPG system [2, 4, 6, 8]. The concept of desynchronisation between bone metabolism and the RANKL/RANK/OPG system in girls with AN seems to be lent support by a negative and significant correlation between CTx and sRANKL observed along with a positive correlation between bone markers and the OPG/sRANKL ratio. In our previous studies, the OPG/sRANKL ratio was identified to be an independent predictor of OC while the OPG/sRANKL ratio and BMI independently predicted CTx [26]. Our investigations in girls with AN did not reveal significant correlations between TGF-β1 and CTx or between TGF-β1 and OPG, sRANKL concentrations [26], indicating that it is rather the OPG/sRANKL ratio and not each of these cytokines separately that determines osteoclast differentiation, activation, and apoptosis.

Just like Corcos et al. [36, 39], we hypothesised that decreased serum TGF-β1 observed in women with AN had resulted from chronic starvation and malnutrition. However, dysregulation between TGF-β1 and the above-mentioned osteotropic factors should also be considered. It seems plausible that alterations in the relationships between TGF-β1, osteotropic agents (whose concentrations are abnormal in AN and which modulate or are modulated by TGF-β1), bone markers, and the RANKL/RANK/OPG system might be involved in the development of osteoporosis in girls with AN.

Conclusions

Changes in bone markers, OPG, sRANKL, and/or the OPG/sRANKL ratio observed in girls with AN are associated with changes in serum TGF-β1.

TGF-β1 suppression in girls with AN might alter the relationship between bone metabolism and the OPG/sRANKL system, which, in turn, might compromise the mechanism compensating for bone remodelling disturbances.

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