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Research Article

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Serum Osteoprotegrin, Total Srankl and Their Determinants in Adolescents with Type 1 Diabetes

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Abstract

Aim: *The aim of the study was to evaluate the levels of osteoprotegerin (OPG), receptor activator of nuclear factor –κB ligand (s-RANKL), OPG/sRANKL ratio and sRANKL/OPG ratio in adolescent patients with type 1 diabetes mellitus (T1DM), and to assess their correlation with the following factors: patients' gender, age, metabolic control, age of the diagnosis and duration of the disease.*

Patients and Methods: *60 T1DM patients (32 girls and 28 boys, mean±SD age: 15.0±1.9 years, diabetes duration: 5.1±3.9 years, age of the diagnosis: 9.9±3.9 years, HbA1c: 7.9±1.4%) and 18 healthy matched controls were included. Osteoprotegerin and total sRANKL (free and bound sRANKL) were measured by ELISA (enzyme-linked immunosorbent assay).*

Results: *There were no statistically significant differences in serum OPG levels between patients and controls (51.56 ± 12.05 vs. 50.98 ± 13.55 pmol/L, p = 0.84. No correlation betweengender and OPG levels both in the study and control group has been reported, although OPG levels were significantly lower in diabetic boys (2.59±0.67 pmol/L) than in control boys (3.30±1.01 pmol/l) (p = 0.031). Tetryl analysis (qualifications) dependent on OPG levels has demonstrated statistically significant correlation between the study group and clinical factors such as: gender, age of the diagnosis and duration of the disease, but not with the age. Only the tendency toward correlation between OPG and metabolic control of diabetes (p=0.093) has been observed. No statistically significant differences in sRANKL levels between the study group and controls has been identified. In patients with T1DM no correlation between RANKL levels and clinical factors such as gender, duration of the disease, age of the diagnosis and metabolic control has been reported. Only negative correlation between RANKL level and patients' age (p = 0.002) has been observed. No correlationbetween OPG and RANKL levels has been demonstrated. No statistically significant differences in OPG/RANKL ratios between the study group and controls has been reported, the only significant difference in these ratios was observed between control females and males (p = 0.019), but not in the study group. A positive correlation between OPG/RANKL ratio and OPG level with the age has been demonstrated in the study group.*

Keywords: Diabetes Mellitus, Osteoprotegrin

Abbreviations

Anorexia nervosa - AN
Body mass density – BMD
Enzyme-linked immunosorbent assay – ELISA
Glycated haemoglobin- HbA1c
Inflammatory Bowel Disease - IBD
Osteoprotegerin – OPG
Receptor activator of nuclear factor –κB ligand - s-RANKL
Type 1 diabetes mellitus - T1DM
The Children's Memoria Memorial Health Institute – CHMHI
Years old – y.o.

Introduction

Diabetes mellitus (DM) is a group of metabolic disorders characterized by chronic hyperglycemia caused by the dysfunction of insulin secretion and /or its function [1].

In children and adolescents, type 1 diabetes (T1DM) is the most common chronic childhood disease together with the bronchial asthma. Poland is classified as a country with one of the highest incidence rates for T1DM in Europe, wherein the greatest disease factor is being observed in the youngest children[2]. Since in most children with T1DM, the disease develops during developmental period, maintenance of good metabolic control is crucial forproper physical development, puberty, and for preventing/postpone late complications in these patients. In T1DM, there is an increased risk for other autoimmune diseases, such as atherogenesis and cardiovascular disease due to micro and macroangiopathy. Prognosis for diabetic patients with these autoimmune is worse than for the population [3,4]. Individuals with T1DM are more likely to develop osteoporosis and pathological fractures with significantly reduced bone mineral density[5]. It is not clear when bone abnormalities/ disorders begin and what is their etiopathogenesis. Bone remodeling, which is a lifelong process, results from the formation and resorption of the bone tissue. Its role is to maintain the proper mass and mineralization of bones and to achieve peak bone mass during childhood. Osteoblasts play a key role in bone formation and in the process of resorption of osteoclasts [6]. The balance between these two processes ensures/provides the maintenance of bone mass. The maturation and activation of osteoclasts is under the control of the OPG / RANK / RANKL system [7-9].OPG, a glycoprotein belonging to the family of TNF receptors, was described for the first time in 1997 [7].

It is a homodimer, does not have an endothelium domain, and is secreted as soluble protein. The highest expression of OPG mRNA is in the bones, but also in many other organs and tissues, such as heart and vessels, liver, and intestines [10]. OPG inhibits the proliferation and terminal stages of osteoclast differentiation and induces mature osteoclast apoptosis [11]. Estradiol, TGF-β, TNF-α, IL-1, bone morphogenetic protein 2 (BMP-2) and decreases: PTH, pTH-like peptide, glucocorticosteroids and PGE2 [12]. OPG is biologically active when combined with a specific ligand - RANKL (activator of nuclear factor κB-ligand). RANKL is also a glycoprotein belonging to the TNF family. Its expression has not only been demonstrated in osteoblasts, but also in the fetal liver, peripheral lymph nodes and other tissues [12]. It is produced by mature osteoblasts, their precursors, and activated T lymphocytes. The ligand is essential for the maturation of osteoclasts, but it also contributes to the development of thymocytes [11]. RANKL has is biologically activated by binding to the specific receptor RANK (activator of nuclear factor κB) located on the surface of osteoclasts, preosteoclasts, T and B lymphocytes, vascular endothelial cells, liver, spleen and other tissues [10]. RANK is a transmembrane receptor with 3 domains belonging to the TNF receptor superfamily [10].

The connection of RANK and RANKL determines the course of osteoclastogenesis at all stages [12]. OPG is a "fake" competitive receptor, binding to RANKL, preventing it from binding to RANK and signaling, causing the opposite effect - preventing bone loss [10]. The disorders of OPG / RANKL system not only affect bone metabolism but also play an important role in cardiovascular, inflammatory, cancer and autoimmune diseases [13-15]. Most available data on that issue come from the research in adults. In children, the OPG / RANK / RANKL system has been studied in children with inflammatory bowel disease, Kawasaki disease, juvenile idiopathic arthritis and type 1 diabetes, cystic fibrosis [13,15-18].OPG is produced and plays its biological role not only in bones but also in the walls of blood vessels. Calcifications are found in the plaques which increase stiffness of the vessels. Calcium deposition also has been observed in the inner wall of the vessel. In the animal model, in mice lacking the gene for OPG, there was not only severe osteoporosis, but also hypercalcificationof the aortic wall and renal arteries [19]. In humans, a positive correlation between OPG and vascular calcification and cardiovascular disease has been demonstrated [20]. OPG is also involved in the formation of atherosclerotic plaque in the blood vessels [21]. It is unclear whether OPG is a marker of endothelial injury or whether it accelerates the progression of vascular disease or it is rather a protective mechanism against vascular injury [20]. The role of RANKL is even less studied/known than OPG. In literature, its contribution to the differentiation of osteoclasts and calcification of smooth muscle cells in the vascular wall has been reported [20,21]. RANKL gene deletion caused osteopetrosis, lack of mature osteoclasts,and incorrect teeth eruption [7]. RANKL has free forms and is bound with OPG (or with other proteins) [8]. This connection of OPG and RANKL is clinically relevant, as the effect of drugs or other substances on OPG can affect RANKL serum levels and the OPG / RANKL ratio. The OPG / RANKL rate/index provides information on the imbalance between the two systems, which may be found in diseases of the skeletal and cardiovascular system [7].

Material and Methods

The study included 60 patients with T1DM treated at the Department of Endocrinology and Diabetology of the Children's Memorial Health Institute (CMHI) in Warsaw (32 girls -53.3% and 28 boys - 46.7%). Patients older than 12 years old (y.o) were included in the study. The average age of girls was 14.7 ± 1.8 y.o. and boys 15.6 ± 1.9 y.o (Table 1). The disease duration was 5.1 ± 3.9 years, and the age of onset was 9.9 ± 3.9 years. Patients were treated with intensive insulin therapy or continuous subcutaneous insulin infusion using a personal insulin pump. Children with other chronic diseases and complications of chronic microangiopathy were excluded (retinopathy was excluded by ophthalmologic examination, and nephropathy by the lack of microalbuminuria, neuropathy was diagnosed on neurological basis). The control group consisted of 18 healthy individuals aged 14.0 ± 1.9 years. The average age of girls (n=32) was 13.8 ± 1.8 y.o. and boys (n=28)14.5 ± 2.1 y.o.

Table 1: Baseline characteristics of the study population

	Diabetic N=60	Control N=18
Gender (% females)	53.3	66.7
Age (y)	15.1 ± 1.9 (14.5 -15.5)	14.0 ±1.9 (13.1 – 15.0)
Girls	14.7 ± 1.8	13.8 ± 1.8
Boys	15.6 ± 1.9	14.5 ± 2.1
OPG (pmol/L)	2.78 ± 0.67	3.10 ± 0.89
	(2.61 - 2.96)	(2.65 - 3.54)
Total sRANKL (pmol/L)	405.4 ± 207.8	456.6 ± 233.2

	(351.7 - 459.0)	(340.6 - 572.6)
sRANKL / OPG (ratio)	152.0 ± 81.5	157.3 ± 76.9
	(131.0 - 173.1)	(119.1 - 195.6)
Age at diabetes diagnosis (y)	9.9 ± 3.9	-
	(9.0 - 11.0)	
Diabetes duration (y)	5.1 ± 3.9	-
	(4.1 - 6.1)	
HbA1c (%) – 1-year period	7.9 ± 1.4	-
	(7.6 - 8.3)	
Body height (cm)	165 ± 11.5	-
	(163.5 – 169.4)	
Body weight (kg)	58,9 ± 11.9	-
	(55.9 – 62.0)	
Body weight (SD-score)	0.29 ± 1.16	-
	(-0.01– 0.59)	
BMI (kg/m^2)	21.1 ± 3.4	-
	(20.2 – 22.0)	
BMI (SD-score)	0.31 ± 1.22	-
	(-0.01 – 0.63)	

mean ± SD (95% CI) or percentage of subjects (%); not significant differences p>0.05
OPG: Osteoprotegerin
Total sRANKL(free and bound): soluble Receptor Activator of Nuclear Factor-κB Ligand

Blood for laboratory testing (HbA1c, OPG, total sRANKL) was collected in the morning (8.00), after night fasting in hospital. In all T1DM patients, anthropometric measurements were performed and the puberty stage was assessed according to Tanner's scale.

The study was conducted with permission and under the supervision of the local Ethical Committee at the CMHI in Warsaw, Poland, and in accordance with the Declaration of Helsinki. Informed consent for the participation in the study was obtained in every case from patient's parents, and, additionally from the patient him-or herself, if he or she was sixteen y.o. or older, according to Polish law.

Laboratory Methods

From each subject, a sample of venous blood was taken in the morning after the night fasting. Following centrifugation, the serum was frozen and stored at -80 ° C until analysis. All analysis were performed strictly according to the manufacturer protocols. The concentration of osteoprotegerin and total sRANKL was determined by enzyme-linked immunosorbent assay (ELISA).

Assay of the total OPG (osteoprotegrin) in the serum samples was performed using MicroVue OPG enzyme immunoassay (EIA) kit from Quidel Corporation, San Diego, CA, USA. Intra- and inter-assay CV was 3.5 and 6.1%, respectively. Sensitivity of the assay was 0.4 pmol/l. There was no cross-reactivity of the antibodies with recombinant human CD40, sTNF RI and TNF RII. Norman range for healthy adults was established by the manufacturer is 5.7 ± 0.42 pmol/l.

Quantitative measurement of the total (free and bound sRANKL) in the serum samples were performed using sRANKL ELISA kit from Bio Vendor, Brno, Czech Republic Intra- and inter-assay coefficient of variation (CV%) was 7.25 and 11.2%, respectively. Sensitivity of the assay was 0.4 pmol/l (55 pg/ml). There was no cross-reactivity of the antibodies applied in the assay with human OPG, RANK, COMP, osteocrin, CRP at 50 ng/ml and with TNF-alpha, IL-6,

IL-11 at 2 ng/ml.Normal range for healthy adults (25-65 years old) has been established by the manufactures at 339 ± 42.3 pmol/l.

Patients' metabolic control was determined on the basis of HbA1c. HbA1c glycated hemoglobin (%) was assessed by turbidimetry using a Roche Diagnostics kit. Patients' metabolic control was classified into 2 groups: the group with HbA1c≤7.5% (good/satisfactory control) or with HbA1c≥7.5% (unsatisfactory metabolic control of diabetes). Poor metabolic control was defined as HbA1c> 9.0%. HbA1c was assessed during the visit and averaged according to the last year's disease history.

Statistical Analysis

Statistical analysis was performed using the SPSS v.19.0 and STATISTICA v.12 package. The arithmetic mean (x) standard deviation (SD) and 95% confidence interval (95% CI) were calculated from the obtained data. Verifications of normal distribution were made using the Shapiro-Wilk test. Homogeneity of variance was assessed using the Fisher-Snedecor test. Comparisons of the values of the parameters tested were carried out using an analysis of covariance with gender and age adjustment (ANCOVA) using Fisher's least significant difference (LSD). The relationships between the tested features were evaluated using Pearson's correlation coefficient and Spearman's correlation. Chi2 test was used for qualitative variables.

In assessing serum OPG, thymus was used as a positional measure that characterizes the distribution of this variable. The OPG concentration has been characterized as low, medium and high. Tertyl first - lower (T1), includes 1/3 of the data set with the lowest concentrations of OPG. In turn, the third upper tertiary (T3) includes 1/3 of the analysis results that have the highest concentrations of OPG. Between these values is the interval defined as the second tertiary center (T2), and it is a set with intermediate levels of OPG. In individual tetryl, sRANKL concentrations and calculated ratios (OPG / sRANKL, sRANKL / OPG) and metabolic control parameters of diabetes, disease time and age were compared. P < 0.05 wasconsidered statistically significant for the results.

Results

General characteristics of studied groups

Table 1 presents the general characteristics of the TIDM adolescent patients and the control group. There were no statistically significant differences between the study and the control group in the values of the investigatedparameters.

Evaluation of osteoprotegerin concentrations

There was no significant impact of the gender on OPG concentrations in either T1DM and control groups (Table 2).Girls suffering from T1DM revealed OPG concentration values of 2.95 ± 0.64 pmol/ LandT1DM boys of 2.59 ± 0.67 pmol / l, respectively (p = 0.146). Among controls, ,OPG concentrations of 2.99 ± 0.85 pmol/L and 3.30 ± 1.01 pmol/L were noted in girls and boys (p = 0.870), respectively.

Lack of significant difference was noted when OPG concentration values were compared in T1DM females control counterparts (p = 0.858). In boys,the OPG concentration of 2.59 ± 0.67 pmol/Lappeared significantly lower in T1DM group compared to controls(3.30 ± 1.01 pmol/L; p = 0.031) (Figure 1 &Table 2).

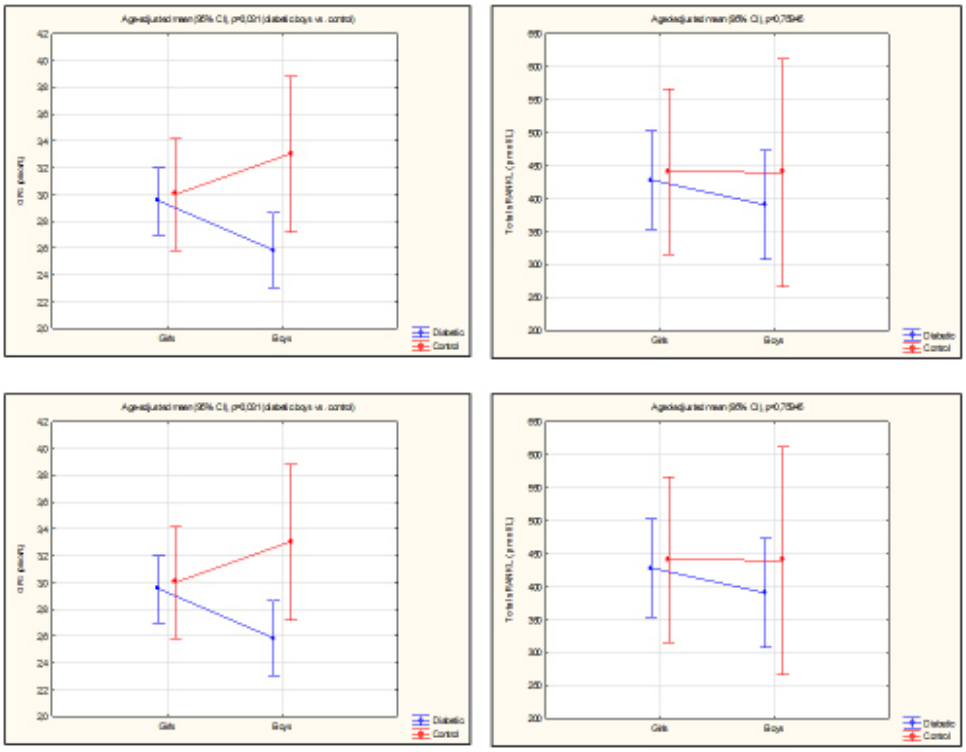

Figure 1: Serum level of osteoprotegerin, total sRANKL, OPG/sRANKL (ratio) and sRANKL/ OPG (ratio) in diabetes patients in comparison with control subjects (adjusted for age).

Table 2: Serum concentration mean ± SD (95% CI) of osteoprotegerin, total sRANKL, OPG/sRANKL (ratio), sRANKL/OPG (ratio) in diabetes patients in comparison with healthy subjects control, and the age-adjusted distribution of diabetes (D), gender (S) and their interaction (D x S)

	Girl		**Boys**		***p value****		
	Diabetic	Control	Diabetic	Control	*pD*	*pS*	*pD x S*
OPG (pmol/L)	2.95 ± 0.64 (2.72- 3.18)	2.99 ± 0.85 (2.45 - 3.53)	2.59 ± 0.67 (2.33 - 2.85)	3.30 ± 1.01 (2.25 - 4.36)	0.066	0.878	0.031 (boys D vs. C)
Total sRANKL (pmol/L)	433.0 ± 210.9 (357.0 - 509.0)	462.1 ± 116.2 (388.3 - 535.9)	373.8 ± 203.3 (294.9 - 452.6)	445.7 ± 393.8 (332.4 - 858.9)	0.614	0.748	0.759
OPG / sRANKL (ratio)	0.0081 ± 0.0039 (0.0067 - 0.0095)	0.0068 ± 0.0026 (0.0052 - 0.0084)	0.0070 ± 0.0114 (0.0027 - 0.0277)	0.0014 ± 0.0246 (0.0035 - 0.0314)	0.228	0.037	0.036 (girls D vs. boys C) 0.019 (girls C vs. boys C)
sRANKL / OPG (ratio)	153.5 ± 80.0 (124.7 - 182.4)	163.9± 55.8 (128.4 - 199.4)	150.3 ± 84.6 (117.5 - 183.1)	144.2 ± 113.8 (124.7 - 263.6)	0.831	0.832	0.683

* age-adjusted p-value for ANCOVA (post-hoc tests using Fisher's Least Significant Difference test); statistically significant differences (p≤0.05)

Analyzes of tertile qualification of OPG concentrations were performed and results are presented in (Table 3 &Table 4). Approx. 59% of T1DM children had OPG levels within lower tertile (2.45 pmol/L); in the group of youngest children (<7 years old at diagnosis) only 14.3% had OPG concentration at this tertile.As shown in Table 4, a significant correlation between OPG concentration and gender (p = 0.038), age of disease onset (p = 0.0034) and disease duration (p = 0.0048)was revealed in T1DM group. In the group of children with diabetes, 21.9%, of T1DM girls and 46.4% T1DM boys had OPG concentrations in the lower tertilium (2.45pmol/L) and 46.9% of girls but only 17.9%of T1DM boys revealed OPG concentrations in the upper tertilium(2.96pmol/L) (p = 0.038). Further, OPG concentrations were thelowest (2.45 pmol/L) in 50% of children with the shortest disease duration (2 years). The correlation between the OPG concentrations and disease duration (T1 vs. T2, p = 0.0048) was also observed (T1 vs. T2, p= 0.0048).

Table 3: Correlation coefficients between serum levels of OPG, sRANKL, OPG/sRANKL (ratio), sRANKL/OPG (ratio), and age, and gender in adolescents with type 1 diabetes and control group

	Diabetic		Control		
	Gender[1])	Age [2])	Gender[1])	Age [2])	
Korelacje Pearsona					
OPG (pmol/L)	R	-0.338*	-0.109	0.148	0.245
	P	0.008	0.408	0.559	0.327
sRANKL (pmol/L)	R	-0.146	-0.231	-0.250	-0.051
	P	0.267	0.076	0.317	0.840
OPG/sRANKL (ratio)	R	0.039	0.264*	0.250	0.182
	P	0.770	0.042	0.317	0.469
sRANKL/OPG (ratio)	R	-0.037	-0.194	-0.250	-0.203
	P	0.781	0.137	0.317	0.419

[1]) Spearman rank correlation coefficients (r)
[2]) Pearson correlation coefficients (r)
* statistically significant correlations (p≤ 0.05)

Table 4: Serum levels of OPG, sRANKL, OPG/sRANKL (ratio), and sRANKL/OPG (ratio) in adolescents with type 1 diabetes and control group according to tertiles of OPG

Tertiles of serum osteoprotegerin (pmol/L)				
	Tertile 1 Low	Tertile 2 Medium	Tertile 3 High	p value*
	<2.45	2.45 – 2.96	> 2.96	
Diabetes				
OPG	2.07 ± 0.27	2.72 ± 0.12	3.55 ± 0.4	0.001 (T1 vs.T2 vs.T3)
(pmol/L)	(1.94 – 2.19)	(2.67 – 2.78)	(3.36 – 3.74)	
sRANKL	346,6 ± 133,8	457,1 ± 274.1	412.4 ± 185.8	0.0901 (T1 vs.T2)
(pmol/L)	(284.0 - 409.2)	(328.8 - 585.4)	(325.4 - 499.3)	
OPG/sRANKL	0.0070 ± 0.0037	0.0085 ± 0.0059	0.0103 ± 0.0044	0.0277 (T1 vs.T3)
(ratio)	(0.0053 - 0.0088)	(0.0058 - 0.0113)	(0.0082 - 0.0123)	
sRANKL/OPG	170.9 ± 69.2	169.1 ± 105.1	116.1 ± 52.5	0.0279 (T1 vs.T3) 0.0335 (T2 vs.T3)
(ratio)	(138.5 - 203.2)	(119.9 - 218.2)	(91.6 - 140.7)	
Age [1])	15.3 ± 2.0	15.1 ± 1.8	14.6 ± 2.2	ns
(y)	(14.4 - 16.3)	(14.3 - 16)	(13.6 - 15.6)	
Controls				
OPG	1.99 ± 0.21	2.79 ± 0.36	3,76 ± 0,66	0.001 (T1 vs.T2 vs.T3)
(pmol/L)	(1.66 - 2.32)	(2.34 - 3.23)	(3.25 - 4.26)	
sRANKL	429.0 ± 124.6	425.6 ± 146.5	486.1 ± 311.6	ns
(pmol/L)	(230.8 - 627,2)	(243,7 - 607,6)	(246,6 - 725,6)	
OPG/sRANKL	0.0050 ± 0,0018	0,0074 ± 0,0036	0,0114 ± 0,009	ns
(ratio)	(0.0021 - 0.0079)	(0,003 - 0.0119)	(0,0045 - 0.0183)	
sRANKL/OPG	220.2 ± 77.5	156,7 ± 59,4	129,7 ± 75,6	0.0629 (T1 vs.T3)
(ratio)	(96.9 - 343.6)	(82,9 -230.5)	(71.6 -187.8)	
Age 1)	13.6 ± 2.5	12.8 ± 0.8	14.9 ± 1.7	0.0540 (T2 vs.T3)
(y)	(9.6 - 17.6)	(11.8 - 13.8)	(13.6 - 16.2)	

Mean ± SD (95% CI); statistically significant differences (p≤0.05)
* age-adjusted p-value for ANCOVA (post-hoc tests using Fisher's Least Significant Difference test)
[1]) p-value for ANOVA (post-hoc tests using Fisher's Least Significant Difference test)

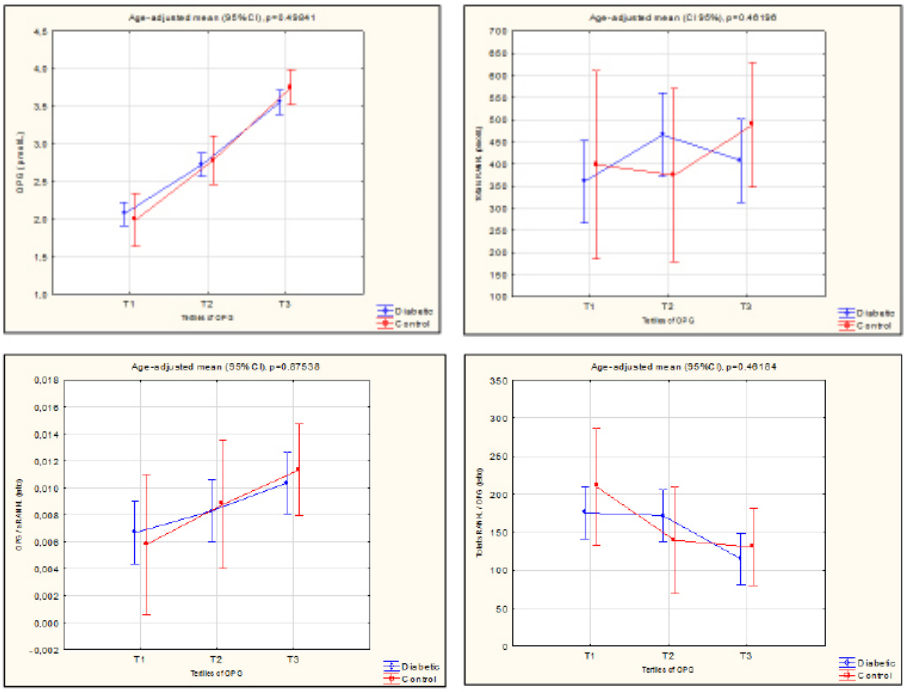

Figure 2: Serum levels of OPG, sRANKL, OPG/sRANKL (ratio), and sRANKL/OPG (ratio) in adolescents with type 1 diabetes and control group according to tertiles of OPG

Negative correlations between OPG concentration values and age were reported, and OPG values appeared lower in older children (T1vs. T2, p = 0.0034 and T1 vs. T3, p = 0.048).

There was no significant correlation between OPG and metabolic control, assessed on the basis of HbA1c. OPG concentrations > 2.96 mmol / L were foundin 50% of patients with HbA1c ≥9% and only in 28.3% with HbA1c <9% (Figure 3). Further, in the lowest OPG tertilethe mean HbA1c (%) was 1.7 ± 1.7%, in the middle one it was 7.8 ± 1.0%, and in the third OPG tertile was 8.2 ± 1.4%.

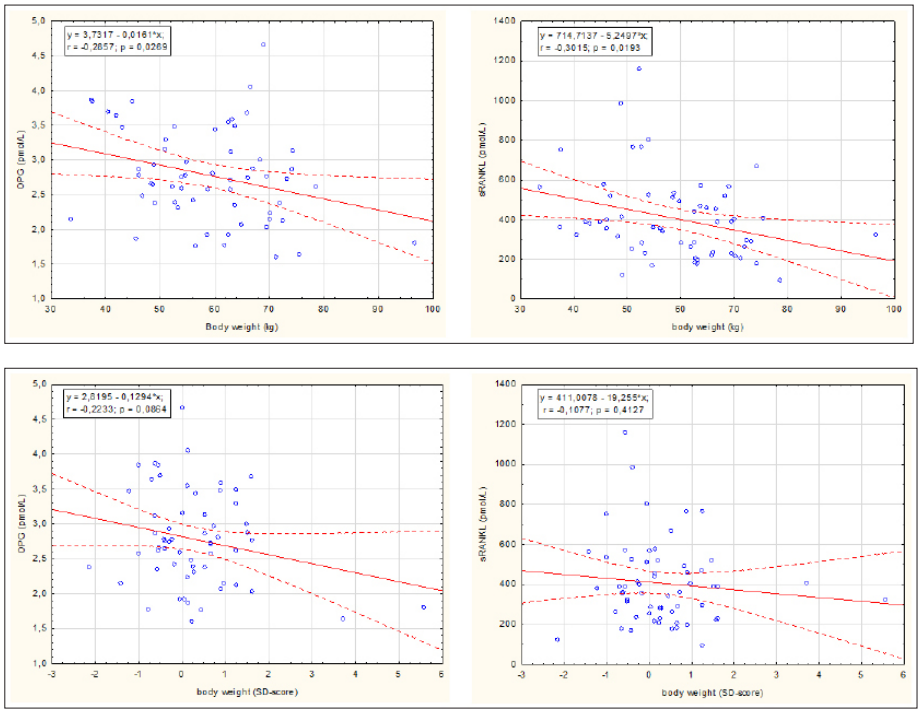

Figure 3: Correlation coefficients between serum levels of OPG, sRANKL and body weight (kg and SD-score) in adolescents with type 1 diabetes

Evaluation of sRANKL concentrations

There were no significant differences in total sRANKL between the study group (405.35 ± 207.8 pmol / l) and control (456.6 ± 233.2 pmol / l) (Table 1). Thecorrelation between OPG and total sRANKLwas not significant (p = 0.090). The highest total sRANKL concentration was found in the middle OPGtertile in the T1DM group and in the upper OPG concentration tertileamong controls (OPG> 2.96 pmol/L).

The total sRANKLvalues did not differ between girls and boys (girls: 440.93 pmol / L, boys: 386.44 pmol / L), however T1DM boys had trended to have slightly lower total sRANKL values (373.8 ± 203.3 pmol / L) when compared to healthy counterparts (445.7 ± 393.8 pmol / L; ns) (Table 2).

Although the OPG/total sRANKL ratio in T1DM group as a whole (0.0086 ± 0.0048)was not significantly different (p> 0.05) from the values observed in healthy (0.0089 ± 0.0070;ns; Table 2), T1DM girls had significantly higher ratio values of 0.0081 ± 0.0039 compared to OPG/total sRANKL ratios noted in healthy girls(0.0068 ± 0.0026; p = 0.019). The same was noted when OPG/total sRANKL ratio values of T1DM and healthy boys were analyzed (0.0070 ± 0.0114 vs 0.0014 ± 0.0246; p = 0.036) (Table 2).

Table 5: Characteristics of determinants (age, gender, age at diagnosis, diabetes duration and metabolic control) in adolescents with type 1 diabetes according to tertiles of serum osteoprotegerin

Tertiles of serum osteoprotegerin (pmol/L)					
		Tertile 1 Low	Tertile 2 Medium	Tertile 3 High	p value*
		<2.45	2.45 – 2.96	> 2.96	
Gender	Girls	21,9	31,3	46.9	0.038
subjects (%)	Boys	46.4	35.7	17.9	
Age (years)	x ± SD	15.3 ± 2.0	15.1 ± 1.8	14.6 ± 2.2	ns
	(95% CI)	(14.4 - 16.3)	(14.3 - 16.0)	(13.6 - 15.6)	
subjects (%)	12-15y	32.3	32.3	35.5	ns
	≥ 15y	34.5	34.5	31.0	
Age atdiagnosis	x ± SD	11.9 ± 3.1	8.4 ± 4.2	9.6 ± 3.7	0.0034 (T1vs.T2)
(years)	(95% CI)	(10.5 - 13.4)	(6.5 - 10.4)	(7.9 - 11.3)	0.0486 (T1vs.T3)
subjects (%)	< 7 y	14.3	50.0	35.7	0,083
	7-12 y	27,6	34.5	37.9	
	≥ 12 y	58.8	17.6	23.5	
Diabetesduration (years)	x ± SD	3.4 ± 3.1	6.7 ± 4.3	5.0 ± 3.8	0.0048 (T1vs.T2)
	(95% CI)	(2.0 - 4.9)	(4.7 - 8.8)	(3.2 - 6.8)	
subjects (%)	< 2 y	50.0	22.2	27.8	0,063
	2-5 y	37.5	25.0	37.5	
	5-10 y	29.4	29,4	41.2	
	≥ 10 y	0.0	77.8	22.2	
HbA1c (%)	x ± SD	7.7± 1.7	7.8 ± 1.0	8.2 ± 1.4	0.093 (T1vs.T3)
	(95% CI)	(7.0 - 8.6	(7.3 - 8.3)	(7.5 - 8.8)	
subjects (%)	< 9%	34.8	37.0	28.3	ns
	≥ 9%	28.6	21.4	50.0	

Mean ± SD (95% CI) or percentage of subjects (%); statistically significant differences (p≤0.05)
*Chi^2 test for categorical variables
*Fisher's Least Significant Difference post-hoc test for continuous variables

Discussion

One of the new aspects of our study was the analysis of their fluctuations as well as total sRANKL children suffering from T1DM. The analyzes included evaluations of impact of age, gender, age of diabetes onset, disease duration and metabolic control on OPG and/or total sRANKL. There has been no significant difference in OPG concentrations between the study groups and the controls. Lambrinoudaki did not observe differences in OPG levels between the control group and those with type 1 diabetes when analyzing the OPG concentrationand endothelial dysfunction [22]. The values of OPG concentrations were neither significantly higher, nor significantly lower in our group. The authors have not found the correlation between OPG levels and diabetes duration or metabolic control. Only thenegative correlations with chronologicalage and BMI has been demonstrated. Following adjustment for age, sex and presence of diabetes using regression analysis, the negative correlation between OPG and BMI has been demonstrated. Age of patients in the group of Lambrinoudaki(12.0 ± 2.7) was lower than

in our group (15.0 ± 1.9 years), duration of diabetes and metabolic rate were comparable.Galluzzi has observed significantly higher OPG levels in TIDM patients than in their healthy peers [23]. In this study, the patient'age was significantly lower (9.8 ± 3.3 years) than in our group, whereas disease duration was comparable and metabolic control was poorer [23].

The authors have not found correlation between OPG concentration and age, diabetes duration, insulin dose, physical activity and calcium intake, age was not analyzed. Using regression analysis, there was a positive correlation between metabolic control (HbA1c) and negative bone mass [23]. Similar results were obtained from Abd El Dayema, who also observed ineffective lowering of OPG levels in children with type 1 diabetes [24]. Xiang found significantly elevated OPG levels in young adults at the onset of type 1 diabetes, decreasing significantly, but higher than in the control group, within 6 months following of insulin therapy commencement [25]. OPG concentrations correlated significantly with vascular endothelial flow and fasting glucose, HbA1c and CRP [25].Wasilewskahas not demonstrated anycorrelation between OPG concentrations and factors such as: sex, age, height, body mass and BMI in healthy Polish children [26]. Ostrowska et al. has shown a negative correlation between OPG and BMI in girls with Anorexia nervosa (AN) with significantly elevated OPG concentrations [27]. In ourstudy, OPG levels in T1DM patients were higher in girls and in boys from the control group. However, when tertial analysis was used, a significant impact of gender has been observed in these patients.

Khosla's has demonstrated an increase in OPG levels with age in healthy adults, and sexual dysmorphism that developed around 50y.o. [28]. An increase in OPG levels at this age is associated with a decrease in estradiol and testosterone levels (29). OPG levels are also affected by medicines such as insulin, glitazones or statins [30]. In children, OPG concentrations can also be affected by other chronic diseases. Ambroszkiewicz and co-workers have demonstrated significantly lower OPG levels in cystic fibrosis patients, and Wasilewska and colleagues had the same observation (significantly lower OPG levels) in children with nephrotic syndrome, whereas no impact of glucocorticoid doses was reported [31,32].

Sanchez et al also observed, that a substantial proportion of patients with IBD had low body mass density (BMD), and levels of bone turnover markers suggested higher bone resorption, possibly in relation to disease activity, without a compensatory increase in bone formation (33).

In our study,no impact of gender on OPG levelshas been reported, but the values were significantly lower in diabetic boys than those in the control group. Age-related OPG levels have notcorrelatedwith age and gender ($p < 0.05$). Over 45% of girls had OPG levels within third tertyl, and more than 45% of boys had these levels within 1st tertyl. No statistically significant correlation between OPG concentration and metabolic control has been observed, however, after the use of titration analysis, such a tendency was demonstrated. Patients with HbA1c > 9% had the highest percentage (50%) of OPG concentrations in the upper tertyl (>2.96 pmpl/l).

Gallazi et al. have demonstrated a positive correlation between OPG concentrations and metabolic control in diabetic patients assessed by HbA1c [23]. Similar outcomes were reported by Xiang and et.Lambrinoudaki et al. did not observe any correlation between HbA1c levels and OPG concentrations [22]. Rassmussen, in the group of adults with T1DMhas demonstrated the correlationbetween OPG and glycemic control, blood pressure, and cardiovascular mortality [34]. Lappin et al, also in T1DM adult patients, has reported higher OPG levels than in the control group,and these concentrations were lower in the group with higher HbA1c levels. g than in the one with lower group [35]. Perhaps, not only the serum OPG level, but also OPG mRNA expression may have an important impacton the direct function of this hormone. Loureirohas observed significantly higher expression of OPG mRNA on peripheral leukocytes of children with T1DM, which did not correspondent with metabolic control [36]. On the other hand, Cunha et al.in their *in vitro* studies, have demonstrated the impact of high glucose level on OPG mRNA expression, significantly higher and preceding RANKL, which, according to the authors, may have an impact on osteoclast activity [37].

In our study,likewise Lambrinoudaki and Galluzzino significant correlation between OPG concentrations and disease duration has been observed [22,23]. This may suggest, that changes in the course of diabetes which influence/have an impact on OPG levels appear with time, which is in contradiction to Singh's observations [38]. However, his study included patients with relatively short-term disease.

After the use of titration analysis, the percentage of children with lowest concentrations of OPG (1 terlyl) was decreasing with the duration of the disease, and the percentage of children with higher OPG levels (3 terlyl) was increasing. Most of the studies assessing OPG levels in diabetic patients have been carried out on adults with either type 1 or type 2 diabetes, therefore both age and disease long-term complications may affect/have an influence/have an impact on the results. Secchiero et al. have shown significantly higher levels of OPG in the population of type 2 diabetes and a positive correlation with duration of the disease [39]. The studies on the adult population with type 2 diabetes have demonstrated the relationship between elevated OPG levels and endothelial dysfunction. Shina et al., also in adults with type 2 diabetes, have observed a significant correlation between endothelial dysfunction and increased OPG levels [40]. Studies in the adult population have shown a correlation between elevated OPG levels and coronary heart disease and cardiovascular mortality [20]. However, according to Xiang and co-workers, elevated OPG levels may be considered protective [25]. According to Bierre, thecorrelation between elevated OPG levels and increased mortality due to cardiovascular complications in adults may indicate a reactive-secondary increase in OPG [41].

According to Singh, endothelial dysfunction in children with T1DN developsas soon as in the first decade of the disease [40]. The increase in OPG levels could serve as a marker of early damage to the vascular wall and increased risk of cardiovascular disease, especially in patients with diabetes mellitus [21].

Alongside with disease duration, another significant parameter, though rarely assessed, is the age of the diseaseonset. This is particularly important in children, in whom the younger the age, the more difficult the management and compliance are,while simultaneously exposing the child's immature organism to chronic disease. In our study, there was no significant correlation between OPG concentrations and onset of the disease, but tertylian analysis has demonstrated, that lower OPG levels were present in children with late-onset disease.

In our study, total serum sRANKL concentration was assessed for both free and bound fractions. In mostpublications, especially earlier ones, other methods were used, therefore not all the results can be comparable, especially in case when the values of free RANKL fractions were very low or even indeterminate. There has been even less data forsRANKL concentration than for OPG - it is not considered as a potential marker in assessment of both bone turnover and endothelial dysfunction. In our study, no significant difference in sRANKL concentrations between the study and the control group was observed, but for both groups and separately for boys and girls, sRANKL levels in T1DM children were insignificantly lower. No significant impact of gender was observed, although in both the examined and control groups, the boys were inferior. This is contrary to the outcomes from the study by Wasilewska, which assessed sRANKL concentrations in healthy Polish children and has demonstrated three times higher values in boys than in girls, and a positive correlation between sRANKLand age and body weight [26]. On the other hand, Buzi and Pastewkohave showed a poor correlation between sRANKL concentrations and age. In our work, such negative correlation was demonstrated only in the group of children with T1DM. These differences may be the due to the age differences in the studied population [42,43]. In our work, both the study and the control group included older teenagers advanced in maturation and growth. Pastewkohas demonstrated increased sRANKL concentrations in girls with puberty, and maximal values were observed before menarche stage III/IV according to Tanner's scale [42].Wasilewskahas observed significantly higher levels of sRANKL in children with nephrotic syndrome which correlated with the dose of steroids [26]. This finding may suggestthe key role of sRANKL in the etiopathogenesis of post-osteoporosis. Similarly, in other pediatric chronic osteoporosis-related diseases such as AN, cystic fibrosis and IBD an increased sRANKL level has been observed [18,27,43]. In the study byLambrinoudakiat all, where total sRANKLin T1DM children was assessed using the same laboratory method as in our study, lower levels of sRANKL in diabetic patients than in control have been reported, but higher than in periodontal disease [22, 34].

No correlation has been found between total sRANKL and OPG concentrations and metabolic control of diabetes mellitus. Similar results were reported by Lambrinoudaki and Lappin [22, 35]. It is surprising, that in vitro studies by Cunha have shown an increased RANKL expression when elevated glucose level [37]. Loureiro et al. also reported elevatedexpression of RANKL mRNA in children with T1DM, which was not statistically significant especially in patients with better metabolic control [36]. This may mean, that serum sRANKL concentrations, especially its soluble isoform, do not strictlycorrespond with tissue concentrations, and thus are not a counterpart of the local paracrine function.

No significant correlation has been found in sRANKL levels and age and disease duration. Lambrinoudaki also has not demonstrated any correlation between sRANKL concentrations and disease duration, these levels were insignificantly lower in children with longer disease duration [22].

The marker of OPG / RANKL / RANK system may be an OPG / sRANKL ratio reflecting the resultant change of both parameters. There was no significant difference in the value of this ratio between the study and the control group,nor theimpact of the gender has been shown. In the control group, the value of the index was significantly higher in the group of girls than boys, but in the T1DM diabetes group, there was insignificant gender dependence. However, a positive correlation between the calendar age and OPG concentration and index value has been observed. The OPG/sRANKLratio could become a marker/indicator of OPG/SRANKL dysfunction.

The OPG / RANKL / sRANKL system still remains unknown though studied for many years. It is an issue of interest to many medical disciplines as a potential marker for complications and treatment outcomes in many chronic diseases. The OPG / RANK / RANKL system impaired bone turnover. Serum levels, especially in the developmental age population, are affected by number of factors, and first of all, it is necessary to establish norms for a healthy population in terms of sex, age and sexual maturity.

Conclusion

There was no difference in OPG or sRANKL concentrations in children with T1DM compared to the control group. There is a correlation between disease duration and OPG level and between disease onset and serum OPG concentration. Duration of diabetes mellitus, disease onsetand metabolic control have no impact onsRANKL concentration. There is a negative correlation between calendar age and sRANKL level. The OPG / sRANKLratiodoes not differentiate between the study and the control group, and is dependent solely on the OPG value and the patient's calendar age.

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