Correlation of OPG/RANKL in patients at the center of Haemoglobinopathy Lushnja, Albania

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KEYWORDS

ABSTRACT:
Osteoporosis is an important cause of morbidity in hemoglobinopathy patients. It is characterized by low bone mass and disruption of bone architecture, resulting in reduced bone strength and increased risk of fractures. Osteoprotegerin (OPG) and receptor activator of NF-kappa-B ligand (RANKL) have been recently implicated in the pathogenesis of various types of osteoporosis. The aim of our study was to determine if there is any correlation between OPG/RANKL and the patients affected by thalassemia major and sickle cell disease in the Center of Haemoglobinopathy in Lushnje.
Methods: We measured in 106 patients with Thalassemia major and Sickle cell disease and in 67 healthy control subject serum OPG and RANKL levels and determined correlations with BMD. We measure T-score and BMD too.
Results: 31.1% of our patients with Thalassemia major and Sickle cell disease have osteoporosis and 21.6 % have osteopenia. We find a correlation between OPG-BMD (r=-0.768, p=0.000 and RANKL-BMD (r=0.468; p=0.000). OPG-T-score (r=0.729. p=0.000) and Rankl-T-score r=-0.409; p=0.000).
Conclusion: OPG and RANKL in Thalassemia major and Sickle cell disease patients should be consider as a main factor responsible for osteoclast activation.

1. INTRODUCTION

The aim of our study was to determine if there was any correlation between OPG/RANKL and the patients affected by thalassemia major and sickle cell disease in the Center of Haemoglobinopathy in Lushnje, Albania. Lushnja is a town in south west of Albania and it's very known for thalassemia and sickle cell disease. Hemoglobinopathies are the most common monogenic diseases worldwide which are characterized by altered hemoglobin synthesis.[1]Beta thalassemia syndromes are mostly autosomal recessive disorders characterized by beta-globin chainssynthesis genetic deficiency[2].[3]SCD is also a major hemoglobinopathy induced by mutations in the β-globin chain of hemoglobin, which results in a substitution of glutamic acid by valine in the sixth position of this chain with consequent formation of HemoglobinS(HbS)[4]Sickle cell disease (SCD) is an autosomal-recessive genetic disorder that affects approximately 100,000 people in the United States and millions worldwide[5]
From the data obtained in 2006, on the screening of thalassemia carriers in the high school of Lushnja district, it resulted that the transferability of thalassemia was quite high in the entire district. In specific areas, a high transferability of sickle cell disease results. The prevalence of thalassemia was higher on plain and costal areas 10-11%, while sickle cell disease was found more in hills 8.57%.
Osteoporosis is an important cause of morbidity in hemoglobinopathies patients.[6][7] It is characterized by low bone mass and disruption of bone architecture, resulting in reduced bone strength and increased risk of fractures. During the last decade, the presence of osteopenia and osteoporosis in well-treated thalassaemics has been described in different studies with high prevalence up to 50% [8]. Osteopenia and osteoporosis represent prominent causes of morbidity in patients of both genders with thalassaemia[9]Osteoprotegerin (OPG) and receptor activator of NF-kappa-B ligand (RANKL) have been recently implicated in the pathogenesis of various types of osteoporosis.[10]

2. **Methods**:

We studied serum OPG/RANKL levels in a total of 106 patients affecting by thalassemia major (58 patients) and Sickle cell disease (48 patients) admitted to the Center of Hemoglobinopathy Lushnje, Albania, 46 male and 60 female, mean age 28.3 ± 13.6 years. Patients presented at the Thalassemia Center every 21 days to receive transfusions.

The control group consisted of 67 patients admitted to the hospital for the routine control (38 female and 29 male) with mean age 32 ±14 years. Five milliliterof fasting pre–transfusion venous blood was collected, and serum was stored at −20°C after separation.

The BMD was determined by dual-energy X-ray absorptiometry (DEXA). Biochemical markers of bone metabolism (serum calcium, phosphorus, alkaline phosphatase, osteocalcin, β-CrossLaps) parameters that affect bone metabolism (serum parathyroid hormone, thyroid-stimulating hormone, 25-hydroxyvitamin D, OPG, soluble RANKL [sRANKL]) were studied.[11]

Date analysis:

We evaluated mean hemoglobin and mean serum ferritin levels.

BMD values were compared with reference values from healthy people with similar age, sex, and ethnicity to calculate a Z score, the number of SDs from the expected mean. Z scores lower than −2.5 were accepted as “low bone mineral density” or osteoporosis. Z-score<2.5 were accepted as osteopenia and z-score -1 to +1 were accepted normal.[12]

According to the 2021 guidelines for the management of transfusion-dependent thalassemia by the Thalassemia International Federation (TIF)[13]assessment of BMD by dual-energy X-ray absorptiometry (DXA) should be performed every 24 months after the age of 10 years, accompanied by vertebral fracture assessment. Osteoprotegerin (OPG) and the receptor activator of nuclear factor-kappa B (RANK)/receptor activator of nuclear factor-kappa B ligand (RANKL) are the major cytokines related to the regulation of bone resorption[14]. The receptor activator of the nuclear factor-kappa B (RANK)/RANK ligand (RANKL)/osteoprotegerin (OPG) pathway has been recently recognised as the final, dominant mediator of osteoclast proliferation and activation [15][16]

Serum osteoprotegerim (OPG) was measured by a commercially available kit (BiovendorELISA,version 17131204).[6] Intra-assay (n=5)≤5% as provided by the manufacturer. The assay for measuring OPG in serum used the sandwich method in ELISSA format with a monoclonal capture and polyclonal antibody detection. The median value, according to the company Biomedica, Vienna was 2.7 pmol/L. OPG was stable at -20 ^{0} in serum and EDTA, citrate, heparin plasma and was stable at +4 ^{0} for 14 days. The assay for measuring RANKL in serum used the sandwich method in Elissa format with a monoclonal capture and polyclonal antibody detection (Biomedica). The lower limit of detection for this assay was 0.08 pmol/L. Accuracy for RANKL (Biomedica) was: intraassay CV from 3%(at 3.2 pmol/L) to 5% (at 1 pmol/L) and interassay CV from 6% (at 1.78 pmol/L) to 9% (at 0.8 pmol/L). The Statistical Package for the Socila Sciences (SPSS) 22 programme was used for statistical analysis. The correlation between changes in various biochemical parameters and BMD were evaluated with Spearman’s correlation coefficient. The Mann-Whitney U test and paired-sample t-test were applied to evaluate the differences between patients and controls. Variables were found to be statistically significant at $p<0.05$

3. **Results**:

The study group included 58 β-TM patients (36 female, 22 male), 48 sickle cell disease (SCD) patients (24 female and 24 male) and 67 controls (38 female and 29 male). The mean age of β-TM group was 25± 13.1(18-64) years, 32±14 (18-54) years, while in control group was 32.3 ±14(19-65) years. The mean age and sex distribution of the groups were not significantly different ($p>0.05$). 48% of our patients had normal BMD, 21% had osteopenia and 31% had osteoporosis. Serum AST,ALT, Creatinine,Urea, calcium, phosphorus, PTH and Vitamin D levels were within normal limits and didn’t differ between patients and control groups. Comparisons of major cytokines related to the regulation of bone resorption between patients with thalassemia major, SCD and healthy controls were presented in table 1.

Table 1. Comparisons cytokines(OPG/RANKL) related with regulation the bone resorption between patients with Thalassemia major, SCD and controls

	β-TM(n=58)	SCD(n=48)	Control(n=67)	p
age(years) (mean± SD)	25 ±13.1	32± 14	32.3 ±14	>0.05
OPG(pmol/L) (mean± SD)	3.2± 1.48	3.26± 1.29	10.2 ±7.5	<0.01
RANKL(pmol/L) (mean± SD)	0.26 ±0.17	0.26± 0.22	0.11± 0.89	<0.01

Serum OPG were significantly lower in thalassemic patients and SCD compared to control group. Serum RANKL were higher in β-thalassemia and SCD compared to controls.

The Tukey HSD test and Anova test were used for the comparisons of categorical variables according to the groups in the study. In table 2, we presented the multiple comparisons of the categorical variables, precisely the difference of their average values between the groups under the study , as well as within the groups in a confidence interval of95% Cl and significance p<0.05.

Table.2 The Tukey test (HSD) The comparisons of categoricalvariables according to the groups in the study

Dependent Variable	**(I) diagnosis**	**(J) diagnosis**	**Difference of avarege (I-J)**	**Standarte rrorr**	**Significance**	**95% Confidence Interval**	
						Low limit	**Upper limit**
B-CrossLaps	TM	SCD	**-.4184***	.1609	.028	-.800	-.037
		Control	.4774	.2274	.094	-.062	1.017
	SCD	TM	**.4184***	.1609	.028	.037	.800
		Control	**.8958***	.2327	.001	.344	1.448
	Control	TM	-.4774	.2274	.094	-1.017	.062
OPG	TM	SCD	0.02691	0.592396	0.999	1.4327	1.37893
		Control	-6.962284*	0.837309	0	-8.9493	4.9752
	SCD	TM	0.02691	0.592396	0.999	1.3789	1.43275
		Control	-6.935374*	0.856851	0	-8.9688	4.9019
	Control	TM	6.962284*	0.837309	0	4.97523	8.94934
RANKL	TM	SCD	-0.012769	0.036683	0.935	-0.0998	0.07428
		Control	146823*	0.051849	0.015	0.02378	0.26987
	SCD	TM	0.012769	0.036683	0.935	-0.0742	0.09982
		Control	.159592*	0.053059	0.009	0.03368	0.28551
	Control	TM	-.146823*	0.051849	0.015	-0.2698	-0.0237

We observed a very significant difference between the control group on the one hand and SCD and TM on the other, with a value of -6.962284 and p<0.00 for OPG.

A significant difference was observed between the values of the control group-TM with a value of -0.1468 and p<0.015 and the control group-SCD with a respective value of -0.1595 and p<0.009 for RANKL.

In table 3, we presented correlation between OPG/RANKL and BMD in our group of the study.

Table 3. Correlation OPG/RANKL and BMD

Variable		OPG	RANKL	BMD
OPG	Coefficient of correlation significance	1	-0.491** 0.000	-0.768** 0.000
RANKL	Coefficient of correlation significance	-0.491** 0.000	1	0.468** 0.000
BMD	Coefficient of correlation significance	-.0768** 0.000	0.468** 0.000	1

**. Correlation was significant at level 0.01

In table 3, we found a strong correlation between OPG-BMD (r=-0.768;p=0.000) and OPG-RANKL(r=-0.491; p=0.000)

4. Discussion:

Haemoglobinopathies are a common cause of skeletal morbidity and increased bone fracture risk in haemoglobinopathies patients[17] Its pathogenesis is multifactorial and mainly includes bone marrow expansion, endocrine dysfunction and iron overload [6]. In this study, we investigated OPG and RANKL values of our patients diagnosed with β-TM and SCD (sickle cell disease). We compared these values with BMD (bone mineral density) to observe whether there was a correlation between them.TheOPG/RANKL system plays an important role in activation and proliferation of osteoclast precursors. Analyzing our dates, the value of OPG and RANKL cytokines, through the analysis of variance ANOVA, we noticed that we had lower mean value of OPG in thalassemia and sickle cell group compared to the control group(OPG in TM 3.2 ±1.48 pmol/L: SCD 3.26± 1.29 pmol/L; control 10.2± 7.5 pmol/L and higher level of RANKL in TM 0.26±0.17; SCD 0.26±0.2; control 0.11±0.89). In our study, a negative correlation was noted between OPG and BMD (r =-0.768; p=0.000) and a positive correlation between OPG and T-score (r=0.729; p=0.000), as in the study [18].In our study, it was observed that patients with hemoglobinopathy associated with osteopenia/osteoporosis had lower OPG levels and a lower OPG/RANKL ration compared to patients with normal BMD.In the study of [19], as far as the OPG/RANKL system is concerned, thalassemic patients showed no differences in plasma levels of OPG compared with controls, that is not the same with our study and significantly higher plasma levels of RANKL, as we found in ours.In the study of [20]the thalassemic patients had significantly higher serum levels of OPG than the controls, not the same in ours, while their higher RANKL levels, were at the threshold of significance as in ours.We found correlation with the study of [6] , where he said that serum OPG levels were significantly lower in thalassemic children than in controls.

In the study of [21]bone turnover was significantly increased in thalassemic patients compared to controls, but OPG was significantly higher in healthy subjects, the same was found in our study too. BMD values didn't correlate with OPG/sRANKL system, in our study, it correlated.

Inthe study of [22]ELISA assay of serum OPG demonstrated significantly higher levels in thalassemia patients than in healthy controls, in our study, it was contrary.

Sixty-four patients with TM (32 men and 32 women) participated in the study of [23]. Almost the same number of patients was in our study (22 men and 36 women). The statistical analysis of the biochemical markers of bone metabolism revealed overall significant differences between the three groups only for RANKL and OPG/RANKL (p=0.049 and p=0.009). RANKL was higher and OPG/RANKL was lower in TM patients compared to osteoporosis group. In our study RANKL was higher too.

In the study of [24] thebiochemical parameters in the (patients/ controls) including calcium and alkaline phosphatase (ALK) were 9.1/10.2 mg/dL and 171.1/310 IU, respectively indicating a significant decrease (P< 0.05) compared to the controls, the same as in our study too. On the contrary, the mean levels of Ferritin and Zinc were 1914.18 µg/L and 113.92 mg/mL, respectively whichwere significantly increased (P= 0.015 and P=0.045, respectively). We measured level of ferritin, which was high too.

The mean age of patients in the study of [25] was 14.86±3.72 years. Normal bone density, osteopenia, and osteoporosis were noted in 2 (5.4%), 21 (56.8%), and 14 (37.08%) patients, respectively. Our study showed 48% of patients normal, 21% osteopenia and 31% osteoporosis. We had more female than male with osteopenia and osteoporosis too. The BMD Z-score was not significantly associated with OPG regarding the total number of participants, whereas in patients with osteoporosis, this association was significant (P=0.001). In all effect modified models, BMD remained statistically non-significant except for body mass index modification (P=0.046).

4.In the study of [26]serum RANKL level was 198.4pg/mL and in controls was 112.25pg/mL. There was a correlation between decrease of femur BMD (p<0.02) and increase of RANKL level.In our study, RANKL was higher than in control group. BMD-RANKL (r=0.468, p=0.000)

The study of[27]β-TM patients showed an altered bone turnover, with an increased resorption phase [shown by significantly high levels of (DPD) (p=0.04)] and a decreased neoformation phase [shown by the low levels of osteocalcin and (CICP) (P=0.0001 for both)]. Moreover, they displayed significantly lower BMD values than controls both at the lumbar and femoral levels (P = 0.0001 for both). The thalassemic patients showed significantly lower serum levels of OPG (P=0.0001), whereas RANKL levels were significantly higher in β-TM patients (P=0.001), who consequently showed a lower OPG/RANKL ratio (P=0.001), the same in our study.

In the study of[28]the mean of spine dual-energy X-ray absorptiometry (DXA) Z-score in patients was −1.66 ± 1.02 standard deviation (SD). Twenty-four of them had low spine DXA Z-scores. The patients showed significantly lower OPG levels and OPG/RANKLs ratios than the control group (3.28 ± 9.11 ng/ml and 11.38 ± 14.93 ng/ml, and 0.01 ± 0.03 and 0.07 ± 0.09, respectively), the same as in our study.

In the study of [29] serum OPG levels were significantly lower in thalassemic children than in controls. The mean ratio of RANKL/OPG was significantly higher in the thalassemic patients than in the control group. Osteoporosis was detected in 10 (3 female and 7 male) of 38 patients (26.3%) according to the femur Z score and in 6 of them (4 male and 2 female) (15.8%) according to the spine Z score. In our study serum OPG was significantly lower in patients compared to control group.

In the study of [30]the results suggested that OPG was significantly and positively correlated with age in the osteoporosis group ($r = 0.29$, $p < 0.05$), while it was inversely correlated with BMD femoral neck left ($r = -0.56$, $p < 0.001$) and BMD femoral neck right ($r = -0.37$, $p < 0.05$) in the same group. Furthermore, the RANKL/OPG ratio had a positive and significant correlation with BMI ($r = 0.34$, $p < 0.05$), BMD femoral neck left ($r = 0.36$, $p < 0.05$) and BMD femoral neck right ($r = 0.35$, $p < 0.05$) in the osteopenia group. By contrast, it showed a significant inverse correlation with waist to hip ratio in the osteoporosis group ($r = -0.38$, $p < 0.05$). Multiple regression analysis showed that OPG contributes to BMD variations in the osteopenia group ($p = 0.03$).

In our study, patients with TM(thalassemia major) and SCD(sickle cell disease)had high ferritin level compared to control group, although they were withferrochelant therapy. Our results are consistent with the results of [21]. Iron overload could induce the formation of osteoclasts through oxidative stress[31][32]or through increased production of RANKL, this might explain the increased ferritin in TM(thalassemia major) and SCD (sickle cell disease)

5. Conclusions:

Serum OPG/RANKL concentrations can be used as a biochemical marker in screening patients with haemoglobinopathy for the development of osteoporosis.

Osteoporosis is a multifactorial disease and may occur early, especially in chronic diseases such as thalassemia and sickle cell disease. Because of the difficulties in diagnosis and follow-up, screening with DEXA and measuring ferritin level and RANKL/OPG ratios on a regular basis is essential. It should be kept in mind that osteoporosis may develop with advancing age in both sexes.

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