

## Evaluation of relationship between biochemical parameters and osteoporosis in patients with $\beta$ -thalassemia major

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Received: 21 November 2020

Accepted: 20 September 2021

### Abstract

**Background:** Osteoporosis is one of the late complications of  $\beta$ -Thalassemia major. The pathogenesis of osteoporosis depends on different factors. Ineffectiveness of hematopoiesis is the major factor, and the other factors are defected by hormonal functions or biochemical parameters. Osteoclasts hyperactivity in thalassemia increases the serum receptor activator of nuclear factor Kappa B ligand (RANKL), which plays a crucial role in bone development. This study aimed to evaluate the biochemical and hormonal parameters in patients with  $\beta$ -thalassemia major and their association with osteoporosis.

**Materials and Methods:** In this case-control study, 52 patients with  $\beta$ -thalassemia major and 23 with thalassemia minor as controls were enrolled. The patients' Bone Mineral Density (BMD) was measured using the Dual Energy X-ray absorptiometry (DEXA) method, and 6 mL peripheral blood of the patients and controls was obtained to detect hormonal and biochemical parameters. Data were analyzed using ANOVA, Spearman correlation coefficient, and T-test.

**Results:** The mean of BMD in patients was  $0.59 \pm 0.01$  and  $0.69 \pm 0.11$  in femur and vertebrae, respectively. The biochemical 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. On the contrary, the mean levels of Ferritin and Zinc were 1914.18  $\mu\text{g/L}$  and 113.92 mg/mL, respectively which were significantly increased ( $P = 0.015$  and  $P = 0.045$ , respectively). There was a negative correlation between the femurs BMD of patients with the RANKL level ( $r = -0.8$ ,  $p = 0.034$ ) and the vertebrae BMD of patients with a Parathormone (PTH) level ( $r = -0.8$ ,  $P = 0.028$ ).

**Conclusion:** The study results indicated that the hyperactivity of RANKL and PTH in thalassemia patients might cause osteoporosis; therefore, detecting biomarkers mentioned above could be useful to diagnose osteoporosis.

**Keywords:** Thalassemia, Biochemical, Osteoporosis, Parameters

### Introduction

$\beta$ -thalassemia is a type of congenital anemia caused by various mutations in the beta-globin genome (1, 2). According to clinical manifestations, patients with  $\beta$ -thalassemia are classified into minima, minor, intermediate, and major. The beta-

thalassemia major is the most severe type (3, 4). As the patient age, this disease's clinical and laboratory symptoms are more visible. By regular blood transfusions and administration of iron chelators (deferoxamine), patients are well managed, and their longevity is increased (5, 6).

However, the accumulation of iron in the tissue of organs, including endocrine glands and bone marrow, leads to destructing these organs or inactivating their function (7). Osteoporosis is a common metabolic disease characterized by its natural structure of the bone and reduced bone density, which is also observed in patients with thalassemia major (7, 8). However, more than 40% of thalassemia patients have osteoporosis (9, 10). Various factors are involved in decreasing the bone density in patients with  $\beta$ -thalassemia major. Osteoblast toxification with iron overload is caused by osteoporosis in these patients (11-14). Bone marrow stromal cell molecules play a crucial role in bone regeneration by binding with different cytokines. The nuclear factor Kappa- $\beta$  ligand (RANKL) receptor activator is a member of tumor necrosis factors (TNF). RANKL binds with its receptor and induces precursor cells to differentiate osteoclast (15, 16). Furthermore, osteoblast produces osteoprotegerin (OPG), a decay RANKL receptor. OPG blocks the RANKL-RANK interaction and thus inhibits osteoblast biological activity through oxidative stress, resulting in decreased bone density and increased osteoporosis via increment of RANKL and reduction of OPG (17). Moreover, the inappropriate calcium, phosphorus, and alkaline phosphatase, which are essential biological agents for bone formation (18), leads to bone weakness. Based on the biochemical inappropriateness in these patients, this study aimed to evaluate the biochemical and hormonal parameters in patients with  $\beta$ -thalassemia major and their association with osteoporosis.

## Materials and Methods

This case-control study was conducted on 52 patients with  $\beta$ -thalassemia major, who were visited at the thalassemia clinic (Zafar, Tehran, Iran) in 2019, and 23 voluntary subjects with  $\beta$ -thalassemia minor as controls.

## Bone Mineral Density (BMD)

The patients' Bone Density Test was measured using the Dual Energy X-ray absorptiometry (DEXA) method in the femur and vertebral bones at the center for Bone Density Assessment.

## Sampling

Six mL of peripheral blood of the patients was obtained to determine the biochemical parameters of sera (Zinc, calcium, phosphorus alkaline phosphatase, and receptor activator of nuclear factor Kappa B ligand (RANKL) Ferritin and Parathormone (PTH).

## Measurement Methods of Biochemical Parameters

The biochemical parameters, including (Zinc, calcium, phosphorus, and alkaline phosphatase) were assayed using Pars Azmoon Biochemical Kits (IRAN) and analyzed by a Hitachi 912 auto-analyzer (Roche Diagnostics, Canada). Ferritin and PTH were detected using a Monobind kit (USA); briefly, 100  $\mu$ l of standard, patient, and control samples were poured into the wells of the separated (Ferritin and PTH) ELISA plates. The plates were incubated at 37°C for 60 minutes. The wells were then washed four times with wash buffer. Then 100  $\mu$ l of the conjugated anti-ferritin were added into the Ferritin ELISA plate, and 100  $\mu$ l of the conjugated anti-PTH antibody were added into PTH ELISA wells; in addition, the plates were incubated at 37°C for 60 minutes. After washing the wells four times with wash buffer, 100  $\mu$ l of the TMB substrate was added and placed in a dark place for 15 minutes. The reaction was stopped by adding 100  $\mu$ l NaOH, and the absorbance was read at 450 nm with an ELISA reader.

## Measurement Methods of Protein RANKL

The measurement of RANKL was performed using the Human Soluble Receptor Activator of the Nuclear Factor-KB Ligand ELISA Kit from Abbeva-UK Company. In summary, RANKL standards were developed with different dilutions (500 pg /ml, 250 pg /ml, 125 pg /ml, 62.5

pg /ml, 31.25 pg /ml, and 15.62 pg /ml), respectively. Then, 100 µl of each standard dilution, patient specimens, and controls were poured into the wells of the ELISA plate. The plate was incubated at 37°C for 90 minutes. Additionally, 100 µl of the anti-RANKL antibody was added to all wells and then incubated at 37°C for 60 minutes. The wells were washed three times with wash buffer. Then 100 µl of the secondary antibody conjugated with HRP (Horse Radish Peroxidase) was poured into the wells and incubated for 37 minutes at 30°C for 30 minutes. Next, the palate was washed five times with wash buffer, and 90 µl of the TMB substrate was added to it, and then it was placed in a dark place for 15 minutes. Using 50 µl NaOH, the reaction was stopped, and the absorbance was read at 450 nm with an ELISA Reader.

### Statistical Analysis

The SPSS 22 software analyzed the results. The independent T-test was applied to determine the difference between the two groups. ANOVA was used to compare multiple data. Spearman correlation coefficient was applied to indicate any correlation between the levels of the biochemical parameter. The chi-square test was applied to compare male and female patients. In all cases, the minimum statistical significance was  $p < 0.05$ .

### Ethical Considerations

The Ethical Committee of Shahid Beheshti University of Medical Sciences approved this study (code: IR.SBMU.REETCH.REC.1395.104), and all the participants provided informed consent following the deceleration of Helsinki.

## Results

### Patients' Profile

Out of 52 patients, 59% were female, and 41% were male ( $p > 0.05$ ). The mean age of all patients was  $35.6 \pm 7.7$  years. Out of 23 controls with thalassemia minor with an average age of  $31.3 \pm 20.8$  years, 15 were female, and 8 were male.

### Investigation of Biochemical and Hormonal Elements

The patients' and controls' sera were examined for biochemical and hormonal parameters. The results indicated that the mean levels of biochemical parameters in the patients, including alkaline phosphatase and calcium, were significantly lower than those in the controls ( $p < 0.05$ ). The phosphorus level in the patients was 4.54 mg/dL, which was equal to the level of controls phosphorus sera. Figure 1 indicates the level of these parameters in the patients and controls. On the contrary, the mean level of Ferritin (1914 ug/L) and Zinc (113 mg/mL) in the patients was significantly increased ( $P < 0.05$ ) compared to the controls (Figure 2). Still, the patients' mean PTH level was 29.5 IU, demonstrating no significant difference between the patients and controls.

### RANKL Samples

The RANKL level in the patients' and controls' sera was measured using the ELISA method. The mean of the RANKL level in the patients and controls was  $198.4 \pm 136.99$  pg/ml and  $70.6\% \pm 112.3$  pg/ml, respectively. As Figure 3 shows, the RANKL level of the patient's sera was significantly increased compared to the controls ( $P < 0.05$ ).

### Patient Bone Density Assessment

The results of the bone density measurement indicated that the patients' mean bone density (MBD) was  $0.59 \pm 0.01$  for femur bone and  $0.69 \pm 0.11$  for vertebral bone, as compared to the control samples' bone density which was more than 1. The patients' bone density in their femoral and vertebrae was significantly ( $p < 0.001$ ) lower than 1. The mean bone density in the patients and controls was also assessed based on the T score, which was  $-2.5 \pm 1.1$  and  $-2.8 \pm 0.9$  in the patients' femur and vertebrae, respectively. Furthermore, the T score in the control subjects' femur and vertebra, the same as normal adults, was approximately 1.5 (WHO 19), suggesting that the bone

density in patients was nearly half of the bone density of the control groups. The T score in the patients' femur and vertebrae was significantly ( $p < 0.001$ ) lower than 1.5. Table I shows the bone density in the patients compared to the controls. Osteoporosis was determined based on the T score and the WHO pattern (19). In this regard, 19 patients with osteopenia (T score - 0.2 to -2.5) and 33 patients with osteoporosis (T score  $> -2.5$ ) were classified. Table 2 shows the rate of osteoporosis in the patients and the classification of T. Based on the WHO pattern, 19 patients in this study had osteopenia or mild bone density, and 33 had osteoporosis, with the potential for fractured bones.

### Relationship between Hormonal and biochemical Parameters and Bone Density

The spearman method demonstrated no significant relationship between the patients' Ferritin level and bone density. Still, there was a significant correlation between the patients' PTH level and vertebral bone density ( $r = -0.8$ ,  $P = 0.028$ ). In other words, the higher serum PTH level is related to the lower score of the patients' vertebral bone density. Moreover, there was a negative correlation between the RANKL protein and the bone density in the femoral of the patients ( $r = -0.8$ ,  $P = 0.034$ ). In other words, the higher level of RANKL in patients is associated with the patients' lower femur bone density.

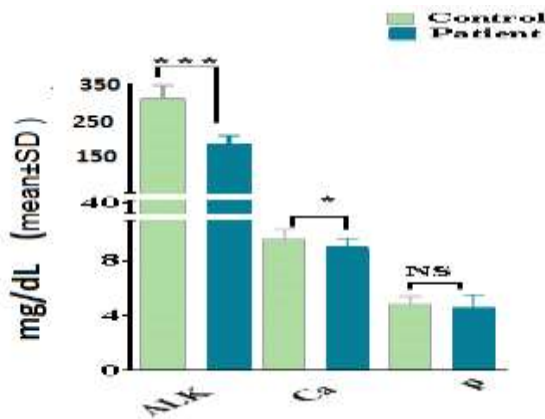


Figure 1. The mean level of alkaline phosphatase (ALK), calcium (ca) and phosphorus (p), of patients and controls. The mean value of chemical parameters demonstrates that the mean of ALk and Ca in patients are significance ( $P < 0.05$ ) lower than control.

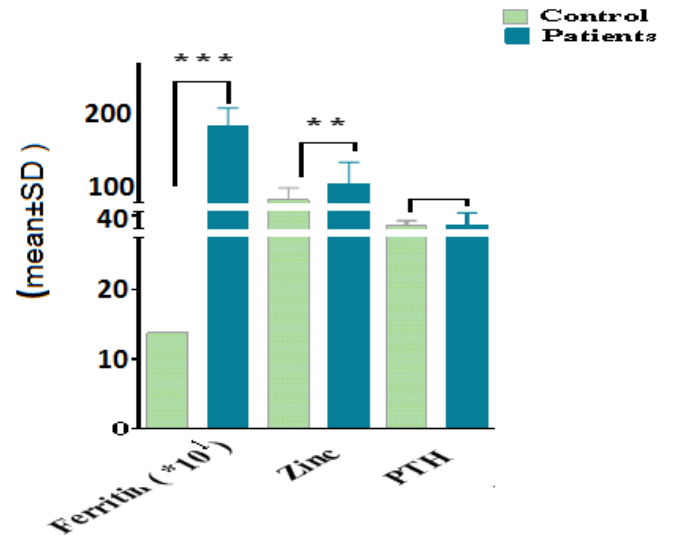


Figure 2. Frequency of biochemical parameters of parathormone (PTH), ferritin and zinc in patients and controls. As the parameters mentioned above are shown to be higher in patients than controls. Regarding ferritin and Zinc, the difference is significant ( $P < 0.05$ ) but there is no significant difference between the parathormone level in two groups.

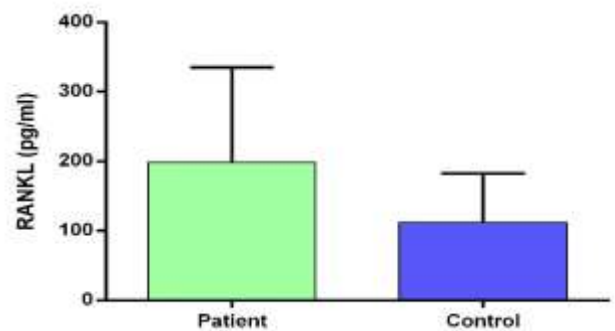


Figure 3. Comparison of RANKL in both patient and control groups, as indicated, the protein content in patients increased to twice as high as the control subjects ( $P < 0.05$ ).

Table I: Bone density based on T score and BMD in patients and controls

Category	T score		BMD	
	Femural	Vertebrae	Femural	Vertebrae
controls	1.5	1.5	1	1
patients	-2.5±1.1	-2.84±0.9	0.59±0.12	<b>0.69±0.01</b>

Table II: Osteoporosis based on T score and WHO pattern

Category	Bone mineral density (BMD)	T score	No of sample	
			Patients	Controls
Normal	1 SD below average peak BMD of young adult	>1	0	<b>23</b>
Osteopenia	.0–2.5 SD below average peak BMD	-0.2 to -2.5	19	<b>0</b>
Osteoporosis	2.5 SD below the mean BMD of young adult	< -2.5	33	<b>0</b>

## Discussion

$\beta$ -Thalassemia is one of the most susceptible inherited blood diseases associated with  $\beta$ -globin chain deficiency. Reports from different countries indicate that the incidence of this disease in some societies is approximately 10% (20). Among the countries of the Middle East, Iran has the highest population of  $\beta$ -thalassemia, and the highest prevalence of  $\beta$ -thalassemia has been reported around the Caspian Sea and the Persian Gulf (2). The results in this study indicated that the patients' levels of alkaline phosphatase and calcium were significantly lower than controls ( $P < 0.001$ ), but there was no significant change in Phosphorus.

An analysis of previous studies revealed decreased serum calcium levels in thalassemia major compared to healthy controls. Still, the Phosphorus in these patients was higher than in healthy subjects (18). Sadia Sultan et al. reported that in 36 thalassemia patients, 66% of people suffered from calcium reduction and 19.4% from reduced Phosphorus

levels (21). A couple of studies by Ayer et al. on 47 patients whose biochemical parameters were assayed found that Ferritin and Phosphorus levels increased significantly. At the same time, alkaline phosphatase and calcium were reduced (22). Calcium, Phosphorus, and alkaline phosphatase are essential biological agents for bone structure. It has been reported that approximately 85% of the total body phosphorus is accumulated in bone tissue (23). Hydroxyapatite is a calcium and phosphorus component making bone hardness. Moreover, alkaline phosphatase is responsible for bone mineralization by increasing the concentration of inorganic Phosphorus (24). In this study, the levels of Zinc and Ferritin in the patients were significantly higher than those in the controls. The calcium level of the patients' sera was significantly lower than controls ( $p < 0.05$ ); however, there was no significant difference in the phosphorus sera levels. There was a significant correlation between the PTH level and vertebral bone density in the patients ( $P =$



0.028); therefore, the increase of PTH increases bone reabsorption (25). Furthermore, frequent blood intake in patients with thalassemia causes the toxicity of citrate and iron deposition in the parathyroid gland, which ultimately causes hyperparathyroidism (26). Owing to frequent transfusion of blood, iron overload occurs in particular parathyroid and bone tissue, resulting in destruction or poisoning of endocrine glands and bone marrow cells with iron radicals (27). Tabatabaei et al. reported that the serum zinc of 40% of 131 patients with thalassemia was significantly reduced (28). The Zinc level in patients with splenectomy has a low Zinc level, and the Zinc level is associated with body mass index (BMI) (29). In this study, the mean of RANKL in thalassemia major was  $198.4 \pm 136.9 \mu\text{g} / \text{ml}$ , which was significantly higher than controls ( $112.3 \pm 70.6$ ). In a study conducted by Hosam Salah et al., an increase in the RANKL protein content of 78 pg/ml was reported in patients with thalassemia major, which had a significant increase compared to the controls (30). In addition, Nicholas et al. claimed that RANKL was associated with age, sex, and serum Ferritin level (31). The BMD of the femur and vertebral fractures was  $0.59 \pm 0.3$  and  $0.69 \pm 0.11$ , respectively, which according to the global (19) and national health pattern, was lower than one of the symptoms of osteoporosis in the patients (32). In the present study, a significant inverse relationship was observed between BMD (T score  $< -1$ ) and femur bone mass ( $P < 0.02$ ). T score is a standard method to calculate bone hardness (33). It should be noted that OPG and RANKL are one of the most important regulators of osteoclastogenesis. By connecting OPG to RANK, RANKL can no longer be connected to the activator of the KB receptor (RANK) on osteoclasts (34). It can be stated that OPG acts as a receptor for the activator of the Nuclear Factor KB (NF-KB) (RANKL) (35). RANKL-RANK signaling influences the

bone remodeling process, which may regulate the activities of bone cells (36). Moreover, the association of BMD and RANKL in the osteoporosis process in patients with thalassemia may be due to a change in the RANK/RANKL/OPG system. Finally, the reason why RANKL-binding molecules stimulate osteoblast differentiation has not been well clarified (37).

## Conclusion

Considering the increased level of RANKL protein and PTH in the serum of thalassemia patients and a significant association with their osteoporosis, this study demonstrated that detection of RANKL and PTH could be helpful to diagnose osteoporosis as a biomarker in thalassemia patients.

## Acknowledgments

The authors would like to thank the Deputy Research of Shahid Beheshti University of Medical Sciences and the Clinic of Thalassemia (Tehran, Iran) for supporting this research.

## Conflict of interest

The authors declare no conflict of interest.

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