Zoledronic acid for the treatment of osteoporosis in patients with β-thalassemia: results from a single-center, randomized, placebo-controlled trial

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Background and Objectives. The aim of this study was to evaluate the effect of zoledronic acid (ZA) on thalassemia-induced osteoporosis.

Design and Methods. We studied 66 thalassemia patients with osteoporosis, who were randomized to receive 4 mg ZA iv, every 6 months (23 patients; group A) or every 3 months (21 patients; group B), or to receive placebo every 3 months (22 patients; group C), for a period of 1 year. Bone mineral density (BMD) of the lumbar spine, femoral neck and wrist was determined before and 12 months after treatment. Pain scores and markers of bone resorption [C-telopeptide of collagen type-I (CTX), 5b-isoform of TRAP], bone formation [bone-alkaline phosphatase (bALP), osteocalcin (OC), C-telopeptide of procollagen type-I (CICP)], and osteoclast stimulators [sRANKL, osteoprotegerin (OPG), osteopontin] were also measured at baseline and before each treatment administration.

Results. The values of CTX, bALP, CICP, sRANKL, and OPG were higher in the all patients than in the controls. Patients in group A showed no differences in BMD of all sites at 12 months, while they had reductions in bone pain, bALP, OC and OPG. Conversely patients in group B had a significant increase in their lumbar spine BMD, which was accompanied by dramatic reductions in bone pain, CTX, bALP, CICP, and OC. Patients in group C showed no alteration in BMD of any studied site or in bone pain, while they had an aggravation in bone resorption.

Interpretation and Conclusions. ZA, at a dose of 4 mg, iv, every 3 months is an effective treatment for increasing BMD and reducing bone resorption in thalassemia-induced osteoporosis.

Key words: thalassemia, osteoporosis, zoledronic acid, bone markers, osteoprotegerin, OPG.

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Life expectancy in patients with β-thalassemia major has greatly improved over the last years as a result of regular transfusions and increased compliance with iron chelation therapy. However, this prolongation is often accompanied by several chronic complications including osteopenia and osteoporosis.[1] Thalassemia-induced osteoporosis is multifactorial rendering its management very difficult. Bone marrow expansion, endocrine dysfunction, iron overload, and genetic factors all seem to play important roles in the development of low bone mass in these patients.[2] However, despite the normalization of hemoglobin levels, adequate hormone replacement and effective iron chelation, patients continue to show an unbalanced bone turnover with an increased resorptive phase resulting in seriously diminished bone mineral density (BMD).[3] The reduced osteoblast function, which is thought to be the major cause of osteopenia/osteoporosis in thalassemia major, is accompanied by a comparable or even greater increase in osteoclast activity.[4] Therefore, bisphosphonates, which are potent inhibitors of osteoclast function, have been used in the management of patients with thalassemia major-induced osteoporosis. Daily administration of oral alendronate seems to increase BMD in such patients, while intramuscular or intravenous administration of clodronate is rather ineffective in this pathology.[5,6] Our group has also shown that pamidronate, a second generation aminobisphosphonate, at a monthly dose of 30 mg, iv, is an effective treatment for thalassemic osteoporosis.[7] Zoledronic acid is the most potent aminobisphosphonate with proven efficacy in post-menopausal osteoporosis,[8] osteoporosis of metabolic diseases,[9] and bone disease of malignancies.[10] However, there are very limited data on its role in the management of thalassemia-induced osteoporosis.[11] The aim of this randomized, placebo-controlled, trial was to evaluate the safety and effect of zoledronic acid on the BMD, bone pain and fracture risk

of patients with β-thalassemia and osteoporosis as well as on a series of biochemical markers of bone remodeling and osteoclast function. The receptor activator of nuclear factor-κ B (RANK)/its ligand (RANKL)/osteoprotegerin (OPG) system has been recognized as the final mediator of osteoclastogenesis, which plays an important role in the pathogenesis of osteoporosis in menopausal women, rheumatoid arthritis and multiple myeloma.[12-14] Recently, this pathway has also been implicated in the pathogenesis of thalassemia-induced osteoporosis.[15] To our knowledge, information about alterations of these molecules in patients with thalassemia and osteoporosis prior- and post-zoledronic acid administration is not available.

Design and Methods

Inclusion criteria

The criteria for recruitment in this study included: (i) the presence of β-thalassemia (major or intermedia); (ii) age above 18 years; (iii) presence of osteoporosis in at least one of the studied sites [lumbar spine (L1-L4), femoral neck, wrist]; (iv) absence of severe cardiac failure (left ventricular ejection fraction >50%); (v) normal liver function (amino transferases < 2x upper normal limit); (vi) normal renal function (serum creatinine <1.5 mg/dL); (vii) absence of pregnancy; (viii) absence of any psychiatric disorder making reliable informed consent impossible and (ix) no previous treatment with bisphosphonates for at least 12 months before the initiation of this study. Patients were advised against becoming pregnant, while patients with aspirin-sensitive asthma were excluded from the trial. Since Novartis Pharmaceutical Corporation has notified dental health professionals of the risk of osteonecrosis of the jaw with the use of bisphosphonates and zoledronic acid (March 2005), all patients were advised to inform the principal investigator of the study if they needed any dental procedure; in such cases a maxillofacial surgeon was to examine the patient.

Osteoporosis was defined using the standard WHO criterion (T-score of BMD lower than -2.5).[16] We used the same criterion for the definition of osteoporosis in males, as the International Society of Clinical Densitometry (2004) has used the same BMD criterion for the definition of osteoporosis in males and recommended that a male database be used for male patients and a Caucasian database for both Caucasian and non-Caucasians.[17,18]

Hypogonadic patients were allowed to continue their hormone replacement therapy. Testosterone enanthate was administered at a dose of 250 mg, im, monthly for males, while females were given estradiol 2 mg, p.o., for the first 16 days and medroxyprogesterone 5 mg, p.o., for the remaining 12 days of their menstrual cycle. The criteria for starting hormone replacement therapy included reduction of follicle-stimulating hormone, luteinizing hormone and testosterone serum levels, as well as decreased libido for males, and reduction of follicle-stimulating hormone, luteinizing hormone, estrogens and abnormal menstrual cycle for females. All patients entering the trial received oral calcium (500 mg) during the protocol period.

Study design

Patients were blindly randomized (1:1 randomization) to receive zoledronic acid at a dose of 4 mg, iv, in a 15 min infusion, every 6 months (group A) or every 3 months (group B), or to receive placebo every 3 months (group C) for a period of one year. The type of β-thalassemia (major or intermedia) and gonadal dysfunction were not taken into account in the randomization process. Only one of the authors (E.V.) who were involved directly with the treatment administration was not blinded.

Informed consent was obtained from all patients prior to inclusion in the study. The study was conducted with the approval of the hospital ethical committee and the Greek National Drug Organization (*Ref. No. Aα-KΔ-79/01/03*) in keeping with the guidelines of Helsinki.

Clinical and laboratory follow-up

The patients were evaluated prior to starting the drug and at each visit thereafter. At baseline, the patients' hemoglobin levels, leukocyte and platelet counts, and complete biochemical profile including calcium and phosphorus levels, were recorded. Furthermore the patients underwent a cardiac evaluation (including a triplex study of the heart) by a cardiologist and had a complete endocrinology study along with liver and renal function tests, and bone remodeling related tests. At each visit a complete physical examination was performed, together with the basic hematology and biochemistry and all bone-related biochemical tests. BMD of the lumbar spine (L1-L4), the femoral neck and the wrist was evaluated before and 12 months after post zoledronic acid administration.

Measurement of BMD

The BMD of L1-L4, femoral neck and wrist was determined by dual energy X-ray absorptiometry (DEXA; LUNAR, PRODIGY Version 8.60.006/SYSTEM GE medical system LUNAR USA 726, Madison, WI, USA).

Measurement of markers of bone remodeling and osteoclast stimulation

After venipuncture serum was separated within 4 hours and stored at –70°C until the day of measurement. An enzyme-linked immunosorbent assay (ELISA) was used for the detection of serum (i) markers of bone resorption: 5b isoenzyme of tartrate resistant acid phos-

phatase (TRACP-5b; BoneTRAP®, SBA, Oulu, Finland), and C-telopeptide of collagen type I (CTX; serum CrossLaps®, Nordic Bioscience Diagnostics A/S, Herlev, Denmark); (ii) markers of bone formation: bone-specific alkaline phosphatase (bALP; Metra® BAP, Quidel Corporation, San Diego, CA, USA), osteocalcin (OC; N/MID® Osteocalcin, Nordic Bioscience Diagnostics A/S, Herlev, Denmark), and C-telopeptide of procollagen type I (CICP; Metra® CICP, Quidel Corporation, San Diego, CA, USA); and (iii) markers of osteoclast stimulation: sRANKL (Biomedica Medizinprodukte, No. BI-20422H, Gesellschaft GmbH & Co KG, Wien, Austria), OPG (Biomedica Medizinprodukte, Gesellschaft GmbH & Co KG, Wien, Austria), and osteopontin (OPN; IBL Co Ltd, Takasaki-shi, Gunma, Japan); according to the manufacturers' instructions. All samples from the same patient were measured on the same ELISA plate.

The above biochemical parameters were also evaluated in a control group of 40, age and gender-matched, healthy blood donors (13 males and 27 females; median age of 40 years, range: 24-64 years). Each control was examined to ensure that there was no evidence of bone disease (osteoporosis or osteoarthritis) and questioned to confirm that he or she had not received any medication that could alter normal bone turnover during the preceding six months.

Measurement of other biochemical parameters

Serum urea, creatinine, uric acid, sodium, potassium, calcium, phosphates, magnesium, protein and albumin were measured using the Bayer-Advia 1650 Clinical Chemistry System (Tarrytown, NY, USA). On commencing the study we also measured in all patients the serum levels of sex hormones (follicle-stimulating hormone, luteinizing hormone, oestrogens, testosterone, sex hormone-binding globulin, di-hydro-epi-androsterone sulphate), as well as the serum and urinary levels of calcium, serum levels of 25-OH- and 1,25-OH_2- vitamin D_3, and serum levels of prolactin and parathormone.

The above sex hormones and prolactin were determined by radioimmunoassay techniques, according to standard methods. Radioimmunoassay methods were also used for the determination of 25-OH- and 1,25-OH_2-vitamin D_3. Parathormone was measured using a immunoradiometric assay (ELSA-PTH, CIS Biointernational, Gif sur Yvelle, France).

Evaluation of bone pain

Patients were asked to quantify the degree of their bone pain on Huskisson's visual analog scale (VAS: 0 cm=no pain; 10 cm=worst pain possible) before entering the trial, and then at 6 and 12 months after zoledronic acid administration. The McGill-Melzack scoring system, a verbal scale with six levels ranging from 0 to 5 (0=no pain; 1=mild pain; 2=troublesome pain; 3=severe pain; 4=very severe pain and 5=excruciating pain) was also measured at the times mentioned.[19]

Statistical analysis

Statistical analysis was performed using the statistical package for the social sciences (SPSS) software (SPSS for Windows, version 12.1, SPSS Inc., Chicago, IL, USA). The Mann-Whitney test and paired samples t-tests were applied to evaluate differences between patients and controls while the Wilcoxon signed rank test was used to evaluate differences between values of the studied parameters at baseline and at the various time points. Differences between patients of the three groups were evaluated using the Mann-Whitney test and one-way ANOVA. The correlation between changes of various biochemical parameters and BMD was evaluated using Spearman's (r_s) correlation coefficient. All p values are two sided with statistically significant levels being ≤ 0.05; the confidence intervals have 95% boundaries.

Results

Patients

Sixty-six patients with thalassemia-induced osteoporosis who are regularly followed-up in the Thalassaemia Centre of Laikon General Hospital entered this trial. Thirty-one patients were regularly transfused (most of them every 15 days), while 35 patients were not transfused or sporadically transfused (during infectious, pregnancy etc; thalassemia intermedia). All regularly transfused patients and 14 thalassemia intermedia patients were under chelation therapy. Thirty-four patients were given oral deferiprone (75/mg/kg/day) and the remaining 11 patients were receiving deferoxamine (40 mg/kg/day for 4-5 days per week, sc, for 8-10h/24h). All hypogonadic patients (n=34) continued their hormone replacement therapy while the study.

Twenty-three patients were randomized to participate in group A, 21 in group B, while 22 patients formed the placebo group C. There was no difference in the presence of gonadal dysfunction between the three groups studied. Table 1 summarizes the main characteristics of the patients. The majority of patients had had pathological fractures due to osteoporosis before entering the trial [29/66 (43.9%) in one site and 10/66 (15.1%) in two different sites]. The wrist, shin, femoral neck and lumbar spine were the commonest sites of fractures. Fractures were diagnosed using plain radiography. There was no difference in the number of fractures prior to zoledronic acid therapy between patients of the three groups studied. Bone pain due to osteoporosis was present in 41 patients (62.1%).

BMD and markers of bone remodeling at baseline

Patients with thalassemia-induced osteoporosis at base-

Table 1. Patients' characteristics (mean values ± SD).

Parameters	*Group A (n=23)*	*Group B (n=21)*	*Group C (n=22)*	*p value (ANOVA)*	*Controls (n=40)*
Age (years)	44.1± 11.7	42.6± 10.7	44.9± 10.7	0.789	43.8± 10.3
Gender (Male/Female)	6/17	9/12	7/15	0.503	13/27
Regularly transfused/ or non-transfused pts (n)	12/11	9/12	10/12	0.462	
Hypogonadic/ non-hypogonadic pts (n)	13/10	10/11	11/11	0.783	
Hemoglobin (g/dL)	8.9± 1.5#	8.3± 1.1#	9.0± 1.4#	0.239	14.7± 2.8
White blood cells ($\times 10^9$/L)	23.6± 23.2#	21.6± 22.2#	23.6± 21.1#	0.936	6.9± 2.4
Platelets ($\times 10^9$/L)	501.4± 256°	511.8± 278.7°	469.3± 232.2°	0.968	241.7± 98.2
Serum creatinine (mg/dL)	0.8± 0.1	0.8± 0.1	0.8± 0.3	0.775	0.9± 0.1
AST (U/L)	47.4±29.3°	39.5±13.4°	49.5±32.2°	0.432	28.4±13.5
ALT (U/L)	50.1±39.6°	37.9±21.8°	53.1±39.7°	0.326	22.7±16.2
BMD (g/cm²)					
L1-L4	0.80±0.12	0.84±0.13	0.89±0.09	0.052	
Femoral neck	0.73±0.12	0.83±0.12	0.77±0.10	0.029	
Wrist	0.36±0.09	0.41±0.06	0.41±0.15	0.254	
BMD (T-score)					
L1-L4	-3.3±0.9	-2.9±0.9	-2.5±0.7	0.017	
Femoral neck	-1.9±1.5	-1.5±0.8	-2.0±0.7	0.423	
Wrist	-4.0±1.7	-3.17±1.1	-3.14±1.4	0.143	
Markers of bone resorption					
CTX (ng/mL)	0.81± 0.56°	0.85± 0.55°	0.83± 0.67°	0.974	0.48± 0.23
TRACP-5b (U/L)	1.29± 0.94	1.20± 1.02	1.35± 0.73	0.872	1.53± 0.81
Markers of bone formation					
bALP (U/L)	34.50± 12.2#	35.0±1 4.0#	38.6± 24.5#	0.713	22.8± 13.8
OC (ng/mL)	7.49± 4.31	9.59± 9.13	8.68± 6.25	0.59	9.66± 5.27
CICP (ng/mL)	81.30±31.0*	82.8±24.4*	100.9±55.4°	0.204	64.1±37.0
Markers of osteoclast activation					
sRANKL (pmol/L)	1.97± 0.77	2.75± 2.64*	2.45± 1.36*	0.334	1.57± 2.70
OPG (pmol/L)	5.37± 1.89°	5.94± 2.08°	5.35± 2.00°	0.556	3.87± 2.85
sRANKL/OPG	0.39± 0.18	0.59± 0.90	0.63± 0.79	0.472	0.74± 1.57
OPN (ng/mL)	12.1± 9.58	18.2± 20.8	16.2± 19.7	0.499	11.1± 10.9

**p<0.05 compared to controls; °p<0.01 compared to controls; #p<0.001 compared to controls. AST: aspartate amino transferase; ALT: alanine amino transferase.*

line had increased values of CTX, bALP, CICP, sRANKL and OPG, compared with controls (Table 1). There were no differences in serum values of bone remodeling markers between the three groups of patients.

Analysis of variance (ANOVA) showed that there were baseline differences between the three groups in T-score of L1-L4 and BMD of the femoral neck. This was mainly due to the group C patients whose values of BMD L1-L4 were higher that those of patients in group A ($p<0.01$) and whose values of BMD of the femoral neck were lower than those of patients in group B ($p=0.04$). No other differences in baseline BMD at the studied sites were observed between the three groups of patients.

Baseline serum levels of CTX, bALP and OC correlated with BMD of L1-L4 (r=-0.34, $p<0.01$; r=-0.32, $p<0.01$; and r=-0.26, $p<0.03$, respectively). Moreover, baseline serum levels of OPG and sRANKL/OPG ratio correlated with BMD of the wrist (r=-0.32, $p<0.01$; r=0.25, $p=0.04$, for OPG and ratio, respectively).

BMD and markers of bone remodeling following administration of zoledronic acid or placebo

Group A patients, who received zoledronic acid every 6 months, had increased BMD at all three evaluated sites after 12 months of treatment, although this did not reach the level of statistical significance (Figure 1A). They did, however, show dramatic reductions in the serum levels of bALP ($p=0.001$), and OC ($p<0.0001$), starting at 6 months and continuing at 12 months post-treatment and OPG ($p<0.0001$) at 12 months after zoledronic acid administration. An increase in TRACP-5b serum levels was observed 12 months post-therapy (Table 2). No change in CTX values was observed after 12 months of treatment.

Group B patients, who received zoledronic acid every 3 months, had a significant increase in lumbar spine BMD after 12 months of therapy ($p=0.028$) (Figure 1B), which was accompanied by dramatic reductions in serum CTX ($p=0.0001$), bALP ($p<0.0001$), CICP ($p<0.03$) and OC ($p<0.01$) as well as a borderline reduction of sRANKL serum levels ($p=0.05$) (Table 3). They also had an increase in the BMD of the femoral neck, although this was of only borderline statistical significance ($p=0.11$); they had no change in the BMD of the wrist.

Group C patients did not show alterations of BMD in any of the three studied sites after 12 months of therapy (Figure 1C), while they had an aggravation in both markers of bone resorption after 12 months of treatment ($p=0.001$ and 0.002, for CTX and TRACP-5b, respectively; Table 4). No other alterations in studied biochemical markers of bone remodeling were observed in group C patients.

The percentage increase of L1-L4 BMD was significantly greater in group B (15.2%) than in group A (5.8%; $p=0.02$) or group C (1.4%; $p<0.001$), while there was only a borderline difference in the percentage increase in L1-L4 BMD between groups A and C ($p=0.09$). Furthermore

there was a significantly greater percentage increase of femoral neck BMD in group B (11.3%) than in group C (2.7%; p=0.03) and a borderline difference between group B and group A (4.8%; p=0.08). No other differences in percentage alterations of BMD were observed between the three groups of patients. The percentage change of L1-L4 BMD in all patients strongly correlated with the percentage change of CTX (r=-0.37, p=0.002; Figure 2), and bALP (r=-0.4, p=0.001), while there was a weak correlation between alterations (%) of L1-L4 BMD and alterations (%) of serum OPG (r=-0.23, p=0.07) and CICP (r=-0.29, p=0.08).

Bone pain and fractures at baseline and during zoledronic acid therapy

At baseline patients had mild to moderate pain according to the pain scoring system used. There were no differences in pain score among the three studied groups (Tables 2-4). Patients in groups A and B had dramatic reductions of pain score after 6 and 12 months of zoledronic acid treatment. In contrast, group C patients had no change in bone pain during the study period. The per cent reduction of the VAS pain score after 6 months of therapy was greater in group B patients than in group A patients (p=0.01); however this difference was not observed after 12 months of zoledronic acid administration. No fractures occurred during the treatment period in any of the patients in the three groups.

Iron status and hemoglobin levels during zoledronic acid administration

The serum ferritin levels of patients with thalassemia major were 1933±1467 mg/L (mean±SD) compared with 863±760 mg/L in patients with thalassemia intermedia. The administration of zoledronic acid had no effect on hemoglobin or ferritin serum levels (Tables 2-4). As regards correlations between hemoglobin and bone markers, we found only a weak correlation between hemoglobin levels and sRANKL (r=0.2, p=0.09).

Side-effects

Diffuse bone pain and fever (up to 39°C) developed in 4/44 (9%) and 9/44 (20%) patients, respectively, after the first infusion of zoledronic acid. The duration of these side effects was less than 24 hours, and the patients were given paracetamol to relieve the symptoms. These effects did not recur after subsequent infusions of the bisphosphonate. No renal abnormalities, hypocalcemia, osteonecrosis of the jaw or other side-effects were observed during the treatment period. Two patients in group A died of causes not related to zoledronic acid administration (lung infection and acute cardiac event); the first patient had received only one dose and the second had been given two doses of zoledronic acid.

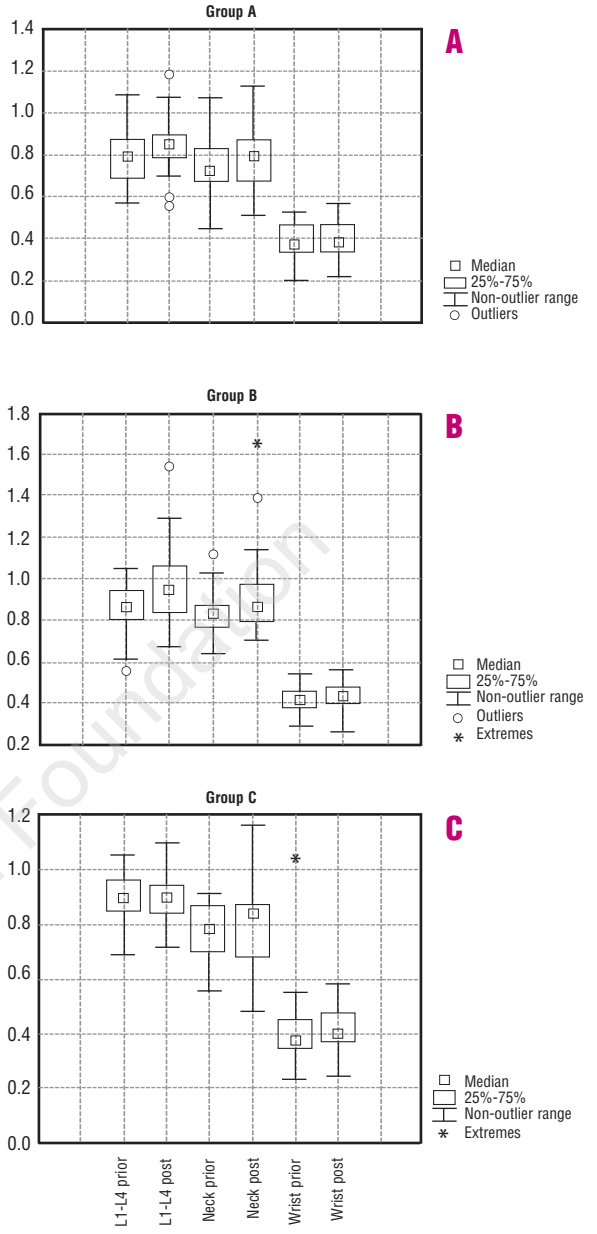

Figure 1. A. Group A patients showed a slight, but not significant, increase of BMD in all studied sites (mean±SD): L1-L4: from 0.80±0.12 g/cm² at baseline to 0.85±0.14 g/cm² at 12 months post treatment (p=0.22); femoral neck: from 0.73±0.12 g/cm² to 0.78±0.13 g/cm² (p=0.26); wrist: from 0.36±0.09 g/cm² to 0.40±0.09 g/cm² (p=0.16). **B.** Group B patients showed a statistically significant increase of L1-L4 BMD (mean±SD: from 0.84±0.13 g/cm² at baseline to 0.97±0.22 g/cm² at 12 months post-treatment (p=0.028), and a borderline increase in femoral neck BMD (from 0.83±0.12 g/cm² to 0.92±0.12 g/cm²; p=0.11). There was no alterations in BMD of the wrist (from 0.41±0.06 g/cm² to 0.43±0.07 g/cm² (p=0.46). **C:** Group C patients showed no significant alterations of BMD in any studied site (mean±SD): L1-L4: from 0.89±0.09 g/cm² at baseline to 0.90±0.09 g/cm² at 12 months post treatment (p=0.75); femoral neck: from 0.77±0.10 g/cm² to 0.79±0.13 g/cm² (p=0.55); wrist: from 0.41±0.15 g/cm² to 0.40±0.08 g/cm² (p=0.81).

Discussion

The pathogenesis of bone loss in thalassemia is multifactorial; underlying mechanisms include mainly bone marrow expansion, iron overload, genetic factors, and hormone deficiency.[1,2,20] Its management is, therefore, difficult. The type of chelation therapy does not interfere with the progression of bone loss in these patients.[21] The role of hormone replacement therapy remains controversial.[3,22] Our group showed that patients who were receiving continuous hormone replacement therapy had comparable osteoporosis to those who were not receiv-

Table 2. Median values (range) of the studied parameters in group A.

Parameter	*Group A (baseline)*	*Group A (6 m. post ZA)*	*Group A (12 m. post ZA)*	*p (baseline vs. 6 m)*	*p (baseline vs. 12 m)*	*p (6 m vs. 12 m)*
Markers of bone resorption						
CTX (ng/mL)	0.60 (0.35-2.40)	0.53 (0.26-1.82)	0.63 (0.12-1.53)	0.16	0.106	0.81
TRACP-5b (U/L)	0.93 (0.31-4.02)	1.20 (0.04-3.35)	1.92 (0.1-5.87)	0.99	0.02	0.02
Markers of bone formation						
bALP (U/L)	33.0 (13.6-57.0)	22.3 (10.5-58.6)	23.5 (8.85-49.6)	0.002	0.001	0.84
OC (ng/mL)	7.96 (0.91-11.9)	4.53 (0-9.93)	4.25 (2.51-5.71)	0.008	<0.0001	0.58
CICP (ng/mL)	76.8 (43.1-167.1)	71.5 (28.3-115.9)	76.4 (36.6-103.6)	0.2	0.39	0.69
Markers of osteoclast stimulation						
sRANKL (pmol/L)	1.85 (0.63-3.48)	2.14 (0-11.5)	0.95 (0-19.7)	0.13	0.77	0.54
OPG (pmol/L)	5.02 (2.15-8.70)	5.37 (0.95-9.78)	2.86 (1.17-6.99)	0.58	<0.0001	0.001
sRANKL/OPG	0.35 (0.18-0.77)	0.39 (0-6.44)	0.30 (0-5.56)	0.06	0.22	0.45
OPN (ng/mL)	10.24 (0-37.4)	8.39 (2-99.3)	13.93 (8.0-26.5)	0.034	0.3	0.26
Pain scoring systems						
VAS (cm)	4.6 (0-7.5)	2.1 (0-3.5)	0.8 (0-2)	<0.0001	<0.0001	<0.01
McGill-Melzack score	2 (0-4)	0 (0-1)	0 (0-1)	<0.0001	<0.0001	0.67
Hemoglobin (g/dL)	9.1 (7.4-12.3)	8.9 (7-11.4)	9.1 (7.3-12)	0.35	0.46	0.32
Ferritin (mg/L)	1300 (210-2360)	1340 (290-1980)	1320 (275-2040)	0.42	0.38	0.44

Table 3. Median values (range) of the studied parameters in group B.

Parameter	*Group B (baseline)*	*Group B (6 m. post ZA)*	*Group B (12 m. post ZA)*	*p (baseline vs. 6 m)*	*p (baseline vs. 12 m)*	*p (6 m vs. 12 m)*
Markers of bone resorption						
CTX (ng/mL)	0.70 (0.32-2.44)	0.57 (0.20-1.52)	0.23 (0.09-0.78)	0.09	0.0001	0.005
TRACP-5b (U/L)	0.96 (0.40-5.35)	1.37 (0.26-1.89)	1.36 (0.32-3.72)	0.92	0.40	0.93
Markers of bone formation						
bALP (U/L)	30.3 (14.0-67.4)	18.7 (10.6-39.4)	17.4 (7.68-34.2)	0.0001	<0.0001	0.52
OC (ng/mL)	6.49 (1.22-31.7)	4.27 (0.10-9.54)	3.98 (1.37-6.40)	0.004	0.006	0.72
CICP (ng/mL)	75.9 (53-132.6)	58.8 (29.7-120.1)	60.6 (44.1-109.7)	0.003	0.03	0.56
Markers of osteoclast stimulation						
sRANKL (pmol/L)	1.93 (0.81-13.1)	1.43 (0-9.31)	0.64 (0-13.5)	0.09	0.05	0.59
OPG (pmol/L)	5.75 (3.01-9.74)	3.84 (1.47-17.5)	2.60 (1.30-27.1)	0.20	0.19	0.99
sRANKL/OPG	0.35 (0.14-4.36)	0.32 (0-1.81)	0.23 (0-5.2)	0.73	0.89	0.89
OPN (ng/mL)	12.8 (0-89.7)	18.6 (1-74)	8.84 (3-44.1)	0.93	0.65	0.52
Pain scoring systems						
VAS (cm)	4.8 (0-10)	1.5 (0-5)	0.3 (0-1)	<0.0001	<0.0001	0.001
McGill-Melzack score	2.5 (0-5)	0 (0-1)	0 (0-1)	<0.0001	<0.0001	0.786
Hemoglobin (g/dL)	8.1 (6.8-10.7)	7.9 (7.0-11.2)	7.9 (7.3-10.3)	0.31	0.33	0.46
Ferritin (mg/L)	1310 (325-1880)	1380 (280-1970)	1300 (250-1870)	0.48	0.56	0.33

Table 4. Median values (range) of the studied parameters in group C.

Parameter	*Group C (baseline)*	*Group C (6 m)*	*Group C (12 m)*	*p (baseline vs. 6m)*	*p (baseline vs. 12 m)*	*p (6 m vs. 12 m)*
Markers of bone resorption						
CTX (ng/mL)	0.62 (0.33-3.15)	0.68 (0.37-3.23)	0.95 (0.56-4.18)	0.89	0.001	0.001
TRACP-5b (U/L)	1.08 (0.23-2.71)	2.21 (0.20-4.09)	2.63 (0.22-5.34)	0.03	0.002	0.17
Markers of bone formation						
bALP (U/L)	32.7 (11.4-117.3)	30.6 (13.9-63.3)	29.3 (12.1-78.3)	0.4	0.47	0.90
OC (ng/mL)	7.31 (1.02-25.8)	6.29 (2.83-24.8)	5.69 (2.76-23.4)	0.87	0.7	0.8
CICP (ng/mL)	89.4 (28.3-246.1)	91.6 (35-248.8)	84.8 (44.1-253.4)	0.82	0.32	0.21
Markers of osteoclast stimulation						
sRANKL (pmol/L)	1.89 (0.86-5.88)	1.45 (0.28-10.2)	1.12 (0-16.6)	0.65	0.82	0.93
OPG (pmol/L)	4.90 (1.50-9.42)	4.41 (1.88-18.3)	4.15 (2.07-12.4)	0.95	0.64	0.71
sRANKL/OPG	0.40 (0.11-3.91)	0.44 (0.03-2.97)	0.46 (0-5.75)	0.59	0.63	0.96
OPN (ng/mL)	8.21 (2-70.2)	13.8 (1-211.8)	16.8 (8.8-361.3)	0.31	0.11	0.19
Pain scoring systems						
VAS (cm)	4.5 (0-7)	4.6 (0-7)	4.8 (0-7)	0.67	0.45	0.83
McGill-Melzack score	2 (0-3)	2 (0-3)	2 (0-3)	0.88	0.78	0.96
Hemoglobin (g/dL)	8.9 (6.9-12.1)	9.1 (6.9-12.1)	8.8 (7.3-12.6)	0.41	0.36	0.44
Ferritin (mg/L)	1390 (390-3200)	1360 (290-3150)	1400 (310-3300)	0.31	0.52	0.22

ing any replacement treatment.[4]

Bisphosphonates, which are potent osteoclast inhibitors, are considered a first line treatment for post-menopausal and male osteoporosis as well as for steroid-induced bone loss.[23] In thalassemia-related osteoporosis only alendronate and pamidronate have been found to be of benefit in terms of increasing BMD of the lumbar spine.[5,7] One concern about administering oral bisphosphonates in thalassemia major is that many patients are unable to take these drugs due to poor tolerability; furthermore, gastrointestinal absorption of oral bisphosphonates is less than 10%, and this absorption is further reduced by food containing milk or iron.[24] Zoledronic acid is the most potent third generation aminobisphosphonate and is very effective in post-menopausal osteoporosis even given once a year at a dose of 4 mg iv.[8] It is normally used for the treatment of osteolytic lesions of malignancies.[25] Knowing from our group's previous experience that thalassemia-induced osteoporosis, due to its multifactorial pathogenesis, is more refractory to bisphosphonates than postmenopausal osteoporosis[7] and taking into consideration the disadvantages of oral bisphosphonates in this disorder, we planned this placebo-controlled, randomized, phase II trial to evaluate the effect of iv zoledronic acid on BMD at different sites, bone pain, fractures, and markers of bone metabolism as well the drug's safety profile in this cohort of patients. According to our knowledge such studies are not available in the literature.

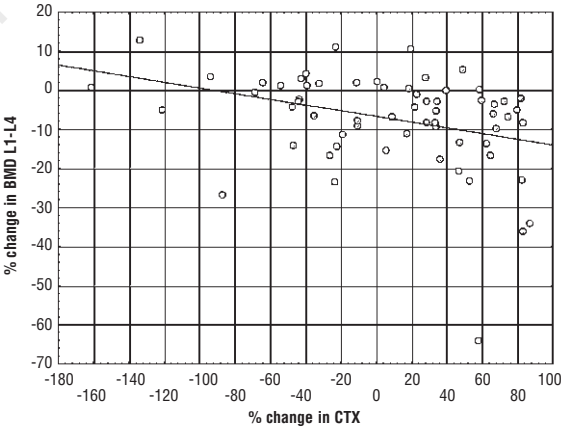

Figure 2. Increase of L1-L4 BMD in all patients correlated with the reduction of CTX.

All patients with thalassemia-induced osteoporosis at baseline had higher values of CTX, bALP, and CICP compared with control values, showing that bone remodeling is increased in thalassemic patients. The finding that CTX, a bone resorption marker, is increased in thalassemia is in accordance with previous results from our group and others who have shown that patients with thalassemia major and osteoporosis have elevated markers of bone resorption, such as urinary levels of NTX, pyridinoline and deoxypyridinoline.[4,15,26,27] The strong inverse correlation between lumbar spine

BMD and CTX in our study further supports the notion that increased bone collagen degradation due to increased osteoclast function is present and associated with osteoporosis in thalassemia patients. The concomitant increase of bALP and CICP, but not of OC (all markers of bone formation) reflects the efforts of osteoblasts to compensate for this increased osteoclastic activity. However, as osteoblasts are rather exhausted in thalassemia[1,28] they cannot fully override the osteoclast activity, the net result being increased bone loss.

What is the reason for the increased osteoclast activity in thalassemia? In this study, thalassemic patients had increased serum levels of both sRANKL and OPG at baseline. The RANK/RANKL/OPG system has been recognized as one of the major pathways of osteoclast activation. RANKL plays an important role in osteoclast formation *in vivo*, and RANKL null mice exhibit a striking osteopetrosis.[29] OPG, the decoy receptor of RANKL, can strongly inhibit osteoclast formation both *in vitro* and in animal models,[30] and OPG serum levels are increased in patients with post-menopausal osteoporosis.[31] This pathway also seems to be important in the pathogenesis of bone degradation in thalassemia. Our group and others have shown that the serum sRANKL/OPG ratio is elevated in patients with thalassemia major.[15,32] However, the increase of sRANKL levels observed in this study was not accompanied by a comparable increase in the sRANKL/OPG ratio due to the concomitantly elevated values of OPG. In previous studies in thalassemia major patients, OPG was not elevated.[15,32] This was possibly due to a disturbed function of osteoblasts as a result of iron deposition, iron chelation therapy, or vitamin D_3 treatment, which downregulates OPG synthesis.[33] However, in our study, only 47% of the patients had thalassemia major while the remaining had thalassemia intermedia with lower iron overload, reduced need for chelation therapy and thus possibly better osteoblast function and higher OPG values. The difference in study populations between the studies is probably responsible for the discrepancy in the results of sRANKL/OPG ratio. However, even in the present study, there was a strong correlation between sRANKL/OPG ratio and BMD of the wrist (the site with the most severe bone loss in thalassemia).[4] This observation, together with the increase of sRANKL values, further suggests that the RANKL/OPG pathway is important in the pathogenesis of bone loss in thalassemia.

Osteopontin is another osteoclast activator which has been implicated in the mechanisms of osteoclast function in malignancies, including myeloma and lung cancer, and its serum levels are elevated in these disorders.[34,35] However, we found no differences in serum osteopontin values between thalassemics and controls and osteopontin levels were not altered following administration of zoledronic acid.

After 12 months of treatment, zoledronic acid produced a significant increase in L1-L4 BMD in patients who received the drug every 3 months, at a dose of 4 mg, iv. This increase was accompanied by dramatic reductions in bone pain and markers of both resorption and formation. Specifically, the reduction of bone resorption, as assessed by CTX levels, was greater (67%) than the reduction of markers of bone formation (42.5%, 38.6%, and 20.1%, for bALP, OC, and CICP, respectively); thus the balance of bone remodeling was in favor of bone formation and led to the increase of BMD in the lumbar spine. Theoretically, this significant reduction of bone remodeling in patients receiving zoledronic acid therapy may create some problems in the long run, such as possible bone fragility; however, this remains to be demonstrated in studies with a longer period (>3 years) of zoledronic acid administration.

Patients who received the same dose of zoledronic acid every 6 months did not have an increase in L1-L4 BMD although they did show reductions in bone pain, bALP, OC, and OPG. The reduction of bone formation markers was not accompanied by a comparable or greater reduction of bone resorption (indeed there was a slight increase of CTX after 12 months); thus no increase in BMD was observed. It may be that a longer period of zoledronic acid administration is needed to produce the same results as those in patients receiving the drug every 3 months. However, this hypothesis needs to be tested in another study. Furthermore, the observation that the percentage increase of L1-L4 BMD was greater in group B than in group A after 12 months of therapy and that the percentage reduction of VAS pain score was also greater in group B than in group A after 6 months of treatment suggest that zoledronic acid needs to be given at least every 3 months in order to have a beneficial effect on BMD in thalassemic patients. In contrast, L1-L4 BMD is increased in patients with post-menopausal osteoporosis who receive 4 mg of zoledronic acid only once a year.[8] This difference confirms our previous experience that thalassemia-induced osteoporosis is more refractory to bisphosphonates than post-menopausal osteoporosis.

The increase in L1-L4 BMD observed in group B was not associated with a similar increase in femoral neck and wrist BMD. This phenomenon may be explained by the faster improvement of BMD in the lumbar spine than in other sites and longer follow-up periods may be needed to reveal improvements in femoral neck or wrist BMD.[36]

Group C patients had a greater than 50% increase in bone resorption markers after 12 months months of following. However, at the same time there was no worsening of BMD at the studied sites. This result suggests that a significant increase in bone resorption markers may be observed earlier than a reduction of BMD. There was no difference in fracture risk among the three groups studied. This may reflect the short period of fol-

low-up. However, zoledronic acid has not yet been licensed for osteoporosis in post-menopausal women because of the necessity of studies clarifying the risk of fractures and the long-term safety of the drug. Therefore studies with larger number of patients evaluating fracture risk in thalassemia are necessary before the broader use of zoledronic acid in thalassemic patients with osteoporosis.

Group A patients had a significant reduction of OPG after 12 months of therapy ($p<0.0001$), while group B patients had a non-significant reduction ($p=0.196$). Moreover, group B patients had a borderline reduction of sRANKL ($p=0.05$). These results are in accordance with those of a previous study we conducted showing that pamidronate reduced the serum level of OPG but did not alter the serum levels of sRANKL in patients with thalassemia major and osteoporosis.[7] The role of bisphosphonates in the regulation of OPG and RANKL synthesis is controversial. Viereck *et al*. showed that both pamidronate and zoledronic acid can increase the production of OPG by primary human osteoblasts, thus antagonizing the osteoclastogenic activity of RANKL.[37] However, Pan *et al.* reported that zoledronic acid did not significantly affect RANKL or OPG gene expression, but markedly increased OPG protein secretion and reduced transmembrane RANKL protein expression in osteoblast-like cells.[38] Our results are more compatible with the second observation since (a) sRANKL levels did not change during zoledronic acid treatment, and (b) the observed increase of the lumbar BMD brought about by zoledronic acid was only weakly correlated with serum OPG levels and not correlated with sRANKL levels. Furthermore, the effect of zoledronic acid on the bone microenvironment may not completely reflect the serum levels of different cytokines, such as RANKL and OPG.

In conclusion, patients with β-thalassemia and osteoporosis have increased bone resorption, which results in low BMD. Zoledronic acid , at a dose of 4 mg, iv, every 3 months is very effective in increasing the BMD of the lumbar spine and reducing osteoclast activity and bone resorption, without causing any severe side-effects. On this background, studies with longer administration of zoledronic acid and longer follow-up periods are warranted to demonstrate the expected decrease of vertebral fractures or other skeletal related events.

EV and ET made major contributions to the conception and design of the study, analysis and interpretation of data; drafting the article; and giving final approval of the version to be published. KK,and CK participated in the acquisition of data, revising the paper critically for important intellectual content; and giving final approval of the version to be published. ES, AA, and ES participated in the analysis and interpretation of data; revising the paper critically for important intellectual content; and giving final approval of the version to be published. ES and ET assayed the biochemical markers of bone metabolism, while AA performed the statistical analyses. This study was supported by Novartis Hellas. The study as a whole is dedicated to the thalassemic patients of Laikon General Hospital whose participation made it possible.

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