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Endocrine and bone disease in appropriately treated adult patients with beta-thalassemia major

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Abstract: The natural history of beta thalassemia major (TM) has significantly changed during the last 2 decades. At present TM patients survive over their thirties and forties, but they have considerable morbidity. Bone demineralization is an important cause of morbidity in older patients; the etiology is multifactorial and partially unknown. We examined cross-sectionally 111 adult TM patients (66 females and 45 males, 32.6 ± 6 years), regularly transfused, properly chelated and replaced for endocrine defects. Bone demineralization was detected in 92.7% of patients, with different severity according to gender and site: osteopenia was the prominent finding at the femur, osteoporosis at lumbar spine ($p < 0.001$), more evident in males. The femoral site was more influenced by biochemical and clinical factors; despite adequate replacement, femoral T-score was lower in the hypogonadic than in the eugonadic group ($p = 0.047$). A significant correlation was found between bone mass and body mass index (BMI), alkaline phosphatase (ALP) and pre-transfusional Hb levels. Multivariate analysis indicated as significant regressors ALP, BMI and hypoparathyroidism (T-score: $p = 0.005, 0.035, 0.002$; Z-score: $0.002, 0.009, 0.003$, respectively) at femur, only ALP at lumbar spine ($p = 0.008$ and 0.045 for T and Z scores, respectively). Statistical significance was reached more frequently by T-score, while Z-score demonstrated to have a lower sensitivity.

Despite best care facilities, bone demineralization in thalassemic patients remains a challenge; further exploration of the relationships between bone loss and endocrine, biochemical and hematologic variables is warranted to find effective measures to reduce bone pain and fracture risk.

Response to Reviewers: We are grateful for the useful suggestions of the reviewers. We have revised our paper following their recommendations.

Reviewers' comments:

1) In the Discussion session they comment that "...the correlation between alkaline phosphatase and bone mineralization indicates a prevalent role of reduced deposition over increased reabsorption, as reported by other Authors, at variance with others. We could not confirm the same relationship with osteocalcin, another marker of osteoblastic activity."

First of all the authors have not measured any of the markers of bone resorption. I believe that it is important for the paper and for the the discussion to include at least NTX or CTX measurements. I hope that they have available serum for such measurement. I suggest that CTX is more sensitive than NTX in the serum.

- In the original paper, we did not include any marker of bone resorption because we had only partial data (different markers, incomplete series). It seems now evident that the importance of these indices to our work has been understated. In effect, we had available serum of most patients, so we decided to measure CTX following the useful suggestion of the reviewer; we recognize that this adjunct increases the scientific interest of our results.

As pointed out in the revised paper (page 8, lines 1 to 3) "no significant differences were found in CTX serum levels between thalassemic patients and controls, nor significant correlations with any of the studied parameters. This result may be due to the wide variability of the values in both cohorts; the problem should probably be obviated by a longitudinal study protocol."

2) Second, in order to state that the correlation of bALP with BMD indicates a prevalent role of reduced deposition, someone could expect a strong correlation. On the contrary the correlation was weak ($r < 0.25$). Furthermore, we do not know if the bALP values were reduced (that will indicate reduced formation) or not.

- The correlation was found between total ALP and BMD, not with the bone isoenzyme; this could be due to the low precision of the electrophoretic method utilized for bALP determination. Unfortunately, these are the only variables in our possession for all the patients at the moment. The values of ALP were reduced in thalassemic patients vs normal controls. The discrepancy between osteocalcin and ALP could be explained as stated in the revised paper, page 7, lines 1 to 3 from the bottom: "In effect these parameters, though both related to bone mineralization, differ as alkaline phosphatase assesses osteoblastic activity, osteocalcin expresses bone mineralization. It is well known that mineralization is defective in thalassemic patients (references no 16 and 17 of the revised paper), while data on bone formation are contrasting."

3) Control values (and not the expected values by the manufacturer of the kits) are needed for all studied parameters.

- A matched control group was included and the values for the measured parameters were compared with those obtained in the thalassemic patients.

4) DEXA has to be substituted by DXA throughout the text.

- The term was substituted throughout the text.

***Conflict of interest**

[Click here to download Conflict of interest: conflitto interessi-1.jpg](#)

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We trust the paper in the present form to be suitable for publication.

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6 **Endocrine and bone disease in appropriately treated adult patients with beta-Thalassemia**
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Abstract

With optimization of transfusional and chelation regimens, beta Thalassemia has changed from a pediatric disease with poor life expectancy into a chronic disease. Bone demineralization is an important cause of morbidity in older patients; the etiology is multifactorial and partially unknown. We examined cross-sectionally 111 adult patients with beta-Thalassemia Major (66 females and 45 males, 32.6 ± 6 years), regularly transfused, sufficiently chelated and replaced for endocrine defects. Bone demineralization was detected in 92.7% of patients, with different severity according to gender and site: osteopenia was the prominent finding at the femur, osteoporosis at lumbar spine ($p < 0.001$), more evident in the males. The femoral site was more influenced by biochemical and clinical factors; despite adequate replacement, femoral T-score was lower in the hypogonadic than in the eugonadic group ($p = 0.047$). A significant correlation was found between bone mass and body mass index (BMI), alkaline phosphatase (ALP) and pre-transfusional Hb levels. The multivariate analysis indicated as significant regressors ALP, BMI and hypoparathyroidism (T-score: $p = 0.005, 0.035$ and 0.002 ; Z-score: $0.002, 0.009, 0.003$, respectively) at the femoral site, only ALP at the lumbar spine ($p = 0.008$ and 0.045 for T and Z scores, respectively). The statistical significance was reached more frequently by T-score, while Z-score seemed to be a less sensitive parameter.

Despite best care facilities, bone demineralization in thalassemic patients remains a challenge; further exploration of the relationships between bone loss and endocrine, biochemical and hematologic parameters is warranted to find effective measures to reduce fracture risk in this disease.

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Key words: Thalassemia, osteoporosis, iron overload, endocrinopathy.

Introduction

1 The life expectancy of patients affected by Thalassemia Major (TM) is significantly increased during the last decade
2
3 due to regular transfusion regimen associated to adequate iron chelation. Today most of the patients enter adult life with
4
5 a relevant improvement of quality of life[1]. Bone deformities, which could not be forecasted 20 years ago are quite rare
6
7 today, nevertheless, low bone mass still remains a significant cause of morbidity in adult patients with TM. An
8
9 increased prevalence of osteoporosis, and consequently of fractures due to bone fragility, continues to be reported [2,3]
10
11 despite transfusions, effective chelation, calcium and vitamin D supplementation and replaced hormonal deficiencies.
12
13 Several concomitant factors may contribute to the pathogenesis of osteoporosis in adult TM patients, making difficult a
14
15 proper and preventing therapeutic approach.
16
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18 We designed a cross-sectional evaluation of adult Thalassemia Major patients followed-up at single tertiary outpatient
19
20 clinic in Milan, with optimal transfusional and therapeutical regimen, in order to assess the prevalence of bone
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22 demineralization and the relationships between bone status and physiological and pathological factors, including
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24 ferritin, pre-transfusional haemoglobin levels, bone metabolism markers and endocrine complications.
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Patients

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2 After the approval of the Ethical Committee of our Institution and after patient's informed consent, 111 adult Caucasian
3 patients affected by beta-Thalassemia Major (homozygous or double heterozygous for severe beta mutations) were
4 enrolled in this study (66 females and 45 males, mean age 32.6 ± 6 years (33, range 19-50). All the patients received
5 regular blood transfusions every 20-25 days, maintaining pre-transfusional haemoglobin around 9-9.5 g/L, according to
6 the international guidelines [4]. Chelation therapy included deferoxamine (DFO) (39 pts), deferiprone (DFP) (7 pts),
7 deferasirox (DFX)(46 pts) or combination DFO/DFP (19 pts). The chelation adequacy was estimated based on mean
8 ferritin levels every 3 months and LIC by MRI when available. Exclusion criteria were medical conditions and
9 pharmacological treatments interfering with the bone mineral metabolism.

10
11 The study design was cross-sectional; a periodic endocrinological evaluation was planned and during the same day bone
12 densitometry, blood and urine biochemical tests and blood hormone evaluations were performed. Data collected
13 included age, gender, BMI, history of auxologic and sexual development, splenectomy, fractures and liver disease,
14 drugs used, transfusion regimen and starting age, mean of pre-transfusional haemoglobin and ferritin in the previous
15 year, liver function, endocrine tests and bone metabolism indices, bone densitometry of femur and lumbar spine. A
16 control group of healthy subjects (24 men and 35 women, mean age 33.7 ± 6.8 years) was recruited and compared with
17 thalassemic patients for bone metabolism indices.

18
19 At the time of the biochemical and densitometric assessment, all hypogonadal patients were on hormonal replacement
20 therapy, and all hypothyroid ones were on replacement with levothyroxine. Patients with hypoparathyroidism were
21 treated with conventional vitamin D and calcium supplements, at a dose sufficient to maintain serum calcium within the
22 normal range or at the lower normal limit.

Methods

23
24 Liver and renal function tests, calcium and phosphorus in serum (n. v. 2.1-2.5 and 0.8-1.4 mmol/L, respectively) and in
25 24-hour urine (n.v. 2.5-7.5 mmol/d and 12.9-42 mmol/d, respectively), intact parathyroid hormone (n.v. 1.5-6.6
26 pmol/L), osteocalcin (n.v. females 5.3-23.6, mcg/l, males 4.4-26.1), 25-hydroxyvitamin D (n.v. > 72 nmol/L), ferritin
27 (n.v. 30-400 mcg/L) and gamma-glutamyltranspeptidase (8-62 U/L) were determined by automated routine procedures.
28 Blood cell count was performed by the Coulter Counter. Alkaline phosphatase was measured by IFCC liquid (n.v. 40-
29 129 U/L), while the bone isoenzyme was assessed by semiquantitative electrophoretic method with scanning
30 densitometer (n.v. 20-75%); serum CTX was determined by the Serum Cross Laps OneStep enzyme-linked
31 immunosorbent assay (ELISA, IDS, Boldon, Tyne & Wear, UK; n.v.: women, post-menopausal 0.142-1.351, pre-
32 menopausal 0.112-0.73; males 0.115-0.748 ng/mL)

1 Iron overload was properly estimated by Liver Iron Content (LIC) measured by MRI [5], that showed a wide standard
2 deviation due to the patients' compliance and the different efficacy of therapies.

3
4 Bone mineral density measurement was performed by means of dual x-ray photon absorptiometry (DXA) using Hologic
5 bone densitometer (Hologic QDR-Discovery A, Version 12.7.3.1).

6
7 BMD values were expressed as T-score and Z-score. T-score was calculated as SDs from normal reference population
8 database, while Z-score as SDs from age and sex matched population.

9
10 Data were classified according to WHO report (WHO Technical Report, ISCD Official Position Paper 2007); T-score >
11 -1: normal. -1 > T-score > -2.5: low bone density (osteopenia). T-score < -2.5: osteoporosis.
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16 17 18 19 20 Statistical analysis

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22 Data are presented as means \pm SD. Intergroup comparison was performed with the Mann-Whitney test. Associations
23 between parameters were determined by Pearson's and Spearman's rank correlation coefficients. To find independent
24 factors affecting bone mineral density in thalassemic patients, a step-wise linear regression analysis was conducted.

25
26 Two-sided exact tests were used and *p* values of less than 0.05 were considered significant. All statistics were
27
28 calculated using the Statistical Package for the Social Sciences 17.0 for Windows software package (SPSS Inc,
29
30
31 Chicago, IL).

Results

The demographic characteristics of the patients studied are presented in Table 1. All the patients were on iron chelating treatment: 35% with deferoxamine (30+5mg/Kg), 42% with deferasirox (20+15mg/Kg), 6% with deferiprone (75mg/Kg) and the remaining 17% with combined therapy (deferoxamine 30mg/Kg + deferiprone 75mg/Kg).

101 patients (91%) were anti-HCV positive, of whom 50.5% were HCV RNA positive. 68% were splenectomised.

Evaluation of bone mineral metabolism and endocrine function

The biochemical indices of bone metabolism are reported in Table 2. All the parameters resulted within the normal range apart from 25OH vitamin D serum levels, that were suboptimal in 21 patients even if 13 of them were supplemented with 10.000 IU/week. The levels of alkaline phosphatase and osteocalcin in the control group (68.1 ± 15.5 U/L and 10.0 ± 4.9 mcg/L respectively) were significant lower (both $p < 0.001$) than in thalassaemic patients (85.4 ± 28.7 U/L and 23.4 ± 30.1 respectively). Conversely, PTH serum concentrations were significantly higher in the control group (3.8 ± 1.2 , $p = 0.007$ vs 3.3 ± 2.2), according to the diagnosis of hypoparathyroidism in 19 out of 111 thalassaemic patients (17.1%). All the hypoparathyroid patients were on treatment with calcium and calcitriol at doses normalizing serum calcium and phosphate concentrations; all of them were also hypogonadic, except one, resulting 16.2% the association of these endocrine diseases. No difference was found for serum levels between thalassaemic and control group (0.42 ± 0.31 vs 0.46 ± 0.28).

Hypogonadism (in all cases hypogonadotrophic) was the most frequent endocrine complication in our cohort of patients, affecting 87 patients (78.4%); all the hypogonadic patients except 4 (95.4%) were on replacement treatment starting from the age of 18.8 ± 3 years. The hypogonadic patients who were not on replacement, being a minority, were excluded from the analysis of densitometric values.

An impairment of glucose metabolism was present in a high proportion of patients, with diabetes mellitus in 20 (18%) and IGT in 24 of them (22%). IGF-1 serum concentrations were evaluated in 78 patients (70%), and resulted lower than normal in 45 of them (41%).

Bone mass measurements

When considering the bone mass measurements in the overall population enrolled, the prevalence of demineralization (including both osteopenia and osteoporosis) was 92.7% (103 out of 111 thalassaemic patients). Indeed, mineralization was normal only in 18 patients (16.2%) at the vertebral site and in 21 patients (18.9%) at the femoral site.

As shown in Table 3, there were no differences in bone mineralization between the hypogonadic and the eugonadic patients at the vertebral site, while at the femoral site the T-score was lower in the hypogonadic group, despite adequate replacement ($p = 0.047$). Further, only at the femoral site, bone mass was significantly different by gender, as females showed lower mean values of T and Z-scores. There were statistically significant differences in T-score at both sites

1 also for the other dicotomic variables examined (hypoparathyroidism, treatment with calcium, treatment with
2 bisphosphonates), while the statistical significance was not reached in all the analyses using Z-score as dependent
3 variable.
4

5 Figure 1 shows the distribution of bone mineralization state (normal / osteopenia / osteoporosis) according to gender
6 and site. Osteopenia was the prominent finding at the femoral site (63 femoral vs 40 vertebral site, $p < 0.001$), while at
7 the vertebral site osteoporosis was more prevalent (53 pts vs 27 pts), the difference was statistically significant
8 ($p < 0.001$). The same distribution was confirmed when the patients were separately considered according to gender,
9 being more evident in the male group ($p < 0.001$). The patient distributions according to bone mineralization were
10 significantly different by gender only at the femoral site.
11

12 Table 4 shows the results of the correlation analysis (correlation indices and statistical significance, $p < 0.05$) between
13 bone mineralization (expressed by T and Z scores) and clinical/biochemical parametric variables. BMI was positively
14 correlated with bone mass at femoral site, while advancing age was negatively correlated only with T-score. No
15 correlation was found between vertebral T-score and the same variables. Among the biochemical indices of bone
16 metabolism examined, alkaline phosphatase was the only one significantly correlated both with femoral and vertebral
17 mineralization, being inversely associated with bone mass. Pre-transfusional Hb levels were significantly associated to
18 vertebral Z-score.
19

20 The correlations between bone mineralization and the other clinical and biochemical characteristics of the patients (age
21 at first transfusion, age at beginning of chelation, diabetes, previous splenectomy, LIC, serum concentrations of ferritin,
22 parathyroid hormone, bone alkaline phosphatase, osteocalcin, 25 OH vitamin D, IGF-1, CTX, and calcium both in
23 serum and urine) were not significant thus were not reported.
24

25 The multivariate analysis indicated as possible regressors alkaline phosphatase, BMI and hypoparathyroidism ($p = 0.005$,
26 0.035 and 0.002 for T-score; 0.002, 0.009, 0.003 for Z-score, respectively) at the femoral site, while alkaline
27 phosphatase was the only one found regressor for T and Z scores (respectively $p = 0.008$ and 0.045) at the lumbar spine.
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Discussion

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2 Despite the longer life expectancy of thalassaemia major patients due to proper transfusional and chelation regimens
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4 [1], endocrine complications and bone disease still remain important causes of morbidity and disability [2,3,6-9] . In
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6 this study we examined adult patients with Thalassemia Major regularly transfused and sufficiently chelated; the
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8 patients were cared for 13 ± 3 years (mean \pm SD) at a tertiary single Italian centre where the Thalassemia International
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10 Guidelines [4] for monitoring and treating Thalassemia and its complications are issued and implemented..

11
12 Despite the availability of the best care facilities, bone demineralization was detected in a high proportion of patients,
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14 involving both sites examined though with different severity: osteopenia prevailed at femoral site, while osteoporosis
15
16 was more prominent at lumbar spine (prevalently constituted by trabecular bone). Overall, the femoral site seemed to be
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18 more likely than the vertebral site influenced by biochemical and clinical factors. The only exception was pre-
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20 transfusional Hb, showing a positive correlation with vertebral mineralization; this could be explained by the high
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22 degree of ineffective erythropoiesis in the spine that can occurs in thalassemic syndromes. This finding is consistent
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24 with the interesting hypothesis that the chronic request for blood cell production can play a role in the etiology of
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26 osteoporosis, through overstimulation of the hematopoietic system increasing the number of osteoclasts and osteoblasts
27
28 resulting in accelerated bone turnover [10].

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30 In our analyses the statistical significance was reached more frequently by T-score, while Z-score seemed to be a less
31
32 sensitive parameter. There is a statistical explanation to this behaviour: the relatively small number of cases can render
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34 the statistical power insufficient to detect smaller changes in Z-score, a parameter with high baseline variability.
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36 However, considering the very low prevalence of the disease, this is a sufficiently large cohort, and quite homogeneous
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38 as regards therapeutical approach and clinical management.

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40 The statistically significant negative association between T-score and treatment with calcium and with bisphosphonates
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42 was expected, and compatible with the prescription of such drugs to the patients with more severe demineralization; the
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44 therapeutic effect of these medications could not be examined in our study, as the protocol did not include a
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46 longitudinal evaluation. Other parameters correlated to demineralization were gender (females are more involved), age
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48 and BMI, similarly to healthy individuals.

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50 Even if we cannot address the issue of the prominent cause of bone disease in Thalassemia [11], the correlation
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52 between alkaline phosphatase and bone mineralization indicates a prevalent role of reduced deposition over increased
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54 reabsorption, as reported by other Authors [12], at variance with others [13, 14] . We could not confirm the same
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56 relationship with osteocalcin, another marker of osteoblastic activity [12, 15]. In effect these parameters, though both
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58 related to bone mineralization, differ as alkaline phosphatase assesses osteoblastic activity, osteocalcin expresses bone
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1 mineralization. It is well known that mineralization is defective in thalassemic patients [16, 17], while data on bone
2 formation are contrasting.

3
4 As regards bone resorption indices, no significant differences were found in CTX serum levels between thalassemic
5 patients and controls nor significant correlations with any of the studied parameters. These results may be due to the
6 wide variability of the values in both cohorts; the problem would probably be obviated by a longitudinal study protocol.
7

8
9 As far as the endocrine complications relevant to bone status are concerned, according to previous reports [18] in our
10 study the hypogonadic patients of both genders were more affected from demineralization than the eugonadic, and,
11 interestingly, the standard hormone replacement therapy (HRT) does not normalize bone condition. A possible
12 explanation may be the concurrence of factors interfering with mineralization, such as vitamin D deficiency, impairing
13 the therapeutic effects of sex steroid hormones and also of bisphosphonates. A recent report [19] stated that an optimal
14 vitamin D repletion is an essential pre-requisite for maximal efficacy of the anti-resorptive drugs in post-menopausal
15 osteoporosis; a similar situation could be hypothesized in thalassemic population. In effect, low serum vitamin D levels
16 have been reported in thalassemic populations; this was ascribed to impaired liver hydroxylation and intestinal
17 absorption, with a causative role of iron overload suggested by a negative correlation between serum 25-OH-vitamin D
18 and ferritin [20]. This correlation was not confirmed in our study ($p = 0.546$). Unexpectedly, in our series the serum
19 vitamin D was often low despite ongoing treatment with metabolites, compatible with a defect in absorption or
20 metabolism [20] but also with low compliance to the therapy; the latter is quite common using associations with
21 calcium salts, badly tolerated. Anyway, the contribution of vitamin D to bone status is probably more complex than
22 presently recognized, and neither vitamin D concentrations nor ongoing treatment did correlate with mineralization
23 status in our and earlier reports [21]. Conversely, impairment of parathyroid function was inversely related to
24 mineralization status, indicating a protective effect on bone of this endocrine complication [21, 22]. In our study the
25 relationship was more evident at the femoral level than at lumbar spine; this was unexpected, as chronic PTH-
26 deprivation should influence more strongly the sites where trabecular content is maximum. Probably, in this peculiar
27 population the superimposed pathologic mechanisms concurring to bone disease can produce net effects difficult to
28 predict in the long-term.
29

30 Finally, an important consideration emerges from our statistical analysis: the model utilized could account for the
31 29.4% of T-score variance at the femur and the 32.03% at the spine. This indicates that the variables significant in the
32 univariate analysis could not explain satisfactorily the mineralization defect. Again, this may be ascribed to insufficient
33 statistical power, linked to the restricted number of cases in a monocentric study, or alternatively to the influence of
34 pathogenetically involved factors beyond those included in our analysis. The indices studied reflect bone marrow
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1 expansion, iron overload, endocrine function and bone metabolism, but further physiological, pathological, genetic and
2 iatrogenic factors can work in this multifactorial disease.

3
4 Furthermore, early preventive intervention and management are needed to influence bone health in adulthood, as
5 demonstrated by normal Z-scores found in children and young adolescents with Thalassemia Major by Christoforidis et
6 al [23]. Our patients are probably comparable for the quality of clinical management; however the different results
7 emerged from our study suggest that transfusion and chelation treatment can prevent bone demineralization only when
8 applied early from childhood.
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12 A deeper learning of the molecular mechanisms causing demineralization, as well as the optimal prevention and
13 treatment of Thalassemia from infancy, could perhaps allow to avoid complications and further ameliorate the quality of
14 life in these patients.
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Fig 1 Bone mineralization state (normal / osteopenia / osteoporosis) of the thalassemic patients as distributed on the basis of site and gender, according to the WHO osteoporosis classification. Osteopenia was the prominent finding at the femur, while at lumbar spine osteoporosis was more prevalent ($p < 0.001$). The same distribution was confirmed when the patients were separately considered according to gender, being more evident in the male group

Table 1 Clinical characteristics of the patients by gender at the time when mineralization was studied

Table 2 Biochemical and hormonal indices related to bone metabolism in 111 patients affected by Thalassemia Major

Table 3 Statistic results (mean \pm standard deviation and p value) of comparisons between clinical/biochemical dicotomic variables and T or Z- scores at femoral and vertebral sites

Table 4 Correlation analysis (Pearson's correlation indices and two-tailed p values) between bone mineralization (expressed by T and Z-scores) and clinical/biochemical parametric variables

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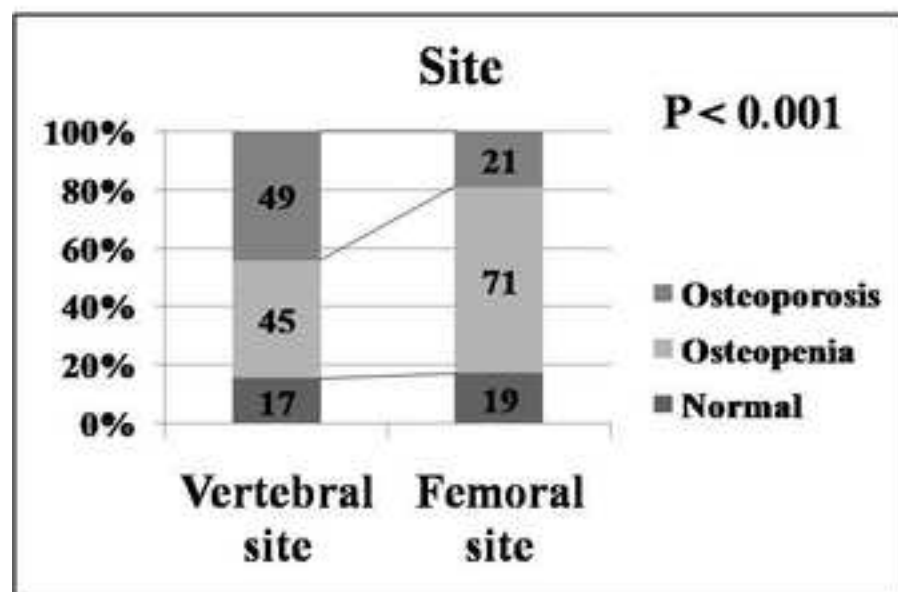
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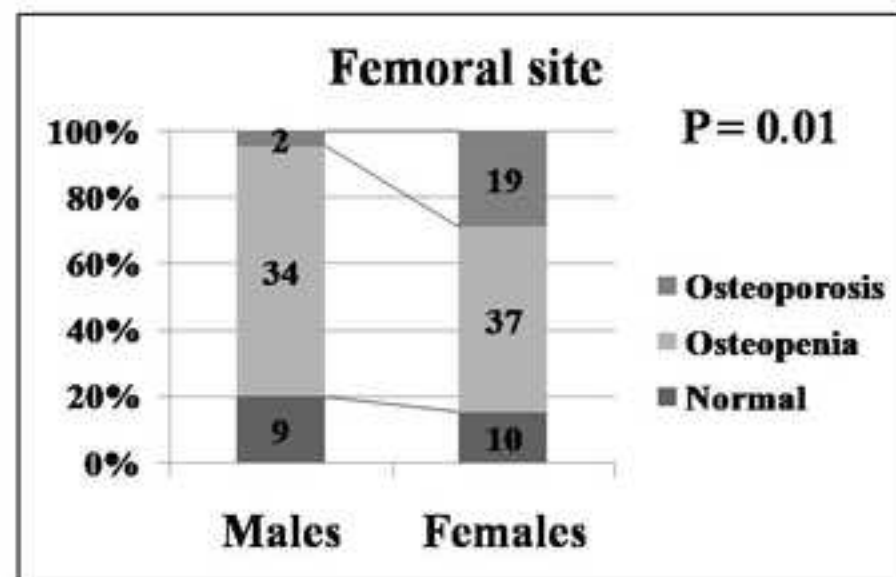
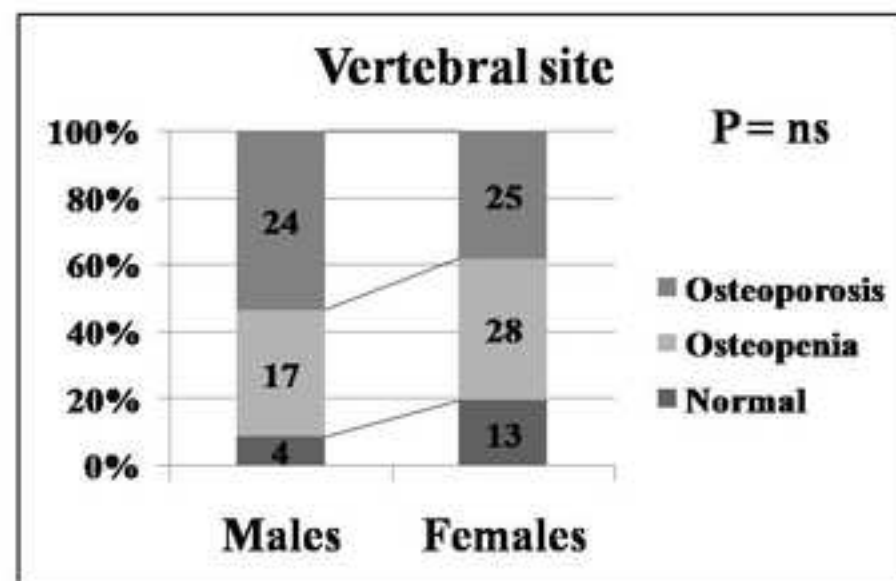
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General



By gender



Table

	males		females	
	Mean \pm SD	range	Mean \pm SD	range
Age (years)	32.5 \pm 4.9	22 - 44	33.9 \pm 6.0	19 - 46
Age at first transfusion (years)	14.3 \pm 11.8	4 - 48	17.3 \pm 16.0	3 - 60
Age at beginning chelation (years)	5.2 \pm 3.7	1 - 15	5.8 \pm 3.5	0 - 15
Body Mass Index (kg/m²)	22.9 \pm 3.3	16 - 29	22.3 \pm 3.0	18 - 31
Pre-transfusion hemoglobin (g/dl)	9.4 \pm 0.6	8.4 - 10.4	9.3 \pm 0.6	8 - 10.4
Serum ferritin (mcg/L)	1267 \pm 1168	431 - 6563	1452 \pm 912	354 - 4509
Liver Iron Content (mg Fe/g liver d.w.)	5.1 \pm 3.9	0.6 - 19.3	5.8 \pm 3.7	0.6 - 15.9

	mean ± SD	range	median
Serum calcium (mmol/L)	2.2 ± 0.1	1.6 – 2.6	9
Serum phosphate (mmol/L)	1.3 ± 0.1	0.7 – 2.2	1.2
Urinary calcium (mmol/d)	6.3 ± 3.7	0.4 – 17	5.8
Urinary phosphate (mmol/d)	22.6 ± 9.7	3.2 – 83.9	22.6
Alkaline phosphatase (U/L)	85.4 ± 28.7	33 - 204	85
Bone alkaline phosphatase (%)	62 ± 17	7 - 95	65
GGT (U/L)	21.2 ± 12.9	7 - 72	35
Osteocalcin (mcg/L)	23.4 ± 30.1	0.3 – 209	14.2
Parathyroid hormone (pmol/L)	3.3 ± 2.2	0.2 – 14.9	2.9
25OH Vitamin D (nmol/L)	46.8 ± 29.3	9.6 - 132	38.6
CTX (ng/ml)	0.4 ± 0.3	0.1 – 1.4	0.3

Table

	T-score				Z-score				
	Femoral		Vertebral		Femoral		Vertebral		
	<i>Mean ± SD</i>	<i>p</i>	<i>Mean ± SD</i>	<i>p</i>	<i>Mean ± SD</i>	<i>p</i>	<i>Mean ± SD</i>	<i>p</i>	
Gender	<i>males</i>	-1.58 ± 0.746	0.046	-2.44 ± 1.119	<i>ns</i>	-1.50 ± 0.830	0.027	-2.28 ± 1.196	<i>ns</i>
	<i>females</i>	-1.90 ± 1.018		-2.12 ± 1.267		-1.87 ± 0.967		-2.04 ± 1.219	
Hypogonadism	<i>no</i>	-1.46 ± 0.772	0.047	-2.20 ± 1.095	<i>ns</i>	-1.65 ± 0.744	<i>ns</i>	-2.12 ± 1.098	<i>ns</i>
	<i>yes</i>	-1.85 ± 0.952		-2.26 ± 1.250		-1.73 ± 0.975		-2.15 ± 1.247	
Hypoparathyroidism	<i>no</i>	-1.87 ± 0.855	0.021	-2.36 ± 1.116	0.039	-1.82 ± 0.850	0.017	-2.21 ± 1.126	<i>ns</i>
	<i>yes</i>	-1.31 ± 1.130		-1.71 ± 1.527		-1.20 ± 1.118		-1.79 ± 1.547	
Calcium treatment	<i>no</i>	-1.64 ± 0.819	0.035	-2.05 ± 1.160	0.036	-1.68 ± 0.795	<i>ns</i>	-2.01 ± 1.192	<i>ns</i>
	<i>yes</i>	-1.93 ± 1.030		-2.49 ± 1.247		-1.75 ± 1.081		-2.31 ± 1.224	
Bisphosphonate treatment	<i>no</i>	-1.67 ± 0.905	0.003	-2.09 ± 1.190	<0.001	-1.66 ± 0.928	0.041	-1.99 ± 1.184	0.001
	<i>yes</i>	-2.35 ± 0.674		-3.23 ± 0.634		-2.14 ± 0.823		-3.16 ± 0.614	

	T - score				Z - score			
	Femoral		Vertebral		Femoral		Vertebral	
	<i>Correlation index</i>	<i>p</i>	<i>Correlation index</i>	<i>p</i>	<i>Correlation index</i>	<i>p</i>	<i>Correlation index</i>	<i>p</i>
Age	-0.218	0.022	-0.032	<i>ns</i>	-0.021	<i>ns</i>	0.063	<i>ns</i>
BMI	0.259	0.006	0.064	<i>ns</i>	0.276	0.006	0.049	<i>ns</i>
Pre-transfusional hemoglobin	-0.088	<i>ns</i>	0.179	<i>ns</i>	-0.058	<i>ns</i>	0.198	0.042
Alkaline Phosphatase	-0.223	0.019	-0.233	0.014	-0.024	0.024	0.137	<i>ns</i>
CTX	0.032	<i>ns</i>	-0.028	<i>ns</i>	-0.024	<i>ns</i>	-0.047	<i>ns</i>

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6 **Endocrine and bone disease in appropriately treated adult patients with beta-thalassemia**
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8 **major**
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Abstract

1 With optimization of transfusional and chelation regimens, beta thalassemia has changed from a pediatric disease with
2 poor life expectancy into a chronic disease. Bone demineralization is an important cause of morbidity in older patients;
3 the etiology is multifactorial and partially unknown. We examined cross-sectionally 111 adult patients with beta-
4 Thalassemia Major (66 females and 45 males, 32.6 ± 6 years), regularly transfused, sufficiently chelated and replaced
5 for endocrine defects. Bone demineralization was detected in 92.7% of patients, with different severity according to
6 gender and site: osteopenia was the prominent finding at the femur, osteoporosis at lumbar spine ($p < 0.001$), more
7 evident in the males. The femoral site was more influenced by biochemical and clinical factors; despite adequate
8 replacement, femoral T-score was lower in the hypogonadic than in the eugonadic group ($p = 0.047$). A significant
9 correlation was found between bone mass and body mass index (BMI), alkaline phosphatase (ALP) and pre-
10 transfusional Hb levels. The multivariate analysis indicated as significant regressors ALP, BMI and hypoparathyroidism
11 (T-score: $p = 0.005, 0.035$ and 0.002 ; Z-score: $0.002, 0.009, 0.003$, respectively) at the femoral site, only ALP at the
12 lumbar spine ($p = 0.008$ and 0.045 for T and Z scores, respectively). The statistical significance was reached more
13 frequently by T-score, while Z-score seemed to be a less sensitive parameter.

14 Despite best care facilities, bone demineralization in thalassemic patients remains a challenge; further exploration of the
15 relationships between bone loss and endocrine, biochemical and hematologic parameters is warranted to find effective
16 measures to reduce fracture risk in this disease.

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43 **Key words:** Thalassemia, osteoporosis, iron overload, endocrinopathy.
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Introduction

1 The life expectancy of patients affected by thalassemia major (TM) is significantly increased during the last decade due
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3 to regular transfusion regimen associated to adequate iron chelation . Today most of the patients enter adult life with a
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5 relevant improvement of quality of life[1]. Bone deformities, which could not be forecasted 20 years ago are quite rare
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7 today, nevertheless, low bone mass still remains a significant cause of morbidity in adult patients with TM. An
8
9 increased prevalence of osteoporosis, and consequently of fractures due to bone fragility, continues to be reported [2,3]
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11 despite transfusions, effective chelation, calcium and vitamin D supplementation and replaced hormonal deficiencies.
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13 Several concomitant factors may contribute to the pathogenesis of osteoporosis in adult TM patients, making difficult a
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15 proper and preventing therapeutic approach.
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18 We designed a cross-sectional evaluation of adult thalassemia major patients followed-up at single tertiary outpatient
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20 clinic in Milan, with optimal transfusional and therapeutical regimen, in order to assess the prevalence of bone
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22 demineralization and the relationships between bone status and physiological and pathological factors, including
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24 ferritin, pre-transfusional haemoglobin levels, bone metabolism markers and endocrine complications.
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Patients

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2 After the approval of the Ethical Committee of our Institution and after patient's informed consent, 111 adult Caucasian
3 patients affected by beta-Thalassemia Major (homozygous or double heterozygous for severe beta mutations) were
4 enrolled in this study (66 females and 45 males, mean age 32.6 ± 6 years (33, range 19-50). All the patients received
5 regular blood transfusions every 20-25 days, maintaining pre-transfusional haemoglobin around 9-9.5 g/L, according to
6 the international guidelines [4]. Chelation therapy included deferoxamine (DFO) (39 pts), deferiprone (DFP) (7 pts),
7 deferasirox (DFX)(46 pts) or combination DFO/DFP (19 pts). The chelation adequacy was estimated based on mean
8 levels every 3 months and LIC by MRI when available. Exclusion criteria were medical conditions and pharmacological
9 treatments interfering with the bone mineral metabolism.

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11 The study design was cross-sectional; a periodic endocrinological evaluation was planned and during the same day bone
12 densitometry, blood and urine biochemical tests and blood hormone evaluations were performed. Data collected
13 included age, gender, BMI, history of auxologic and sexual development; splenectomy, fractures and liver disease,
14 drugs used, transfusion regimen and starting age, mean of pre-transfusional haemoglobin and ferritin in the previous
15 year, liver function and endocrine tests, bone densitometry of femur and lumbar spine.

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17 At the time of the biochemical and densitometric assessment, all hypogonadal patients were on hormonal replacement
18 therapy, and all hypothyroid ones were on replacement with levothyroxine. Patients with hypoparathyroidism were
19 treated with conventional vitamin D and calcium supplements, at a dose sufficient to maintain serum calcium within the
20 normal range or at the lower normal limit.
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Methods

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40 Liver and renal function tests, calcium and phosphorus in serum (n. v. 2.1-2.5 and 0.8-1.4 mmol/L, respectively) and in
41 24-hour urine (n.v. 2.5-7.5 mmol/d and 12.9-42 mmol/d, respectively), intact parathyroid hormone (n.v. 1.5-6.6
42 pmol/L), osteocalcin (n.v.14-42 mcg/L), 25-hydroxyvitamin D (n.v. > 72 nmol/L), ferritin (n.v. 30-400 mcg/L) were
43 determined by automated routine procedures. Blood cell count was performed by Coulter Counter; alkaline phosphatase
44 was measured by IFCC liquid (n.v. 40-129 U/L).
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51 Iron overload was properly estimated by Liver Iron Content (LIC) measured by MRI [5], that showed a wide standard
52 deviation due to the patients' compliance and the different efficacy of therapies..
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54 Bone mineral density measurement was performed by means of dual x-ray photon absorptiometry (DEXA) using
55 Hologic bone densitometer (Hologic QDR-Discovery A, Version 12.7.3.1).
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58 BMD values were expressed as T-score and Z-score. T-score was calculated as SDs from normal reference population
59 database, while Z-score as SDs from age and sex matched population.
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1 Data were classified according to WHO report (WHO Technical Report, ISCD Official Position Paper 2007); T-score >
2 -1: normal. -1 > T-score > -2.5: low bone density (osteopenia). T-score < -2.5: osteoporosis.
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8 **Statistical analysis**
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10 Data are presented as means \pm SD. Intergroup comparison was performed with the Mann-Whitney test. Associations
11 between parameters were determined by Pearson's and Sperman's rank correlation coefficients. To find independent
12 factors affecting bone mineral density in thalassemic patients, a step-wise linear regression analysis was conducted.
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14 Two-sided exact tests were used and *p* values of less than 0.5 were considered significant. All statistics were calculated
15 using the Statistical Package for the Social Sciences 17.0 for Windows software package (SPSS Inc, Chicago, IL).
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Results

The demographic characteristics of the patients studied are presented in Table 1

All the patients were on iron chelating treatment: 35% with deferoxamine (30+5mg/Kg), 42% with deferasirox (20+15mg/Kg), 6% with deferiprone (75mg/Kg) and the remaining 17% with combined therapy (deferoxamine 30mg/Kg + Deferiprone 75mg/Kg).

101 patients (91%) were anti-HCV positive, of whom 50.5% were HCV RNA positive. 68% were splenectomised.

Evaluation of bone mineral metabolism and endocrine function

The biochemical indices of bone metabolism are reported in Table 2. All the parameters resulted within the normal range apart from 25OH vitamin D serum levels, that were suboptimal in 21 patients even if 13 of them were supplemented with 10.000 IU/week.

Nineteen out of 111 patients (17.1%) were affected by hypoparathyroidism and treated with calcium and calcitriol at doses normalizing serum calcium and phosphate concentrations. All the hypoparathyroid patients were also hypogonadic, except one, resulting 16.2% the association of these endocrine diseases.

Hypogonadism (in all cases hypogonadotropic) was the most frequent endocrine complication in our cohort of patients, affecting 87 patients (78.4%); all the hypogonadic patients except 4 (95.4%) were on replacement treatment starting from the age of 18.8 ± 3 years. The hypogonadic patients who were not on replacement, being a minority, were excluded from the analysis of densitometric values.

An impairment of glucose metabolism was present in a high proportion of patients, with diabetes mellitus in 20 (18%) and IGT in 24 of them (22%). IGF-1 serum concentrations were evaluated in 78 patients (70%), and resulted lower than normal in 45 of them (41%).

Bone mass measurements

When considering the bone mass measurements in the overall population enrolled, the prevalence of demineralization (including both osteopenia and osteoporosis) was 92.7% (103 out of 111 Thalassemic patients). Indeed, mineralization was normal only in 18 patients (16.2%) at the vertebral site and in 21 patients (18.9%) at the femoral site.

As shown in Table 3, there were no differences in bone mineralization between the hypogonadic and the eugonadic patients at the vertebral site, while at the femoral site the T-score was lower in the hypogonadic group, despite adequate replacement ($p = 0.047$). Further, only at the femoral site, bone mass was significantly different by gender, as females showed lower mean values of T and Z-scores. There were statistically significant differences in T-score at both sites also for the other dicotomic variables examined (hypoparathyroidism, treatment with calcium, treatment with bisphosphonates), while the statistical significance was not reached in all the analyses using Z-score as dependent variable.

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Figure 1 shows the distribution of bone mineralization state (normal / osteopenia / osteoporosis) according to gender and site. Osteopenia was the prominent finding at the femoral site (63 femoral vs 40 vertebral site, $p<0.001$), while at the vertebral site osteoporosis was more prevalent (53 pts vs 27 pts), the difference was statistically significant ($p<0.001$). The same distribution was confirmed when the patients were separately considered according to gender, being more evident in the male group ($p<0.001$). The patient distributions according to bone mineralization were significantly different by gender only at the femoral site.

Table 4 shows the results of the correlation analysis (correlation indices and statistical significance, $p<0.05$) between bone mineralization (expressed by T and Z scores) and clinical/biochemical parametric variables. BMI was positively correlated with bone mass at femoral site, while advancing age was negatively correlated only with T-score. No correlation was found between vertebral T-score and the same variables. Among the biochemical indices of bone metabolism examined, alkaline phosphatase was the only one significantly correlated both with femoral and vertebral mineralization, being inversely associated with bone mass. Pre-transfusional Hb levels were significantly associated to vertebral Z-score.

The correlations between bone mineralization and the other clinical and biochemical characteristic of the patients (age at first transfusion, age at beginning of chelation, diabetes, previous splenectomy, LIC, serum concentrations of ferritin, parathyroid hormone, bone alkaline phosphatase, osteocalcin, 25 OH vitamin D, IGF-1, and calcium both in serum and urine) were not significant thus were not reported.

The multivariate analysis indicated as possible regressors alkaline phosphatase, BMI and hypoparathyroidism ($p=0.005$, 0.035 and 0.002 for T-score; 0.002, 0.009, 0.003 for Z-score, respectively) at the femoral site, while alkaline phosphatase was the only one found regressor for T and Z scores (respectively $p= 0.008$ and 0.045) at the lumbar spine.

Discussion

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2 Despite the longer life expectancy of thalassaemia major patients due to proper transfusional and chelation regimens
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4 ([1], endocrine complications and bone disease still remain important causes of morbidity and disability[2,3,6-9] . In
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6 this study we examined adult patients with thalassemia major regularly transfused and sufficiently chelated; the patients
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8 were cared for 13 ± 3 years (mean \pm SD) at a tertiary single Italian centre where the Thalassemia International
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10 Guidelines [4] for monitoring and treating Thalassemia and its complications are issued and implemented..

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12 Despite the availability of the best care facilities, bone demineralization was detected in a high proportion of patients,
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14 involving both sites examined though with different severity: osteopenia prevailed at femoral site, while osteoporosis
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16 was more prominent at lumbar spine (prevalently constituted by trabecular bone). Overall, the femoral site seemed to
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18 be more likely than the vertebral site influenced by biochemical and clinical factors. The only exception was pre-
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20 transfusional Hb, showing a positive correlation with vertebral mineralization; this could be explained by the high
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22 degree of ineffective erythropoiesis in the spine that can occurs in thalassemia syndromes. This finding is consistent
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24 with the interesting hypothesis that the chronic request for blood cell production can play a role in the etiology of
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26 osteoporosis, through overstimulation of the hematopoietic system increasing the number of osteoclasts and osteoblasts
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28 resulting in accelerated bone turnover [10].

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30 In our analyses the statistical significance was reached more frequently by T-score, while Z-score seemed to be a less
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32 sensitive parameter. There is a statistical explanation to this behaviour: the relatively small number of cases can render
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34 the statistical power insufficient to detect smaller changes in Z-score, a parameter with high baseline variability.
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36 However, considering the very low prevalence of the disease, this is a sufficiently large cohort, and quite homogeneous
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38 as regards therapeutical approach and clinical management.

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40 The statistically significant negative association between T-score and treatment with calcium and with bisphosphonates
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42 was expected, and compatible with the prescription of such drugs to the patients with more severe demineralization; the
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44 therapeutic effect of these medications could not be examined in our study, as the protocol did not include a
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46 longitudinal evaluation. Other parameters correlated to demineralization were gender (females are more involved), age
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48 and BMI, similarly to healthy individuals.

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50 Even if we cannot address the issue of the prominent cause of bone disease in Thalassemia [11], the correlation
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52 between alkaline phosphatase and bone mineralization indicates a prevalent role of reduced deposition over increased
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54 reabsorption, as reported by other Authors [12], at variance with others [13, 14] . We could not confirm the same
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56 relationship with osteocalcin, another marker of osteoblastic activity [12, 15].

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58 As far as the endocrine complications relevant to bone status are concerned, according to previous reports [16] in our
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60 study the hypogonadic patients of both genders were more affected from demineralization than the eugonadic, and,
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1 interestingly, the standard hormone replacement therapy (HRT) does not normalize bone condition. A possible
2 explanation may be the concurrence of factors interfering with mineralization, such as vitamin D deficiency, impairing
3 the therapeutic effects of sex steroid hormones and also of bisphosphonates. A recent report [17] stated that an optimal
4 vitamin D repletion is an essential pre-requisite for maximal efficacy of the anti-resorptive drugs in post-menopausal
5 osteoporosis; a similar situation could be hypothesized in thalassemic population. In effect, low serum vitamin D levels
6 have been reported in thalassemic populations; this was ascribed to impaired liver hydroxylation and intestinal
7 absorption, with a causative role of iron overload suggested by a negative correlation between serum 25-OH-vitamin D
8 and ferritin [18]. This correlation was not confirmed in our study ($p = 0.546$). Unexpectedly, in our series the serum
9 vitamin D was often low despite ongoing treatment with metabolites, compatible with a defect in absorption or
10 metabolism [18] but also with low compliance to the therapy; the latter is quite common using associations with
11 calcium salts, badly tolerated. Anyway, the contribution of vitamin D to bone status is probably more complex than
12 presently recognized, and neither vitamin D concentrations nor ongoing treatment did correlate with mineralization
13 status in our and earlier reports [19]. Conversely, impairment of parathyroid function was inversely related to
14 mineralization status, indicating a protective effect on bone of this endocrine complication [19, 20]. In our study the
15 relationship was more evident at the femoral level than at lumbar spine; this was unexpected, as chronic PTH-
16 deprivation should influence more strongly the sites where trabecular content is maximum. Probably, in this peculiar
17 population the superimposed pathologic mechanisms concurring to bone disease can produce net effects difficult to
18 predict in the long-term.

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36 Finally, an important consideration emerges from our statistical analysis: the model utilized could account for the
37 29.4% of T-score variance at the femur and the 32.03% at the spine. This indicates that the variables significant in the
38 univariate analysis could not explain satisfactorily the mineralization defect. Again, this may be ascribed to insufficient
39 statistical power, linked to the restricted number of cases in a monocentric study, or alternatively to the influence of
40 pathogenetically involved factors beyond those included in our analysis. The indices studied reflect bone marrow
41 expansion, iron overload, endocrine function and bone metabolism, but further physiological, pathological, genetic and
42 iatrogenic factors can work in this multifactorial disease.

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50 Furthermore, early preventive intervention and management are needed to influence bone health in adulthood, as
51 demonstrated by normal Z-scores found in children and young adolescents with Thalassemia Major by Christoforidis et
52 al [21]. Our patients are probably comparable for the quality of clinical management; however the different results
53 emerged from our study suggest that transfusion and chelation treatment can prevent bone demineralization only when
54 applied early from childhood.

A deeper learning of the molecular mechanisms causing demineralization, as well as the optimal prevention and treatment of thalassemia from infancy, could perhaps allow to avoid complications and further ameliorate the quality of life in these patients.

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Fig 1 Bone mineralization state (normal / osteopenia / osteoporosis) of the Thalassemic patients as distributed on the basis of site and gender, according to the WHO osteoporosis classification. Osteopenia was the prominent finding at the femur, while at lumbar spine osteoporosis was more prevalent ($p < 0.001$). The same distribution was confirmed when the patients were separately considered according to gender, being more evident in the male group

Table 1 Clinical characteristics of the patients by gender at the time when mineralization was studied

Table 2 Biochemical and hormonal indices related to bone metabolism in 111 patients affected by Thalassemia Major

Table 3 Statistic results (mean \pm standard deviation and p value) of comparisons between clinical/biochemical dicotomic variables and T or Z- scores at femoral and vertebral sites

Table 4 Correlation analysis (Pearson's correlation indices and two-tailed p values) between bone mineralization (expressed by T and Z-scores) and clinical/biochemical parametric variables

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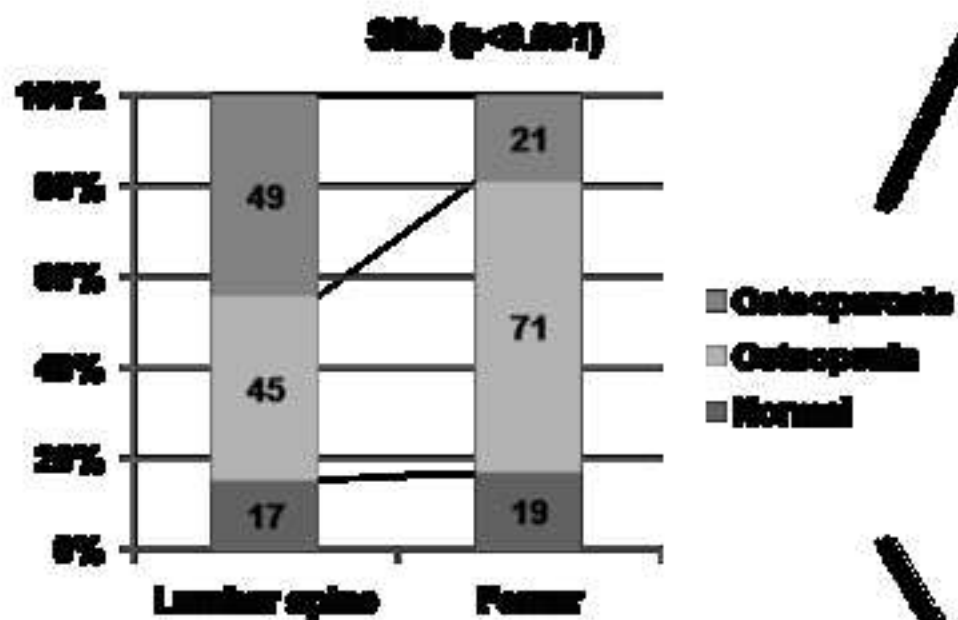
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