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ORIGINAL ARTICLE

Bone turnover and mineral density in adult thalassemic patients: relationships with growth hormone secretory status and circulating somatomedins

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Abstract Previous evidence supports a role for growth hormone (GH)–insulin-like growth factor (IGF)-I deficiency in the pathophysiology of osteopenia/osteoporosis in adult thalassemia. Moreover, serum IGF-II has never been studied in this clinical condition. Thus, we elected to study the GH secretory status and the levels of circulating somatomedins, correlating these parameters with bone mineral density (BMD) and biochemical markers of bone turnover. A hundred and thirty-nine normal weight adult thalassemic patients (72 men and 67 women) were studied. Lumbar and femoral neck BMD were measured in 106/139 patients. Sixty-eight patients underwent growth hormone releasing hormone plus arginine testing. Measurement of baseline IGF-I and IGF-II was performed in all patients, while osteocalcin, C-terminal telopeptide of type I collagen (CTx), and urinary cross-linked *N*-telopeptides of type I collagen (NTx) were assayed in 95 of them. Femoral and lumbar osteoporosis/*Z* score below the expected range for age were documented in 61.3 and in 56.6 % of patients, respectively. Severe GH deficiency (GHD) was demonstrated in 27.9 % of cases, whereas IGF-I SDS was low in 86.3 %. No thalassemic patients displayed circulating levels of IGF-II below the reference range. GH peaks were positively correlated with femoral, but not lumbar, *Z* score. No correlations were found between GH peaks and osteocalcin, CTx and NTx. GH peaks were positively correlated with IGF-I values, which in their turn displayed a positive correlation with osteocalcin, CTx, and NTx. No correlations emerged between IGF-I values and either femoral or lumbar *Z* scores. No correlations were found between IGF-II and any of the following parameters: GH peaks, osteocalcin, CTx, NTx, femoral *Z* score, and lumbar *Z* score. Our study, besides providing for the first time evidence of a normal IGF-II production in thalassemia, contributes to a better understanding of the involvement of the somatotropin-somatomedin axis in the pathophysiology of bone demineralization in this disease. In particular, the contribution of GHD to femoral osteoporosis appears to be likely mediated by locally produced rather than circulating IGF-I.

Keywords Growth hormone · IGF-I · IGF-II · Thalassemia

This paper is dedicated to the memory of Professor Francesco Minuto.

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Introduction

Among the systemic comorbidities affecting adult thalassemic patients, bone demineralization is extremely frequent [1]. Genetic, hematological, iatrogenic, and endocrine factors are thought to contribute to this complication [2–5]. Previous data from our group suggested a role for growth hormone (GH)–insulin-like growth factor (IGF)-I deficiency in the pathophysiology of osteopenia/osteoporosis in thalassemia, in particular at the femoral level [1].

In vitro, both IGF-I and IGF-II are known to stimulate human osteoblast proliferation, differentiation, and collagen synthesis via IGF-I receptor [6]. Recent in vivo findings point to IGF-II decrease as a major contributor to the reduced calcaneal bone mineral density (BMD) observed in elderly women [7]. Serum levels of IGF-II have never been determined in patients with β-thalassemia.

Based on these considerations, we elected to further investigate, in a large series of thalassemic adults, the GH secretory status and the levels of circulating somatomedins, correlating these parameters with BMD and biochemical markers of bone turnover.

Patients and methods

Patients

A total of 139 adult thalassemic patients (72 men and 67 women), aged 32.3 ± 7.87 years (range 28–58), were studied. Mean height of the patients was 159.2 ± 10.21 cm, their mean height SDS was −1.06 ± 1.55, and mean body mass index (BMI) was 21.3 ± 3.04 kg/m^2. One hundred and thirty-two of them were affected by β-thalassemia major and 7 by β-thalassemia intermedia. All Cooley's patients were on stable transfusion regimen. At the time of the study, hemoglobin levels were >7.5 g/dl. All subjects, with the exception of the 7 patients with thalassemia intermedia, were receiving adequate iron chelation, i.e., infusions of desferrioxamine (Desferal, Novartis, Origgio, Italy) 30–50 mg/kg body weight 5 days a week or oral deferasirox (Exjade, Novartis, Origgio, Italy) 20–30 mg/kg body weight daily. Fifty-six of them had undergone splenectomy in childhood. None of the patients had been investigated for GH secretion and had received GH replacement therapy during childhood.

Twenty-four male and thirty-eight female subjects were affected by hypogonadotropic hypogonadism and were on sex steroid replacement therapy [testosterone enanthate (Testo Enant, Geymonat SpA, Anagni, Italy) at the mean dose of 250 mg i.m. monthly in men and standard estrogen-progestogen association in women]. Primary hypothyroidism was present in 4 patients, who were receiving 50–175 μg daily levothyroxine (Eutirox, Bracco SpA, Milan, Italy). None of the patients had been diagnosed with adrenal insufficiency. Sixteen patients had impaired glucose tolerance and were following a proper diet regimen. Thirty-eight patients were affected by hepatitis C virus-related chronic liver disease: 26 of them had been previously treated with interferon while, at the moment of the study, no patient was on such treatment; all patients displayed normal or slightly elevated transaminase values and none of them had severe liver disease. Finally, 10 patients had experienced clinical fractures but none of the subjects, at the moment of the evaluation, was receiving bisphosphonate therapy or vitamin D-calcium supplementation; unfortunately no data on morphometric fractures were available for this series.

All patients gave their informed consent to participate in the study, which was approved by the Ethics Committee of our Institution.

Measurement of BMD

BMD was assessed by dual energy X-ray absorptiometry (DEXA) using a Hologic QDR 2000 Plus densitometer (Hologic Italia srl, Rome, Italy). Both lumbar (L1–L4) and femoral neck BMD were measured in 106 (55 men and 51 women) out of 139 patients. In agreement with the 2015 official position of the International Society for Clinical Densitometry, in patients aged 50 years or older (only 5 out the 106 undergone DEXA) osteoporosis was defined by a *T* score lower than −2.5, while in the vast majority of the patients, the so-called condition of BMD below the expected range for age was defined by a *Z* score lower than −2.0.

Test procedures

In a subset of 68 patients (36 men and 32 women), GH was measured before and after stimulation with growth hormone releasing hormone (GHRH) plus arginine. An indwelling catheter was placed into a forearm vein between 7.00 and 8.00 AM and kept patent by saline infusion. Two baseline samples were collected at least 15-min apart prior to the injection of 1 μg/kg body weight GHRH (Ferring SpA, Milan, Italy) as an i.v. bolus and the infusion of 0.5 g/kg body weight (maximal dose 30 g) arginine (S.A.L.F., Turin, Italy) over 30 min through a separate i.v. access. Blood samples were collected at 15, 30, 45, 60, and 90 min after the bolus and the beginning of the infusion and centrifuged at 1000×*g* for 10 min at 4 °C: serum was then collected by aspiration and stored at −20 °C until assayed for GH. IGF-I, IGF-II, osteocalcin, and C-terminal telopeptide of type I collagen (CTx) values were measured on blood samples collected at baseline. Cross-linked *N*-telopeptides of type I collagen (NTx) were assayed on 24-h

urinary collections, and values were corrected for urinary creatinine. In particular, values of both somatomedins were measured in 139 patients, while biochemical markers of bone turnover were available from 95 subjects (49 men and 46 women).

Biochemical assays

GH serum concentrations were measured by chemiluminescent assay (CLIA, Nichols Institute Diagnostics, San Juan Capistrano, CA, USA). Intra-assay coefficients of variation for mean GH levels of 0.8, 5.9, and 17.1 μg/l were 2.8, 3.7 and 5.4 %, respectively. Inter-assay coefficients of variation for mean GH levels of 0.1, 6.2 and 16.1 μg/l were 7.5, 6.2 and 8.7 %, respectively. The sensitivity of the assay is 0.02 μg/l. The assay was calibrated against the IS 98/574 recombinant standard. Severe GH deficiency (GHD) was defined by a GH peak lower than 9 μg/l, while partial GHD was diagnosed with a peak ranging from 9 to 16.5 μg/l; GH peaks greater than 16.5 μg/l were considered normal.

IGF-I serum concentrations were assayed by CLIA (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA) after IGF-I separation from insulin-like growth factor binding proteins (IGFBPs) by sample acidification. Intra-assay coefficients of variation for mean IGF-I levels of 63, 208, and 766 μg/l were 4.8, 5.2, and 4.4 %, respectively. Inter-assay coefficients of variation for mean IGF-I levels of 62, 215, and 811 μg/l were 7.1, 5.7, and 7.4 %, respectively. Sensitivity is 6 μg/l. Data are expressed as IGF-I standard deviation score (SDS) values, calculated according to Brabant et al. [8]. IGF-I SDS values lower than −1.88 (3rd percentile) were considered abnormally low.

Serum IGF-II was determined by radioimmunoassay (RIA, Mediagnost GmbH, Reutlingen, Germany) using specific polyclonal antibodies with high affinity. Intra- and inter-assay coefficients of variation were 5.4 and 7.9 %, respectively. Cross-reactivity with IGF-I is below 0.05 % and sensitivity is 0.1 μg/l. Values between 360 and 880 μg/l (3rd and 97th percentile in a healthy population aged 20–40 years, respectively) were considered normal.

Serum osteocalcin was measured by electrochemiluminescence immunoassay (ECLIA, Roche Diagnostics GmbH, Mannheim, Germany). The sensitivity of the assay is 0.5 μg/l, while intra- and inter-assay coefficients of variation are 3 and 4 %, respectively. Reference range is 12.0–52.1 μg/l for men and 6.5–42.3 μ/l for women.

Serum CTx was measured on the Elecsys 2010 automated analyzer (Roche Diagnostics GmbH, Mannheim, Germany) using the β-Crosslaps/serum reagents. This assay is specific for cross-linked β-isomerized type I collagen C-telopeptide fragments and uses two monoclonal antibodies [9]. The sensitivity of the assay is 70 ng/l. The upper limit of normal range is 580 ng/l for men and 540 ng/l for premenopausal women.

Urinary NTx was determined by enzyme-linked immunosorbent assay (ELISA, Wampole Laboratories, Princeton, NJ, USA). The lower limit of detection is 20 nmol BCE, while intra- and inter-assay coefficients of variation are 5 and 5 %, respectively. Reference range for men and premenopausal women is 5–65 nmol BCE/mmol creatinine.

Serum ferritin was measured by ECLIA (Roche Diagnostics). Intra- and inter-assay coefficients of variation were 1.8 and 2.9 %, respectively. The sensitivity of the assay is 0.5 μg/l. Reference range is 30–400 μg/l in males and 13–150 μg/l in females.

Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). Normal distribution of data was assessed by the Kolmogorov–Smirnov test. Student's *t* test for unpaired data was used for comparison between groups. Chi-squared test was used for comparison of quantitative variables. Linear regression analysis was used to evaluate associations between variables. Statistical analyses were performed with a commercially available software package (STATVIEW, Abacus Concepts, Berkeley, CA, USA). A p value lower than 0.05 was considered statistically significant.

Results

Short stature, classically defined as a height SDS below −1.88 (third percentile), was present in 48 out of the 139 (34.5 %) patients studied and, in particular, in 35 out of the 106 (33 %) patients who underwent BMD measurement.

Femoral osteoporosis/*Z* score below the expected range for age was documented in 65 of the 106 (61.3 %) patients undergone hip DEXA, while lumbar osteoporosis/*Z* score below the expected range for age was demonstrated in 60 of the 106 (56.6 %) patients submitted to L1–L4 DEXA. In particular, the prevalence of femoral osteoporosis/*Z* score below the expected range was comparable in men (34/55, i.e., 61.8 %) and women (31/51, i.e., 60.7 %), and the same held true for lumbar osteoporosis/*Z* score below the expected range (33/55, i.e., 60 %, in men and 27/51, i.e., 52.9 %, in women).

Height SDS was not correlated with lumbar *Z* score; the correlation between height SDS and femoral *Z* score approached statistical significance ($p = 0.07$), without attaining it. Mean femoral *Z* score was not significantly different between short subjects and the remaining patients (−2.41 ± 0.79 vs.

−1.9 ± 1.55, NS, respectively), and the same held true for lumbar *Z* score (−2.31 ± 0.97 vs. −2.0 ± 1.25, NS, respectively). Likewise, the prevalence of osteoporosis/*Z* score below the expected range for age at both sites was comparable in short subjects and the remaining patients (23/35, i.e., 65.7 %, vs. 42/71, i.e., 59.1 %, respectively, at femoral level and 19/35, i.e., 54.2 %, vs. 41/71, i.e., 57.7 %, respectively, at lumbar level).

The mean GH peak in response to GHRH plus arginine was 18.0 ± 11.98 μg/l. According to the established criteria for the interpretation of this test [10], 19 of 68 (27.9 %) adult thalassemic patients displayed severe GHD. In particular, the prevalence of GHD was similar in male (10/36, i.e., 27.7 %) and female (9/32, i.e., 28.1 %) patients. IGF-I SDS was below −1.88 in 120 out of 139 (86.3 %) patients: the prevalence of IGF-I deficiency was significantly greater in women than in men (63/67, i.e., 94.0 %, vs. 57/72, i.e., 79.1 %, $p < 0.05$, respectively). Serum concentrations of IGF-II ranged in our series from 665 to 2247 μg/l, with a mean value of 893.7 ± 254.01 μg/l: in particular, none of the 139 patients in whom IGF-II was measured displayed circulating levels of the growth factor below the reference range, while the same parameter was above the upper limit of the range in 16 out of 139 (11.5 %) subjects.

Mean serum osteocalcin was 18.8 ± 16.75 μg/l in male patients and 13.8 ± 12.75 μg/l in female patients, exceeding the upper limit of normal range only in 4 % (2/49) of men and 2.1 % (1/46) of women. Mean serum CTx was 767.5 ± 434.95 ng/l in male patients and 610.3 ± 566.02 ng/l in female patients, exceeding the upper limit of normal range in 59.1 % (29/49) of men and 45.6 % (21/46) of women. Mean urinary NTx was 76.9 ± 61.65 nmol BCE/mmol creatinine in the whole group of patients, exceeding the upper limit of normal range in 49.4 % (47/95) of them.

GH peaks were positively correlated with femoral neck ($r = 0.273$, $p < 0.05$), but not lumbar, *Z* score (Fig. 1). No correlations were found between GH peaks on the one hand and osteocalcin, CTx and NTx on the other hand. On the contrary, GH peaks were positively correlated with IGF-I values ($r = 0.31$, $p < 0.01$), which in their turn displayed a positive correlation with osteocalcin ($r = 0.58$, $p < 0.0001$), CTx ($r = 0.56$, $p < 0.0001$), and NTx ($r = 0.54$, $p < 0.0001$) as well (Fig. 2), and a negative correlation with the age of patients ($r = -0.53$, $p < 0.0001$). No correlations emerged between IGF-I values and either femoral or lumbar *Z* scores. Likewise, no correlations were found between IGF-II and any of the following parameters: GH peaks, osteocalcin, CTx, NTx, femoral *Z* score, and lumbar *Z* score. Like IGF-I, IGF-II was negatively correlated with age ($r = -0.19$, $p < 0.05$).

Interestingly, no correlations emerged between height SDS or BMI values and any of the following parametres: GH peaks, IGF-I SDS, and IGF-II.

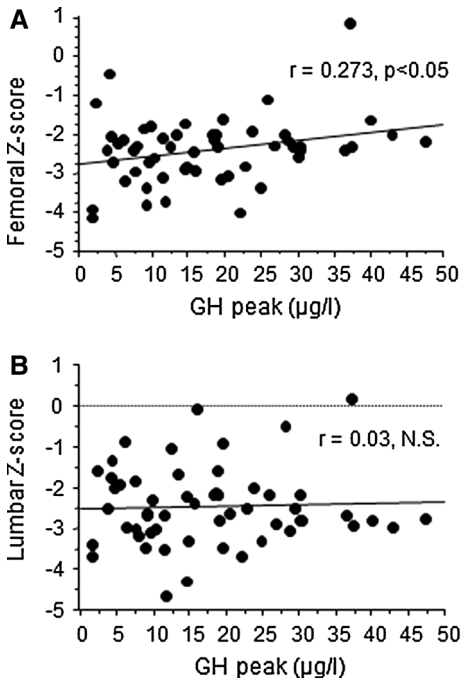

Fig. 1 Correlations between stimulated GH secretion and femoral (**a**) and lumbar (**b**) *Z* scores in adult thalassemic patients

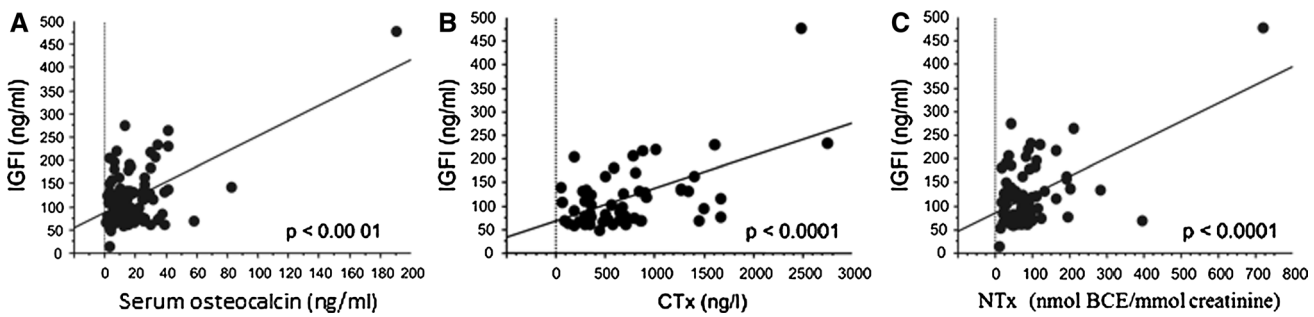

Fig. 2 Correlations between IGF-I values and serum osteocalcin (**a**), serum CTx (**b**) and urinary NTx (**c**) in adult thalassemic patients

Finally, mean serum ferritin was 1686.7 ± 462.73 μg/l, and no correlations were found between ferritin levels and *Z* score values at both skeletal sites.

The same pattern of correlations described in the whole group of patients was also evident in the subset of "osteoporotic" subjects.

Discussion

The present study provides further insights into the relationships between GH and somatomedin axis and bone mineralization and turnover in adult thalassemic patients. The merging of original data with previous knowledge has allowed us to propose new hypotheses for the interpretation of this aspect of the complex pathophysiology of bone disease in this clinical condition.

Our data confirm the high prevalence of both GHD and bone demineralization in adults with thalassemia, but both these epidemiological findings are matters of scientific debate. By performing a single-challenge test, i.e., GHRH + arginine, we have repeatedly reported a prevalence of severe GHD ranging from one-fifth to one-fourth in our series of thalassemic patients [1, 11], and this figure is confirmed in the current group of subjects. The literature on this topic, however, is quite discordant with papers describing both higher and lower prevalence. De Sanctis et al. [12] found severe GHD in up to 55 % of their patients after glucagon stimulation. On the contrary, with the use of two provocative tests, Pincelli et al. [13] could confirm the presence of severe GHD in only 8 % of patients. The figure of 55 % of the former study is very likely overestimated, since the period of observation after glucagon injection, a test known to elicit physiologically delayed GH responses, was too short. On the other hand, the possibility that in this clinical condition the prevalence of GHD is lower than generally appreciated with the use of a single test is worth investigating, with the perspective of avoiding inappropriate GH replacement.

The lack of correlations between height SDS values and GH peaks/IGF-I SDS values is not surprising when considering the role commonly assigned to factors other than or additional to somatotropin-somatomedin deficiency, such as gonadotropin deficiency, hypoxia, bone marrow expansion, and nutritional deficits, in the pathophysiology of growth retardation in these patients [11]. Likewise, the lack of correlation between BMI values and GH peaks is not unexpected in our subjects, given the widely accepted hypothesis that factors other than anthropometric parameters, such as iron overload in the early phase of life or cell damage deriving from hypoxia, contribute to GHD in thalassemia [11].

The real prevalence of osteoporosis in this clinical condition is also debated, because different techniques for measuring BMD yield discordant results. In particular, values measured by traditional DEXA resulted lower when compared with those determined by quantitative-computed tomography (QCT) in these patients. Therefore, the prevalence of bone demineralization in thalassemia appears to be lower in studies performed with QCT. However, the reliability of this technique in this clinical condition has recently been challenged by a study demonstrating higher BMD values, measured by QCT, in patients than in controls [14].

Stature may influence BMD measurement: indeed, a 10-cm difference in height has been estimated to correspond to a difference in *T* score of about 0.35 at the femoral neck and 0.40 at the spine, and this may lead to an overdiagnosis of osteoporosis in small subjects. However, studies performed in both pediatric and adult populations point to low height as a predictor of low BMD [15, 16]. Our findings, not supporting a major role of short stature as a determinant of bone demineralization in thalassemia, underline the peculiarity of this complication. Indeed, several factors are thought to contribute to bone disease in this clinical condition, including genetic susceptibility, partial persistence of ineffective erythropoiesis, negative effects of iron overload and/or chelation therapy on osteoblast function [2–5]. The iron chelators desferrioxamine, deferiprone, and deferasirox have also been shown to decrease both tartrate-resistant acid phosphatase and cathepsin *K* expression, as well as osteoclast activity [17]. In vivo, in the experience of Wong et al. [18], the decline in femoral neck *Z* score was accelerated after transition from desferrioxamine to deferasirox in a large group of young thalassemic patients. As regards endocrine factors, according to our previous experience [1], both delayed replacement of hypogonadism and somatotropin-somatomedin deficiency may play a role. Indeed, in non-thalassemic untreated GH deficient adults an increased fracture rate is well established [19, 20].

To the best of our knowledge, the present study is the first to address the issue of circulating IGF-II in thalassemia. In contrast with serum IGF-I, which was abnormally low in the vast majority of our patients, IGF-II was normal in most of them, even exceeding the upper limit of reference range in about one tenth of patients. Furthermore, given the lack of correlation between GH peaks and IGF-II values, the production of this growth factor appears to be largely independent of GH secretion in thalassemia, as well as reported in the normal population, where physical activity and mechanical loading seem to be the chief regulators. On the contrary, the finding of a positive correlation between GH peaks and IGF-I levels confirms that in thalassemia the GH secretory status retains its influence on IGF-I production. According to our previous experience [11], another relevant factor influencing IGF-I production

in thalassemia is represented by reduced liver protidosynthetic activity.

IGF-II, traditionally considered as a growth factor involved in growth of fetal tissues and cancer cells, is now emerging as a potential regulator of metabolism and body composition. Indeed, this growth factor has been shown to stimulate glucose uptake and to exert antilipolytic activity in cell cultures [21], as well as to stimulate preadipocyte proliferation, skeletal myoblast differentiation, and myofiber hypertrophy [22–24]. Furthermore, both IGFs promote osteoblast proliferation, differentiation, and function [6] and, when administered to humans and rodents, they increase biochemical markers of osteoblast function [25, 26]. Most studies carried out in pre- and post-menopausal women of different ethnic groups have failed to demonstrate an association between circulating IGF-II and BMD [27–30], but such a correlation has been recently described in women after adjusting for age [7, 31] and in men [32]. In particular, in the experience of Sittadjody et al. [7], circulating IGF-II was positively correlated with calcaneal BMD and serum osteocalcin in post-menopausal Indian women, suggesting an influence of this growth factor on bone density also in female gender.

As regards thalassemia, we failed to demonstrate a correlation between serum IGF-II and femoral or lumbar *Z* score in our current series. Furthermore, at variance with what previously reported by ourselves in a smaller group of patients [1], we also failed to find an association between serum IGF-I and BMD. This lack of correlation militates against a major role of circulating IGFs in the pathophysiology of bone demineralization in thalassemia. On the contrary, given the positive correlations between serum IGF-I and markers of bone resorption and formation, the ability of the growth factor to promote bone turnover seems to be preserved in these patients.

The positive correlation between GH stimulated release and femoral, but not lumbar, *Z* score confirms our previous observations [1] and points to GH secretory status as a determinant of bone health in this clinical condition: the discrepant finding at the two skeletal sites might be accounted for by the well established greater negative influence of GHD on cortical than on trabecular bone [33].

In conclusion, our study contributes to a better understanding of the involvement of the GH-somatomedin axis in the pathophysiology of osteoporosis in this disease. In particular, the contribution of somatotropin deficiency to femoral demineralization is confirmed; however, this effect appears to be likely mediated by locally produced rather than circulating IGF-I. Along this line of interpretation, a reduced bone content of IGF-I had been described in the past by other investigators [34] in non-thalassemic post-menopausal women. If, on the one hand, our data allow new hypotheses for the interpretation of the puzzling issue of bone disease in thalassemia, on the other hand, the discrepancy between the rate of BMD lower than expected and the frequency of clinical fractures remains to be elucidated. Furthermore, our findings encourage studies on the prevalence of morphometric fractures in these patients.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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