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ORIGINAL ARTICLE

A 2-year prospective densitometric study on the influence of Fok-I gene polymorphism in young patients with thalassaemia major

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Abstract
Summary This study is to estimate the degree of genetic contribution of Fok-I gene polymorphism of Vitamin D receptor to bone mass in patients with thalassaemia. Results indicate a protective role of the f allele of the Fok-I gene polymorphism when found in homozygosity on bone mineral density of young thalassemic patients.
Introduction The purpose of this study is to estimate prospectively the degree of genetic contribution of Fok-I gene polymorphism of vitamin D receptor (VDR) to the evolution of bone mass in patients with beta-thalassemia major (b-TH).
Methods Sixty-four children and young adults (33 males and 31 females) with mean decimal age of 23.20±5.41 (range 9.25–32.41 years) were recruited in this study. All patients were genotyping for Fok-I gene polymorphism and were assessed with dual energy X-ray absorptiometry (DXA) at baseline and 2 years after. Z-scores were calculated based on normal age and sex matched Caucasian population. Metabolites of vitamin D, intact PTH, total calcium, inorganic phosphorous, and alkaline phosphatase were measured at the serum pre-transfusion.
Results A moderate proportion of patients had decreased DXA Z-scores (Z-score ≤−2) predominately in total hip (31 %) and secondary in lumbar spine (15.6 %). Patients being homozygous for the f allele had apparently higher BMD Z-scores compared with those carrying the F allele in homo- or heterozygosity, however, with a difference that did not reached significance. Interestingly enough, a significant deterioration in BMD Z-scores measured at femur (FF: P=0.004 Ff: P<0.001, ff: P=0.024) and total hip (FF: P=0.022, Ff: P=0.005) was recorded for all type of genotypes, except for ff genotype and with regard to the total hip DXA values. An increased prevalence of serum 25(OH)D_3 deficiency (59.4 %) and 25(OH)D_3 borderline (12.5 %) was recorded.
Conclusion Our study indicates a protective role of the f allele of the Fok-I gene polymorphism when found in homozygosity on bone mineral density of young patients with b-TM.

Keywords Dual energy X-ray absorptiometry · Fok-I gene polymorphism · Thalassaemia · Vitamin D · Vitamin D receptor

Introduction

Beta-thalassemia major (b-TM) represents the most frequent inherited hemoglobinopathy in our country, although the adoption of prenatal diagnostic techniques has drastically reduced the incidence of new cases [1]. Relatively, recent advances in the conventional management of b-TM have significantly increase life expectancy and lead to the emergence of additional conditions that are associated with aging, such as osteoporosis [2]. Several factors such as ineffective erythropoiesis with bone marrow expansion, iron overload, desferioxamine toxicity, endocrinopathies, vitamin deficiency, and inadequate physical activity have been implicated in the pathogenesis of impaired bone metabolism [3].

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As genetic influence accounts for a significant percentage of variation in bone mass, genetic factors are implicated as well in the pathogenesis of low bone mass in patients with b-TM [4]. Vitamin D is strongly related to the development and maintenance of bone mass, and most of its biological actions are mediated by a high-affinity intracellular receptor (vitamin D receptor (VDR)) that acts as a nuclear transcription factor, regulating the synthesis of proteins involved in bone mineral homeostasis and cell proliferation [5]. The VDR gene is located on chromosome 12 (q12–q14); several single-nucleotide polymorphisms (SNP) that could potentially modify the expression and activation of VDR have been identified. One of the most frequently studied VDR polymorphism is Fok-I, located near the 5′ end coding region of the gene [6]. The Fok-I polymorphism is represented by two allelic variants of the VDR gene; the f allele leads to the production of a 3 amino-acid longer and less functional VDR protein, compared to the product of the F allele.

We sought to estimate the degree of genetic contribution of Fok-I gene polymorphism of VDR to the evolution of bone mass in children and young adults with b-TM assessed with imaging techniques and biochemical parameters of bone metabolism over a period of 2 years.

Materials and methods

Sixty-four children and young adults (33 males and 31 females) with mean decimal age of 23.20±5.41 (range 9.25–32.41 years) were recruited in this study. All patients were treated conventionally with regular blood transfusions in order to maintain pre-transfusional Hb levels above 9 g/dl and adequate iron-chelation therapy with deferiprone, deferasirox, or combined therapy with deferiprone and desferrioxamine. None of them suffered from other disease entities or was receiving any medication known to affect vitamin D status and bone metabolism. The protocol was designed in accordance with the Helsinki Declaration and was approved by the Institutional Review Board and Ethics Committee. Written informed consent was collected from parents or legal guardians of each participant.

All patients were genotyping for Fok-I gene polymorphism through a procedure of DNA extraction from peripheral blood leukocytes with a DNA extraction kit (Promega, Madison, WI, USA). Primers used for genotyping the Fok-I polymorphism were as follows: forward, GATGCCAGCTGGCCCTGGCACTG; reverse, ATGGAAACACCTTGCTTCTTCTCCCTC. PCR conditions included an initial denaturing step at 94 °C for 5 min, followed by 25 cycles of 94 °C for 30 s, 60–50 °C touch-down (−0.5 °C/cycle) for 30 s, 72 °C for 45 s, and a final extension step at 72 °C for 10 min. The amplified product was digested with the Fok-I restriction enzyme (New England Bilolabs, Beverly, MA, USA) producing fragments of 272 bp for the F allele and 198 and 74 bp for the f allele. Digestion products were resolved on a 2 % agarose gel stained with ethidium bromide and visualized with ultraviolet light.

Bone mineral density was assessed by dual energy X-ray absorptiometry (DXA) technique using Cronos bone densitometer (DMS, Montpelier, France) in the following sites: lumbar spine vertebrae (L_2–L_4), femoral neck, and total hip. The mean value was expressed as grams per centimeter square (g/cm^2). Z-score were calculated based on BMD measurements of normal age- and sex-matched Caucasian population [7]. As abnormal DXA scan measurements were considered those with BMD Z-score values below <−2. DXA values were measured twice, at the beginning of the study and 2 years after. Anthropometric values were also measured using standard techniques and commercial devices. Values were expressed as Z-scores compared to normal Greek sex- and age-matched population [8, 9].

Metabolites of vitamin D, intact PTH, total calcium, inorganic phosphorous, and alkaline phosphatase were measured at the serum collected prior to a scheduled transfusion. The concentrations of $25(OH)D_3$ and $1,25(OH)_2D_3$ in the serum were quantified using commercial radio-immuno assays (DRG Instruments GmbH, Germany). Intra-assay coefficients of variance (CVs) were 3.3 and 9.3 %, whereas inter-assay CVs were 5.2 and 12.7 % for $25(OH)D_3$ and $1,25(OH)_2D_3$, respectively. Ferritin levels were measured every trimester, and a mean value of four consecutive measurements was calculated at the beginning and at the end of the study.

The statistical package for the social sciences (SPSS) for Windows version 20 and Microsoft® Excel® for Mac 2011 version 14.0 were used for data analysis and graphical demonstration. Deviation from Hardy-Weinberg equilibrium [10] and comparison of percentages was tested with the chi-square test. Results are expressed as mean±SD. The Kolmogorov-Smirnov and the Shapiro-Wilk tests were employed to identify parameters with skewed distribution. Comparison of means between groups was performed with the use of independent samples student *t* test or Mann-Whitney *U* test for parametric and non-parametric data, respectively, whereas comparison of the change of means within the same group during the study were tested with paired student *t* test or one-sample Wilcoxon signed rank test for parametric and non-parametric data, respectively. Comparison of the means for more than two groups was performed with analysis of variance (ANOVA) followed by Bonferroni's post-hoc comparisons and Kruskal-Wallis tests for parameters with normal or skewed distribution, respectively. Bivariate correlations were tested using Pearson's or Spearman's correlation coefficient for normally or skewed distributed parameters, respectively. A P value<0.05 was considered statistically significant.

Results

Anthropometric characteristics and number of abnormal DXA scans both at the beginning of the study and 2 years after, categorized by gender and in total, are presented in Table 1. With the exception of a significant increase in BMI Z-scores during the 2-year follow-up in the totality of the patients (−0.16±0.75 versus −0.06±0.76, P=0.013), mainly attributed to male patients (−0.28±0.66 versus −0.14±0.68, P=0.017), no other statistically significant difference was observed in any anthropometric parameter when genders were compared or in the evolution of time.

Despite their young age, a significant percentage of patients showed abnormal DXA measurements at lumbar spine and total hip, both at the beginning and the completion of the study (Table 1). Femoral neck was a site with no abnormal DXA value observed at the beginning of the study, however, with a significantly increase percentage during the study in total patients (0 versus 17.2 %, P=0.001), something which is mainly attributed to females (0 versus 29 %, P=0.002). In general, females had greater percentages of pathological DXA values in all sites and in every instance but without reaching statistical significance compared to males with the exception of DXA values measured at femoral neck and at the completion of the study (29 versus 6.1 %, P=0.015).

When DXA values were quantified as BMD Z-scores, lumbar spine showed low values in general, however with no statistically significant difference throughout the study period or between genders (Table 2). On the contrary, a remarkable deterioration was recorded in femoral BMD both in males (−0.13±1.05 versus 0.50±1.17, P<0.001) and in females (−0.85±1.70 compared to 0.26±1.59, P <0.001) and subsequently in total (−0.47±1.48 versus 0.39±1.38, P<0.001) (Table 2, Fig. 1). At the end of the study, mean BMD Z-scores measured at femoral neck were statistically significantly lower in females compared to males (−0.85±1.70 versus −0.13±1.05, P=0.044) (Fig. 1). Finally, BMD of total hip recorded low values during the study and with significant deterioration in both males (−1.44±1.10 versus −1.18±1.28, P=0.029) and females (−1.68±1.54 versus −1.32±1.60, P=0, 005) and of course in totality (−1.60±1.33 versus −1.24± 1.42, P<0.001).

An increased prevalence of serum $25(OH)D_3$ deficiency (<20 ng/ml) (59.4 %) and $25(OH)D_3$ borderline (20–30 ng/ml) (12.5 %) was recorded. When stratified according to $25(OH)D_3$ levels, no statistically significant difference in the evolution of DXA parameters was observed between groups (Table 3). Regarding DXA Z-scores measured at total hip, a significant deterioration was recorded in all groups, independently of the status of $25(OH)D_3$, whereas for femoral neck, BMD Z-scores were significantly decreased in both patients with deficiency and sufficiency of $25(OH)D_3$, and for lumbar spine, significant decreased BMD values were observed in patients with borderline levels of $25(OH)D_3$.

No deviation of the genotype distribution from the Hardy-Weinberg equilibrium was noticed: FF genotype accounted for 44.29 %, ff genotype for 12.86 %, and heterozygosity (Ff) for 42.85 %. When patients were stratified according to

Table 1 Anthropometric characteristics (expressed as Z-scores) and numbers and percentages of abnormal DXA measurements at the beginning of the study and 2 years after categorized by gender and in total

Parameter		Boys	Girls	P	Total
Height Z-score	Beginning	−0.91±1.31	−0.73±1.13	0.555	−0.82±1.22
	End	−0.78±1.29	−0.58±1.18	0.504	−0.68±1.23
	P	0.304	0.103		0.063
Weight Z-score	Beginning	−0.72±1.42	−0.04±1.52	0.214	−0.38±1.50
	End	−0.60±1.30	−0.01±1.44	0.089	−0.31±1.39
	P	0.161	0.435		0.129
BMI Z-score	Beginning	−0.28±0.66	−0.03±0.83	0.191	−0.16±0.75
	End	−0.14±0.68	0.03±0.83	0.624	−0.06±0.76
	P	0.017	0.237		0.013
DXA L_2–L_4	Beginning	3/33 (9.1 %)	7/31 (22.6 %)	0.137	10/64 (15.6 %)
	End	6/33 (18.2 %)	8/31 (25.8 %)	0.461	14/64 (21.9 %)
	P	0.281	0.767		0.365
DXA Femoral neck	Beginning	0/30 (0 %)	0/27 (0 %)	-	0/57 (0 %)
	End	2/33 (6.1 %)	9/31 (29 %)	0.015	11/64 (17.2 %)
	P	0.171	0.002		0.001
DXA Total hip	Beginning	8/31 (25.8 %)	10/27 (37 %)	0.356	18/58 (31 %)
	End	10/33 (30.3 %)	16/31 (51.6 %)	0.083	26/64 (40.6 %)
	P	0.689	0.266		0.271

Table 2 Mean BMD Z-scores measured at various sites at the beginning of the study and 2 years after categorized by gender and in total

Parameter		Boys	Girls	*P*	Total
BMD L_2–L_4 Z-score	Beginning	−0.83±1.19	−0.82±1.51	0.361	−0.83±1.34
	End	−0.81±1.18	−0.85±1.59	0.383	−0.83±1.38
	P	0.820	0.816		0.986
BMD Femoral neck Z-score	Beginning	0.50±1.17	0.26±1.59	0.516	0.39±1.38
	End	−0.13±1.05	−0.85±1.70	0.044	−0.47±1.48
	P	<0.001	<0.001		<0.001
BMD Total hip Z-score	Beginning	−1.18±1.28	−1.32±1.60	0.717	−1.24±1.42
	End	−1.44±1.10	−1.68±1.54	0.476	−1.60±1.33
	P	0.029	0.005		<0.001

the presence of the polymorphism Fok-I of the vitamin D, three groups were formed (Table 4). Patients being homozygous for the f allele had apparently higher BMD Z-scores compared with those carrying the F allele in homo- or heterozygosity, however, with a difference that did not reached significance. Interestingly enough, a significant deterioration in BMD Z-scores measured at femoral neck (FF: P=0.004 Ff: P<0.001, ff: P=0.024) and total hip (FF: P=0.022, Ff: P=0.005) was recorded for all type of genotypes, except for ff genotype and with regard to the total hip values (Fig. 2).

Discussion

Since osteoporosis is an important and increasing cause of morbidity in patients with b-TM, with a prevalence reaching up to 40–50 % [11, 12], even among those with adequate compliance to conventional treatment, early and accurate identification of patients at increased risk is of major importance. Despite the multifactorial pathogenesis of osteoporosis associated with b-TM, genetic factors appear to play an crucial role in the imbalance of bone architecture and metabolism [2]. The highlight of this prospective study is that Fok-I gene polymorphism of VDR seems to play a determinant role on the accrual and maintenance of bone mass in young patients with b-TM. According to the results at the beginning of our study, patients being homozygous for the f allele had better DXA measurements compared to the rest of the genotypes, although this difference did not reach statistical significance. Over and above, during the 2-year follow-up, BMD measured at total hip in patients with ff genotype did not deteriorate significantly, unlike other subpopulations (FF and Ff). These results indicate a protective effect of the f allele on bone mass of patients with b-TM when found in a homozygosity. This observation has not been replicated at other measuring sites and as far as it concerns the lumbar spine (L_2–L_4); no significant difference has been prospectively recorded in any of the genotypes, whereas at femoral neck, BMD Z-scores decreased significantly in all groups, even in ff patients, although to a lesser extent compared to the rest.

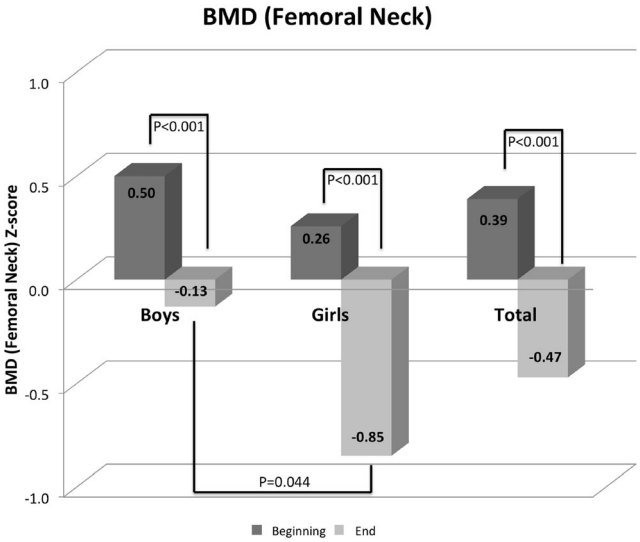

Fig. 1 Significant decrease in femoral neck BMD Z-scores both in males and females and subsequently in total. At the end of the study, females had significantly lower values compared to males

In partial agreement with our reuslts, Ferrara et al. showed that FF genotype was associated with short stature and low bone density in both lumbar spine and femoral neck in 108 prepubertal patients with b-TM, concluding that homozygosity of the F allele makes the VDR less active in the action of vitamin D [13]. In addition, Singh et al., revealed the protective effect of the allele f in bone density at the lumbar spine but not at the hip when studing 40 Indian thalassemic patients [14]. Our study supports and enhances these results with its prospective nature. Just opposite effects arised from Tantawy et al., who showed that FF correlated with significantly increased bone density at femoral neck but not lumbar spine of adult patients with b-TM [15]. The diverse results in different measuring sites, just as in our study, suggest that factors affecting bone density act individually from place to place. The same authors in 2010 connected heterozygosity Ff, in 31 prepubertal patients with beta-TM, with significantly lower bone density in both the hip and spine but only in male sex [16]. Authors tried to attribute this sexual dimorphism to a

Table 3 Study parameters at the beginning and at the end of the study in patients categorized by $25(OH)D_3$ levels measured at the beginning of the study

Parameter		Deficiency <20 ng/ml	Borderline 20–30 ng/ml	Sufficiency >30 ng/ml	P^*
N		38	8	18	
Age		22.06±5.77	26.00±5.24	24.35±4.08	0.222
Male (*n*, %)		22 (57.9)	3 (37.5)	8 (44.4)	0.447
PTH (pg/ml)	Beginning	35.48±20.37	41.78±19.03	30.89±13.05	0.243
	End	36.39±15.81	30.11±11.87	35.65±14.28	0.561
	P	0.514	0.208	0.679	
Ferritin (ng/ml)	Beginning	1538.79±1309.76	2071.50±2293.56	1156.44±1016.82	0.255
	End	1623.37±1399.53	1607.38±1321.467	1400.39±975.18	0.617
	P	0.185	0.161	0.396	
BMD L_2–L_4 Z-score	Beginning	−0.59±1.28	−1.15±1.12	−1.19±1.52	0.956
	End	−0.55±1.25	−1.58±1.26	−1.09±1.57	0.389
	P	0.643	0.025	0.332	
BMD Femoral neck Z-score	Beginning	0.61±1.49	−0.05±1.06	0.11±1.23	0.137
	End	−0.33±1.51	−0.82±1.09	−1.64±1.42	0.579
	P	<0.001	0.036	0.005	
BMD Total hip Z-score	Beginning	−1.00±1.43	−2.05±1.28	−1.35±1.39	0.178
	End	−1.29±1.31	−2.37±1.12	−1.76±1.30	0.08
	P	0.037	0.068	0.027	

*Statistical significance $P<0.016$

presumable greater impact that genetic influence may have in younger ages as their male patients were much younger than females. Similar conclusions cannot be drawn from our study as the number of prepubertal patients was limited. Finally, Gaudio et al. showed no influence of Fok-I and Bmsl polymorphisms in the apparently low bone mass in a cross-sectional study of 40 patients with b-TM [17].

This diversity of the results regarding Fok-I gene polymorphism and its effect on bone mass is broad and refers to either specific groups of population, e.g., thalassemic patients, as in our study, or general population. In agreement with our

Table 4 Study parameters at the beginning and at the end of the study in patients categorized by Fok-I genotyping

Parameter *n* (%)		FF 29 (45.3)	Ff 27 (42.2)	ff 8 (12.5)	P^*
25(OH)D (ng/ml)		18.23±12.30	23.02±18.30	18.36±13.78	0.825
$1,25(OH)_2D$ (ng/ml)		28.36±17.67	32.99±29.34	24.84±17.59	0.462
PTH (pg/ml)	Beginning	38.91±4.04	30.33±13.12	36.43±19.53	0.253
	End	39.55±2.75	30.48±14.44	36.96±13.29	0.041
	P	0.738	0.981	0.779	
BMD L_2–L_4 Z-score	Beginning	−0.88±1.27	−0.91±1.46	−0.16±1.44	0.271
	End	−0.86±1.32	−0.96±1.51	−0.12±1.35	0.294
	P	0.674	0.778	0.779	
BMD Femoral neck Z-score	Beginning	0.37±1.46	0.25±1.29	0.86±1.44	0.559
	End	−0.38±1.63	−0.72±1.37	0.02±1.32	0.516
	P	0.004	<0.001	0.024	
BMD Total hip Z-score	Beginning	−1.10±1.30	−1.52±1.43	−0.72±1.77	0.339
	End	−1.54±1.32	−1.84±1.37	−0.94±1.22	0.462
	P	0.022	0.005	0.499	

*Statistical significance $P<0.016$

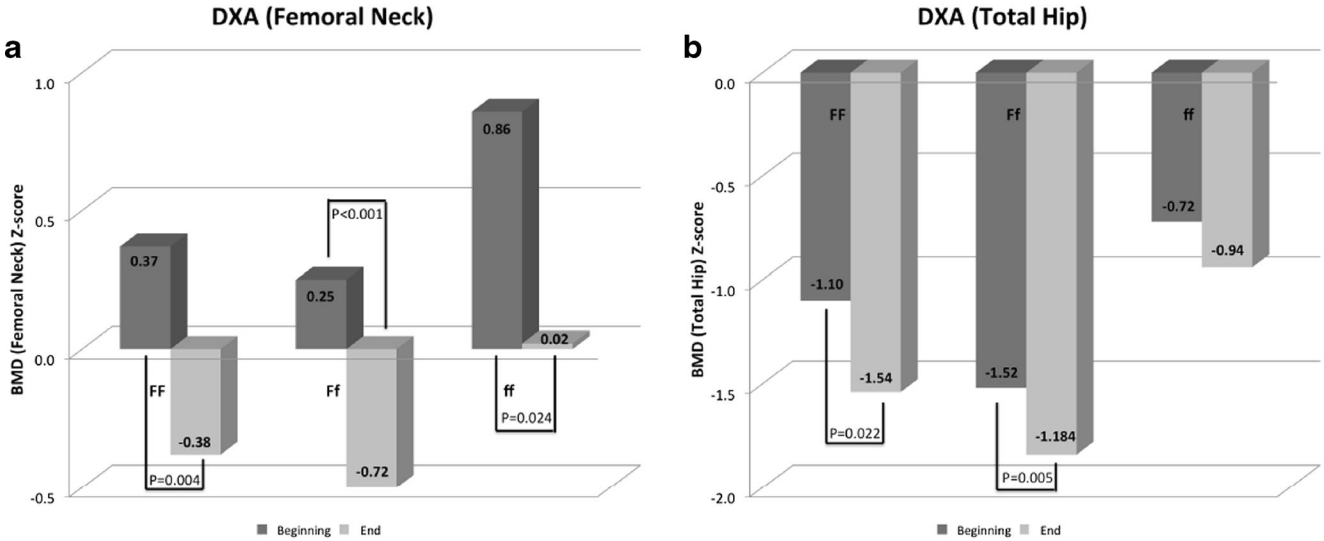

Fig. 2 Significant deterioration in BMD Z-scores measured at femoral neck (**a**) and total hip (**b**) in all type of genotypes, except for ff genotype and with regard to the total hip values

results, demonstrating a positive effect of the f allele in a homozygous state on bone density, are the results of Jakubowska et al. [18] and those by Terpstra et al. [19], both referring to normal Caucasian pediatric population. In fact, the latter study, demonstrate this finding only in boys, supporting the sexual dimorphism effect shown by the study of Tantawy et al. in thalassemic population [16]. On the contrary, several studies on general population, mainly on postmenopausal women, override our results exhibiting a correlation of ff genotype with significantly lower bone density measured either at lumbar spine [20–23], total hip [24], and even at all measuring sites [25]. This effect is attributed to the lower rate of calcium absorption observed in population carrying the ff genotype [25, 26], probably due to a larger but less active protein that is produced compared to the FF genotype [27]. However, this theory is disputed by later studies that failed to find a difference in receptor binding affinity of VDR, stability of mRNA, or activity of transactivation induced by the two different protein derivatives depending on Fok-I polymorphism [28]. Finally, few studies show no correlation, concluding that not only the combined heterogeneity in age groups, race or nationality, and environmental factors associated with diet and exercise but also issues related to the statistical power of the samples contribute to the weak and poor extraction of safe and reliable conclusions.

According to our results, it appears that a substantial but not particularly high percentage of patients with b-TM shows abnormal bone mineral density values at the beginning of the study. These data are consistent with the latest evidence reported in the literature that shows a delayed onset of bone disease in patients with b-TM following regular surveillance and sufficient compliance to treatment since early age [29, 30], in contrast to the majority of both past and relatively recent reports, exhibiting high rates of bone disease, even in young thalassemic patients treated adequately [31]. However, a significant worsening of DXA values is evident in all patients of our study and in a rather small period of just 2 years, implying the complexity nature of bone disease in these patients. Similar adverse results have been recorded in other prospective studies in patients with b-TM as well. In our study, lowest bone density values were recorded at total hip, followed by lumbar spine and femoral neck and in contrast to the majority of the published literature, mainly older one, where the lumbar spine was mostly affected. Lumbar spine as is mainly consisted of cancellous bone is prone to the expansion of the bone marrow in the context of ineffective erythropoiesis [32, 29, 33]. In our study, females preceded in the incidence of low bone mineral density at all measurement sites at all times but without reaching statistical significance, except for the femoral neck site and at the completion of the study. Sexual dimorphism in bone accrual in thalassemic patients is commonly demonstrated by other studies, some of them agree with our findings [34, 35] while others don't [12, 30, 31].

Another interesting finding of this study is the extremely low levels of 25(OH)D_3, a finding confirmed by the majority of relevant studies in patients with b-TM [36–38]. The fact that samples were collected during winter months could justify, in part, these low levels, as seasonal variation is well documented in the literature [39]. However, Moulas et al. demonstrated significantly decrease levels of 25(OH)D_3 in thalassemic patients compared to controls even during summer months [36]. Potential causative factors for this phenomenon have been proposed: (a) reduced intestinal absorption, (b) reduced skin synthesis due to subicteric tint or due to iron-induced increased pigmentation, or (c) reduced hepatic hydroxylation due to hepatic dysfunction and increased liver siderosis. Low levels of 25(OH)D_3 were not accompanied

by expected variations in levels of serum calcium, phosphorus, alkaline phosphatase, and i-PTH. Also, no correlation between vitamin D levels and bone density were shown in our study in contrast with previous reports [40, 41, 33]. Hypocalcemia following vitamin D deficiency and a compensatory PTH increase to stimulate osteoclastic activity and maintain calcium homeostasis can partially interpret previous data. In our study, patients deficient to $25(OH)D_3$ exhibited higher levels of parathyroid hormone as compared to patients with normal levels of the hormone, but the difference was not statistically significant. The compensatory hyperparathyroidism is probably less apparent in patients with b-TM due to the development of secondary iron-induced hypoparathyroidism [42]. Authors claim normal DXA values within a hypoparathyroid state attributing to this endocrinological complication a relative protective role [43, 42].

In conclusion, our study being the first prospective study to examine the role of Fok-I gene polymorphism of the VDR indicates a protective role of the f allele when found in homozygosity on bone mineral density of young patients with b-TM. Identification of genetic factors that play a role in bone mass accrual and bone metabolism could move prevention and management of low bone density to an advanced level of individualized approach.

Conflicts of interest None.

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