Predictors of bone disease in Egyptian prepubertal children with β-thalassaemia major

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Abstract

Introduction: Thalassaemic osteopathy is a multifactorial disorder and limited information exists about bone accrual and bone mineral density (BMD) in prepubertal thalassaemic children. The study aimed to investigate some potential genetic and biochemical bone markers as possible early predictors of BMD variations in children with β-thalassaemia major (TM) before puberty.
Material and methods: Thirt-one prepubertal children with β-TM, and 43 matched controls were subjected to BMD assessment by dual energy X-ray absorptiometry (DEXA). Vitamin D receptor (VDR) gene polymorphisms (Bsm1, Fok1) and the biochemical bone markers serum osteocalcin and propeptide I procollagen (CPIP) and urinary deoxypyridinoline (DPD) excretion were assessed.
Results: Bone mineral density was reduced in 25% of thalassaemics at the spine and 15.4% at the hip region. Significantly higher levels of urinary DPD and lower serum osteocalcin and CPIP levels were found in the studied thalassaemic children compared to controls ($p < 0.001$). A significant negative correlation was present between BMD in spine and hip and the patients' age ($r = -0.6367$, $p = 0.0002$ and $r = -0.616$, $p = 0.00079$, respectively). There was a significant reduction in BMD in males compared to females. Reduced BMD was more frequent in male patients with genotypes bb and Ff but not in females. Bone mineral density was not related to the studied biochemical bone markers, mean pre-transfusion haemoglobin or serum ferritin.
Conclusions: Routine BMD screening with DEXA is proposed to be a sensitive predictor for early bone changes, particularly at the lumbar spine. DR gene polymorphisms of Bsm1 and Fok1 polymorphisms may be determinants of BMD in Egyptian prepubertal male thalassemics

Key words: β-thalassaemia, bone mineral density, gene polymorphism vitamin D receptor.

Introduction

Thalassaemia is a hereditary disease that causes chronic anaemia and increased erythropoietin. Consequently, an expansion of bone marrow spaces may contribute to osteopenia/osteoporosis [1]. Thalassaemic osteopathy is a multifactorial disorder. The underlying disease, its complications and management can contribute to its pathogenesis [2, 3]. The skeletal changes associated with untreated patients include osteoporosis, growth failure, bone age delay and spondylometaphyseal abnormalities [4]. Some published studies have confirmed deferoxamine-

induced bone dysplasia [5]. Hypothalamic-pituitary-gonadal axis and growth hormone dysfunction may be responsible for the development of osteoporosis in thalassaemic children and adolescents [6]. Regular transfusion and subcutaneous deferoxamine chelation therapy have improved the clinical picture and quality of life of thalassaemic patients. However, osteopenia with cortical thinning, increased trabeculation of the spine and osteoporosis remain serious complications even in well transfused and iron-chelated patients [7, 8]. Despite calcium and vitamin D administration, effective iron chelation and normalization of haemoglobin levels, patients with thalassaemia major continue to lose bone mass [9]. Moreover, twin studies have shown that osteoporosis is a polygenic disorder. It is determined by the effects of several genes, which account for 80% of the variance in bone mineral density [10, 11]. Some loci, such as the vitamin D receptor (VDR) gene as well as collagen type I α I, are promising genetic determinants of bone mass [12]. Vitamin D receptor gene polymorphisms' association with osteoporosis is highly controversial. In humans, the VDR gene has been located on the chromosome locus 12q13-14. The gene is composed of a minimum of nine exons. Vitamin D receptor gene start codon polymorphisms and 3 end region polymorphism may modulate bone density [13]. Clinical studies have identified a range of regulatory and structural VDR gene loci which are proposed to be involved in bone density determination as length polymorphism of COLI αI, Bsm1 and FokI [14]. The VDR gene codes for the VDR protein which regulates intestinal calcium absorption. However, studies of the relationship between VDR gene polymorphisms and bone density have been found to be inconsistent and poorly reproducible in different populations, suggesting genetic heterogeneity of osteoporosis [15]. Fok1 and Bsm1 polymorphisms are closely related to low bone mineral density (BMD) and can be used as useful genetic markers in determining risk of low BMD and osteoporosis [16]. Bsm1 VDR gene polymorphism is associated with osteopenia in adult thalassaemic patients [2]. Fok1 VDR gene polymorphism seems to directly affect bone mineral accretion during pubertal growth through an effect on calcium absorption. The relationship between different genetic polymorphisms and bone mineral metabolism may vary with life stage [17]. Ferrari *et al*. [18] reported that BMD is significantly associated with VDR gene polymorphism only before puberty. Limited information exists about bone accrual and low bone mass in prepubertal β-thalassaemic children. Therefore, this study was designed to evaluate the skeletal changes in prepubertal Egyptian children with β-thalassaemia major and

the possible role of Bsm1 and Fok1 polymorphisms in the gene encoding the vitamin D receptor, and the biochemical indices of bone metabolism were studied as early predictors of BMD variation in this young population.

Material and methods

The present study is a case control study and was carried out during the period from March 2005 to June 2007. The study was conducted on 31 children with β-thalassaemia major (14 females and 17 males) at the prepubertal age (mean age 9.74 ±2.85 years), on transfusion therapy and chelated with subcutaneous deferoxamine. They were recruited from regularly treated patients in the Paediatric Haematology Unit, Children's Hospital, Ain Shams University and Paediatric Haematology Clinic of the National Research Centre in Egypt. Forty-three age- and sex-matched children were included as controls.

The included thalassaemic children and controls were subjected to detailed history and clinical examination. Data files of patients were revised for determination of mean pre-transfusion haemoglobin and mean serum ferritin level in the last year prior to the study.
- Assessment of BMD using dual energy X-ray (DEXA) absorptiometry technique (Norland XR-46, version: 3.9.6/2.3.1.) was done on three sites (lumbar spine, hip and femoral neck) in a medical services unit in the National Research Centre; a *Z*-score 2.5 to –1 SD is referred to as normal BMD, *Z*-score < –1 to –2.5 SD is diagnoses osteopenia and *Z*-score < –2.5 means osteoporosis (WHO 2003).
- Blood and urine samples were assayed at the Clinical Pathology Department in the National Research Centre (Egypt).

Assessment of bone turnover markers

Five millilitres of blood was withdrawn under aseptic conditions by venipuncture from every child, 3 ml on EDTA for DNA extraction, 2 ml of blood was centrifuged and sera were obtained and stored at –20°C for assay of:
- osteocalcin as a marker of osteoblastic activity and bone formation was measured using the host ELISA kit (Biosource Europe S.A.), a quantitative sandwich enzyme-linked immunosorbent assay;
- carboxy-terminal propeptide of type I procollagen (CPIP) as a marker of bone formation. It was measured using the METRA CICP EIA Kit (QUIDEL Corp.)

All children were asked to void urine in special tubes and urine samples were stored at –20°C. Deoxypyridinoline cross links (DPD) level was measured using the METRA DPD EIA Kit (QUIDEL Corp.), as a marker of osteoclastic activity and bone resorption.

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Study of gene polymorphism of vitamin D receptors Bsm1 and Fok1

DNA was extracted by using a spin column kit (supplied from Qiagen): polymerase chain reaction (PCR) amplification and enzymatic digestion of the products with Bsm1 and Fok1.

For the Bsm1 polymorphism 2 mg of genomic DNA was amplified with each of forward primer 5"AAGACTACAAGTACCGCGTCAGTG and reverse primer 5"AACCAGCGGGAAGAGGTCAAGGG (supplied by Biosynthesis). Polymerase chain reaction was performed with a Biometra thermoblock, under standard conditions, for 35 cycles, and with 65°C as the annealing temperature. After amplification, the PCR product (0.825 kb) was digested with restriction endonuclease BsmI and electrophoresed in a 1.2% agarose gel. With the enzyme $Bsm1$ (Fermentas, Lithuania), the respective genotypes were defined as B (indicating the absence of the restriction site) or b (indicating the presence of the restriction site). The PCR product for the Bsm1 polymorphism was 825 bp, and the restriction fragments were 650 bp and 175 bp.

For the Fok1 polymorphism 2 mg of genomic DNA was amplified with each of forward primer 5"AGCTGGCCCTGGCACTGACTCTGGCTCT and reverse primer 5"ATGGAAACACCTTGCTTCTTCTCCCTC. Products were digested with restriction enzyme BseGI (Fermentas, Lithuania), an isoschizomer of the FokI enzyme, at 55°C for 120 min. Fragments were electrophoresed through a 2% agarose gel containing ethidium bromide, visualized, and photographed. The presence of the FokI restriction site on both alleles (defined before as ff) generates 196-bp and 69-bp fragments, whereas the absence (FF) yields one undigested 265-bp fragment. Heterozygous Ff exhibits fragments of 265 bp, 196 bp, and 69 bp [19].

Statistical analysis

Data were expressed as mean and standard deviation. Student's t-test was used for parametric data and Wilcoxon rank sum test (Z value) for non-parametric data.

Differences between levels of osteocalcin, CPIP and DPD in different Fok1 genotypes and Bsm1 genotypes were tested using the Kruskal-Wallis test in all genotypes. Mann-Whitney test was used to assess the difference among different Bsm1 and Fok1 genotype subgroups and between cases with Z score for spine and for hip less than –1 and those with Z score more than –1 as regards the level of osteocalcin, CPIP or DPD (data were presented as median and IQR – inter-quartile range). Pearson correlation coefficient, r, defines the strength and direction of the linear relationship between Z score and other parameters. Chi-square test, χ^2, and Fisher's exact test were used to test the difference in proportions of cases with Z score for spine and for

hip less than –1 and those with Z score more than –1, between Fok1, and Bsm1 genotypes, in total, in male and female cases. Value of $p < 0.05$ was taken as significant. The data were analysed using SPSS (version 15).

Results

The results are illustrated in Tables I-VI and Figures 1, 2.

Age and sex distribution in the control group did not significantly differ from the patient group (Table I). There were significantly higher concentrations of urinary deoxypyridinoline (DPD) levels in thalassaemic children (157.41 ±116.92 mmol/mmol creat) than controls (53.96 ±39.37 mmol/mmol creat) ($p < 0.001$). Lower levels of both serum osteocalcin (5.68 ±8.85 ng/ml) and CPIP (193.57 ±225.42 ng/ml) were found in thalassaemic patients compared to controls (45.04 ±28.83, 398.13 ±201.85 ng/ml respectively) ($p < 0.001$).

Among the thalassaemics, the spine Z score had a mean value of –0.48 ±0.93 (median –0.54); the hip Z score had a mean value of –0.08 ±0.70 (median 0.17). Twenty-five percent of the studied thalassaemics (7/28) showed a Z score < –1 at the spine and 15.4% (4/26) had a Z score < –1 at the hip site. Table II and Figures 1, 2 show a highly significant negative correlation between spine Z score and hip Z score in relation to age at examination and a non-significant correlation between Z score and the studied laboratory markers of bone turnover. There was no correlation between the BMD and serum ferritin or pre-transfusion haemoglobin levels. Comparison between thalassaemic children with fair and bad chelation shows a non-significant difference in BMD and serum levels of osteocalcin, CPIP, and urinary levels of DPD in relation to chelation adequacy ($p > 0.05$).

No statistical significant difference in osteocalcin, CPIP and DPD was found between cases with either Z score for spine or hip < –1 and those with Z score > –1 ($p > 0.05$) (Table III).

Significant younger mean age of studied male patients (8.7 ±2.93 years) was found than that of female patients (11 ±2.25 years) ($p < 0.05$). Lower Z score of the lumbar spine was detected in males (0.49 ±0.06) than that found in females (0.55 ±0.08) ($p < 0.05$).

Tables IV, V reveal no statistically significant difference in the distribution of either Fok1 or Bsm1 genotypes between thalassaemic patients with Z score (spine and/or hip) < –1 and those with Z score > –1 ($p > 0.05$). A significantly higher frequency of Ff alleles (66.7%) was found in thalassaemic males with Z score of spine and/or hip < –1 than those with corresponding Z score > –1 ($p = 0.04$ and $p = 0.03$ respectively). All male thalassaemic children (100%) with bb genotype had

Table I. Comparison between patients and controls as regards clinical and laboratory data (using Student's *t*-test and Wilcoxon rank sum test)

Parameter		Patients (*n*=31)	Controls (*n*=43)	Test of significance
Age [years]	Mean ± SD	9.74 ±2.85	9.33 ±2.99	*t* value = 0.607
	Range	4-14	5-15	*p* = 0.54
Male : female ratio		17 : 14	22 : 21	
Mean pre-transfusion haemoglobin [gm/dl]	Mean ± SD	7.2 ±1.9		
	Range	6.9-8.2		
Mean serum ferritin [ng/ml]	Mean ± SD	910 ±823		
	Range	550-1750		
Osteocalcin [ng/ml]	Mean ± SD	5.68 ±8.85	45.04 ±28.83	*Z* value= −5.84
	Range	1-48.5	0.3-105	*p* < 0.001
CPIP [ng/ml]	Mean ± SD	193.57 ±225.42	398.13 ±201.85	*Z* value= −4.02
	Range	1-892	49-731	*p* < 0.001
DPD [mmol/mmol creat]	Mean ± SD	157.41 ±116.92	53.96 ±39.37	*Z* value = −4.62
	Range	30-459	8-208	*p* < 0.001

Z score (spine and/or hip) < −1 vs. none with BB or Bb alleles (*p* = 0.004, *p* = 0.002 respectively).

Table VI shows no statistically significant difference between osteocalcin, CPIP or DPD in different Fok genotypes (*p* > 0.05) in males and females. Thalassaemic children with bb genotype had significantly higher osteocalcin levels than detected in those with either BB or Bb alleles (*p* = 0.02).

Discussion

We carried out a study on BMD, biochemical and VDR (Bsm1, Fok1) genetic profiles in prepubertal children with β-thalassaemia major with the hope of gaining new insight into establishing early predictors for these bone changes.

The study revealed a highly significant increase in concentration of urinary deoxypyridinoline and lower levels of serum CPIP and osteocalcin in studied thalassaemic children compared to controls

Table II. Correlation between BMD in thalassaemic patients and different clinical and laboratory parameters (Pearson correlation coefficient)

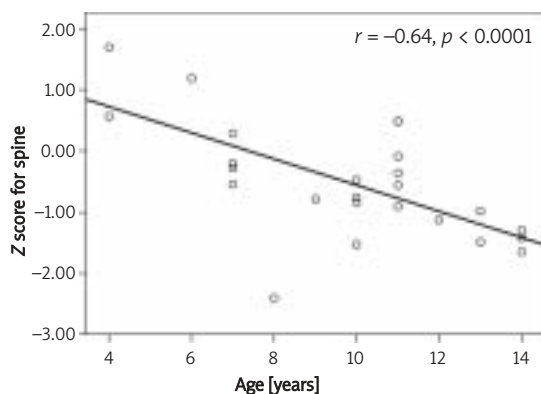| Parameter	Spine *Z* score	Femur *Z* score	Hip *Z* score
Age [years]	*r* = −0.6367 *p* < 0.0001	*r* = −0.3236 *p* = 0.10	*r* = −0.616 *p* < 0.0001
Mean pre-transfusion Hb [gm/dl]	*r* = −0.15 *p* =0.43	*r* = −0.127 *p* = 0.53	*r* = −0.098 *p* = 0.63
Mean serum ferritin [ng/ml]	*r* = −0.35 *p* = 0.06	*r* = −0.369 *p* = 0.06	*r* = −0.368 *p* = 0.06
Osteocalcin [ng/ml]	*r* = −0.041 *p* = 0.83	*r* = −0.14 *p* =0.49	*r* = −0.05 *p* =0.79
CPIP [ng/ml]	*r* = −0.018 *p* = 0.92	*r* = −0.04 *p* = 0.84	*r* = −0.02 *p* = 0.91
DPD [mmol/mmol creat]	*r* = 0.218 *p* = 0.27	*r* = 0.115 *p* = 0.58	*r* = 0.312 *p* = 0.12

Figure 1. Pearson correlation study between *Z* score of the spine by DEXA and the age of thalassaemic patients
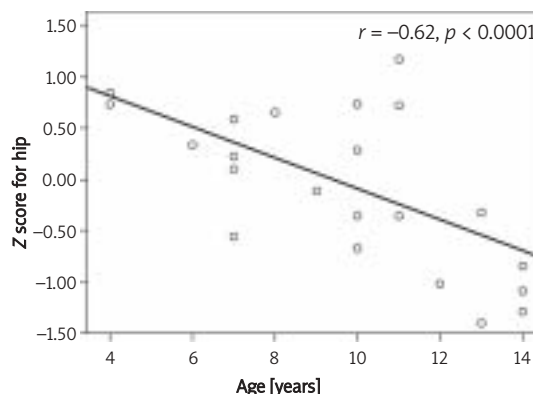

Figure 2. Pearson correlation study between *Z* score of the hip by DEXA and the age of thalassaemic patients in years

Table III. Comparison of biochemical markers between thalassaemic patients with *Z* score less than –1 and those with *Z* score more than –1 for either spine or hip (Mann-Whitney test)

Bone marker	*Z* score for spine						Value of *p*
	BMD < –1 (*N* = 7)			BMD > –1 (*N* = 20)			
	Median	IQR		Median	IQR		
Osteocalcin	3.70	1.30	7.85	1.65	1.20	4.60	0.57
CPIP	180.00	92.50	298.00	159.00	13.00	374.50	0.92
DPD	70.80	64.25	142.00	150.50	83.50	254.00	0.22
	Z score for hip						
Bone marker	BMD < –1 (*N* = 4)			BMD > –1 (*N* =23)			Value of *p*
	Median	IQR		Median	IQR		
Osteocalcin	4.10	1.30	8.65	1.70	1.20	4.60	0.58
CPIP	180.00	92.50	247.50	171.00	13.00	374.50	0.98
DPD	75.25	64.25	246.50	100.50	81.50	237.00	0.68

• *IQR – inter-quartile range*

Table IV. Comparison of the distribution of Fok1 genotypes between thalassaemic patients with *Z* score for spine or hip less than –1 and *Z* score more than –1 (Mann-Whitney test)

Fok genotypes	Total patients									
	Z score for spine (27)				Value of *p*	*Z* score for hip (28)				Value of *p*
	< –1 (7)		> –1 (20)			< –1 (4)		> –1 (24)		
	N	%	*N*	%		*N*	%	*N*	%	
FF	2	18.2	9	81.8	0.12	1	9.1	10	90.9	0.25
Ff	5	45.5	6	54.5		3	27.3	8	72.7	
ff			5	100.0				6	100.0	
	Male patients									
Fok genotypes	*Z* score for spine (11)				Value of *p*	*Z* score for hip (12)				Value of *p*
	< –1 (2)		> –1 (9)			< –1 (2)		> –1 (10)		
	N	%	*N*	%		*N*	%	*N*	%	
FF			6	100.0	0.04			6	100.0	0.03
Ff	2	66.7	1	33.3		2	66.7	1	33.3	
ff			2	100.0				3	100.0	
	Female patients									
Fok genotypes	*Z* score for spine (16)				Value of *p*	*Z* score for hip (16)				Value of *p*
	< –1 (5)		> –1 (11)			< –1 (2)		> –1 (14)		
	N	%	*N*	%		*N*	%	*N*	%	
FF	2	40.0	3	60.0	0.43	1	20.0	4	80.0	0.71
Ff	3	37.5	5	62.5		1	12.5	7	87.5	
ff			3	100.0		0	0	3	100.0	

(*p* < 0.001). These results are in agreement with Morabito *et al.* [20]. These results reflect derangement of bone metabolism with increased bone turnover in thalassaemic children which started early in the prepubertal period [21]. This is supported by the finding of a significant negative correlation between reduced BMD at studied sites (hip and spine) and increased patient age. These findings are in parallel with the data published by Christoforidis *et al.* [22], who demonstrated a delay in bone mass acquisition with advancing age in the thalassaemic group compared to controls.

Reduced BMD (*Z* score < –1) was detected in 25% at the spine and 15.4% at the hip of studied

Table V. Comparison of the distribution of Bsm1 genotypes between thalassaemic patients with *Z* score for spine or hip less than –1 and *Z* score more than –1 (Mann-Whitney test)

Total patients										
Bsm1 genotypes	*Z* score for spine (28)				Value of *p*	*Z* score for hip (29)				Value of *p*
	< –1 (7)		> –1 (21)			< –1 (4)		> –1 (25)		
	N	%	N	%		N	%	N	%	
BB	3	25.0	9	75.0	0.84	1	8.3	11	91.7	0.30
Bb	2	20.0	8	80.0		1	9.1	10	90.9	
bb	2	33.3	4	66.7		2	33.3	4	66.7	
Male patients										
Bsm1 genotypes	*Z* score for spine (11)				Value of *p*	*Z* score for hip (12)				Value of *p*
	< –1 (2)		> –1 (14)			< –1 (2)		>-1 (14)		
	N	%	N	%		N	%	N	%	
BB			4	100.0	0.004			4	100.0	0.002
Bb			5	100.0				6	100.0	
bb	2	100.0				2	100.0			
Female patients										
Bsm1 genotypes	*Z* score for spine (17)				Value of *p*	*Z* score for hip (17)				Value of *p*
	< –1 (5)		> –1 (12)			< –1 (2)		> –1 (15)		
	N	%	N	%		N	%	N	%	
BB	3	37.5	5	62.5	0.33	1	12.5	7	87.5	0.65
Bb	2	40.0	3	60.0		1	20.0	4	80.0	
bb	0	0	4	100.0		0	0	4	100.0	

Table VI. Comparison between the studied biochemical markers in different studied Fok1 and Bsm1 genotypes of thalassaemic children (Kruskal-Wallis test)

Fok1 genotype									Value of *p*
	FF			Ff			ff		
	Median	IQR		Median	IQR		Median	IQR	
Osteocalcin	1.40	1.20	2.75	2.20	1.45	7.85	9.10	1.20	9.50	0.11
CPIP	187.00	89.50	424.00	180.00	14.50	380.00	59.30	12.00	147.00	0.55
DPD	97.30	68.00	290.80	82.00	68.15	142.00	177.25	51.00	254.00	0.64
Bsm1 genotype									Value of *p*
	BB			Bb			bb		
	Median	IQR		Median	IQR		Median	IQR	
Osteocalcin	1.50	1.20	2.95	1.90	1.25	7.25	10.40	10.40	11.70	0.02
CPIP	175.50	14.50	454.00	180.00	11.00	292.50	100.60	100.60	281.00	0.66
DPD	85.00	75.90	204.50	218.00	68.00	308.80	65.50	65.50	85.00	0.46

prepubertal thalassaemic children. This result reflects prominent and more frequently detected reduced BMD at the lumbar spine, which is proposed to be an interesting site for screening for early bone changes in those cases to call for more intensive and preventive treatment at this age period [23]. Significantly lower BMD of spine was found in male than in female cases. A similar gender difference of BMD was found by Christoforidis *et al.* [22].

Reduced BMD was found more frequently in male cases with genotypes bb and Ff, but a non-significant correlation was found between either Bsm1 or Fok1 allele distribution in female cases and BMD. This result is confirmed by the finding of significantly higher osteocalcin levels

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in thalasseamic males with bb genotype than levels detected in those with either BB or Bb alleles, which reflects increased bone turnover in those cases. These findings support the involvement of Bsm1 and Fok1 genotypes as a determinant of BMD in male prepubertal thalassaemic children [18]. The significant gender difference within a particular genetic pattern may be attributed to the significant younger age of studied males than that of females based on the concept that BMD is genetically determined in early age before puberty.

Moreover, no significant difference of studied biochemical indices was detected between thalassaemic children with reduced BMD and those with normal BMD. These results are contradictory to Angelopoulos *et al.* [24]. These findings suggest that BMD assessment by DEXA may be a sensitive predictor for early bone changes in this particular age [25].

No significant difference of either BMD or studied biochemical indices was found between thalassaemic children with fair and bad chelation. In addition to the insignificant correlation of reduced BMD with the mean pre-transfusion haemoglobin, these results suggest a weak contribution of anaemia and chelating therapy in early bone derangement in prepubertal patients [21].

In conclusion, the present study indicates a delay in bone mass acquisition with advancing age in prepubertal thalassaemic children. Studied Egyptian male thalasseamic children with genotypes bb and Ff had a higher rate of bone turnover, supporting the involvement of Bsm1 and Fok1 polymorphisms as determinants of BMD before puberty with a gender difference. Early diagnosis should be done during childhood to improve quality of life in adulthood. We recommend early routine BMD screening before puberty, which is proposed to be a sensitive predictor for early bone changes, in particularly at the lumbar spine. Further prospective studies on a wider scale are required to fully clarify the precise environmental and genetic mechanisms underlying bone metabolism derangement in thalassaemic children.

References

1. Mahachoklertwattana P, Pootrakul P, Chuansumrit A, et al. Association between bone mineral density and erythropoiesis in Thai children and adolescents with thalassemia syndromes. J Bone Miner Metab 2006; 24: 146-52.
2. Dresner Pollack R, Rachmilewitz E, Blumenfeld A, Idelson M, Goldfarb AW. Bone mineral metabolism in adult with beta-thalassaemia major and intermedia. Br J Haematol 2000; 111: 902-7.
3. Lala R, Chiabotto P, Distefano M, Isaia GC, Garofalo F, Piga A. Bone density and metabolism in thalassaemia. J Pediatr Endocrinol Metabolism 1998; 11 Suppl 3: 785-90.
4. Filosa A, Di Maio S, Vocca S, Saviano A, Esposito G, Pagano L. Longitudinal monitoring of bone mineral density in thalassaemic patients. Ggenetic structure and osteoporosis. Acta Pediatrica 1997; 86: 342-6.
5. Naselli M, Vignolo M, Di Battisto E, et al. Long term follow up of skeletal dysplasia in thalassaemia major. J Pediatr Endocrinol Metabolism 1998; 11 Suppl 3: 817-25.
6. Dundar U, Kupesiz A, Ozdem S, et al. Bone metabolism and mineral density in patients with beta-thalassaemia major. Saudi Medical Journal 2007; 28: 1425-9.
7. Wonke B, Jensen C, Hanslip JJ, et al. Genetic and acquired predisposing factors and treatment of osteoporosis in thalassaemia major. J Pediatr Endocrinol Metabolism 1998; 11 Suppl 3: 795-801.
8. Vogiatzi MG, Autio KA, Mait JE, Schneider R, Lesser M, Giardina PJ. Low bone mineral density in adolescents with beta-thalassaemia. Ann NY Acad Sci 2005; 1054: 462-6.
9. Gaudio A, Morabito N, Xourafa A, et al. Bisphosphonates in the treatment of thalassemia-associated osteoporosis. J Endocrinol Invest 2008; 31: 181-4.
10. Carbonell Sala S, Masi L, Marini F, et al. Genetics and pharmacogenetics of osteoporosis. J Endocrinol Invest 2005; 28 (10 Suppl): 2-7.
11. Stewart TL, Ralston SH. Role of genetic factors in the pathogenesis of osteoporosis. J Endocrinol 2000; 166: 235-45.
12. Giguère Y, Rousseau F. The genetics of osteoporosis: "complexities and difficulties". Clin Genet 2000; 57: 161-9.
13. Tokita A, Hisada K, Nishzawa K. Bone disease with vitamin D receptor abnormality. Nippon Rinsho 2002; 60: 385-90.
14. Ames SK, Ellis KJ, Gunn SK, Copeland KC, Abrams SA. Vitamin D receptor gene Fok1 polymorphism predicts calcium absorption and bone mineral density in children. J Bone Miner Res 1999; 14: 740-6.
15. Ongphiphadhanakul B. Osteoporosis: the role of genetics and environment. Forum Nutr 2007; 60: 158-67.
16. Ivanova J, Doukova P, Boyanov M, Popivanov P. Fok1 and Bsm1 polymorphisms of VDR gene and bone mineral density in a random Bulgarian population sample. Endocrine 2006; 29: 413-8.
17. Abrams SA, Griffin IJ, Hawthorne KM, et al. Vitamin D receptor Fok1 polymorphisms affect calcium absorption, kinetics and bone mineralization rates during puberty. J Bone Miner Res 2005; 20: 945-53.
18. Ferrari SL, Rizzoli R, Slosman DO, Bonjour JP. Do dietary calcium and age explain the controversy surrounding the relationship between bone mineral density and VDR gene polymorphisms? J Bone Miner Res 1998; 13: 363-70.
19. Quesada JM, Casado A, Diaz C, Barrios L, Cuenca-Acevedo R, Dorado G. Allele-frequency determination of Bsm1 and Fok1 polymorphisms of the VDR gene by quantitative real-time PCR (QRT-PCR) in pooled genomic DNA samples. J Steroid Biochem Molecul Biol 2004; 89-90: 209-14.
20. Morabito N, Russo GT, Gaudio A, et al. The "lively" cytokines network in beta-thalassemia major-related osteoporosis. Bone 2007; 40: 1588-94.
21. Vogiatzi MG, Autio KA, Schneider R, Giardina PJ. Low bone mass in prepubertal children with thalassemia major: insights into the pathogenesis low bone mass in thalassemia. J Pediatr Endocrinol Metab 2004; 17: 1415-21.
22. Christoforidis A, Kazantzidou E, Tsatra I, et al. Normal lumbar bone mineral density in optimally treated children and young adolescents with beta-thalassemia major. Hormones (Athens) 2007; 6: 334-40.
23. Eren F, Yilmaz N. Biochemical markers of bone turnover and bone mineral density in patients with beta-thalassaemia major. Int J Clin Pract 2005; 59: 46-51.

24. Angelopoulos NG, Katounda E, Rombopoulos G, et al. Evaluation of bone mineral density of the lumbar spine in patients with beta-thalassemia major with dual-energy x-ray absorptiometry and quantitative computed tomography: a comparison study. J Pediatr Hematol Oncol 2006; 28: 73-8.
25. Karimi M, Ghiam AF, Hashemi A, Alinejad S, Soweid M, Kashef S. Bone mineral density in beta-thalassaemia major and intermedia. Indian Pediatr 2007; 44: 29-32.