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Original Article

Association of *VDBP* rs4701 Variant, but not *VDR/RXR*-α Over-Expression with Bone Mineral Density in Pediatric Well-Chelated β-Thalassemia Patients

Shaimaa Sahmoud[1], Mostafa S. Ibrahim[2], Eman A. Toraih[3,4], Noha Kamel[5], Manal S. Fawzy[6,7*] and Samar Elfiky[1].

[1] Pediatric Department, Faculty of Medicine, Suez Canal University, Ismailia, Egypt
[2] Diagnostic Radiology Department, Faculty of Medicine, Suez Canal University, Ismailia, Egypt.
[3] Genetics Unit, Histology and Cell Biology Department, Faculty of Medicine, Suez Canal University, Ismailia, Egypt.
[4] Department of Surgery, Tulane University, School of Medicine, New Orleans, Louisiana, USA.
[5] Clinical Pathology Department, Faculty of Medicine, Suez Canal University, Ismailia, Egypt.
[6] Medical Biochemistry and Molecular Biology Department, Faculty of Medicine, Suez Canal University, Ismailia, Egypt.
[7] Biochemistry Department, Faculty of Medicine, Northern Border University, Arar, Saudi Arabia.

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Abstract. ***Background:*** **The reduced rate of bone formation despite the availability of vitamin D has been reported in β-thalassemia. Genetic factors, together with environmental ones, could be implicated in this condition. Since vitamin D binding protein (VDBP) maintains bioavailability of vitamin D which binds to vitamin D receptor (VDR)-retinoid X receptor alpha (RXRA) heterodimer to exert its molecular actions, we speculated that vitamin D metabolic-axis expression signature and variants could be potential molecular candidates for bone turnover/disease in thalassemia. To this end, this study aims to analyze *VDR*/*RXRA* expression signature, and two *VDBP* variants in a pilot sample of Egyptian β-thalassemia children in correlation with bone mineral density (BMD).**

Patients and methods: **Forty-four well-chelated β-thalassemia children and 40 unrelated controls were enrolled. The serum bone chemistry profile was measured. Peripheral blood mononuclear cells (PBMN) *VDR*/*RXRA* expression levels were quantified by Real-Time quantitative reverse transcription-polymerase chain reaction (qRT-PCR). *VDBP* rs7041 and rs4588 variants were identified by Real-Time allelic discrimination assay. All patients were subjected to lumbar-spine Dual-energy X-ray absorptiometry (DEXA).**

Results: ***VDR*/*RXRA*** **expressions were significantly higher in β-thalassemia children compared to controls (*P* = 0.001 and <0.001, respectively) and showed higher values in β-thalassemia major relative to β-thalassemia intermedia. Expression levels of both genes were not associated with sex or BMD. However, *VDBP* rs4701 genotyping revealed lower BMD-L4 and a higher frequency of osteoporosis.**

Conclusions: **β-Thalassemia children had higher expression levels of PBMN *VDR*/*RXRA*. *VDBP* rs4701 variant was associated with osteoporosis in our β-thalassemia patients on vitamin D supplementation. Further large-scale studies in other ethnic populations are warranted.**

Keywords: Bone mineral density; Gene expression; Genotyping; Real-Time PCR; RXRA; Single nucleotide polymorphism; Thalassemia; VDBP; VDR.

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Correspondence to: Prof. Manal S. Fawzy, Tel: + (2) 01015180786 (ORCID: 0000-0003-1252-8403). E-mail: manal2_khashana@ymail.com manal_mohamed@suez.med.edu.eg

Introduction. As an emerging global health burden, carriers of hemoglobin disorders approach 7% worldwide, and nearly 50,000-100,000 children with beta (β)-thalassemia major die each year in low- and middle-income countries.[1] In Egypt, β-thalassemia is considered the most common monogenic disorder with a carrier rate of almost 5.3 to 9%,[2] representing the most common genetically determined chronic hemolytic anemia (85.1%).[3]

Vitamin D deficiency has been reported to be prevalent among children and adolescents with thalassemia[4] in several countries,[5-7] including upper Egypt.[8] Vitamin D is essential for calcium hemostasis and bone mineralization, and 25 (OH) vitamin D is considered the major circulating vitamin D metabolite and the best indicator of vitamin D deficiency.[9] The main carrier protein which supports the bioavailability of circulating vitamin D and its metabolites is vitamin D binding protein (VDBP).[10] By maintaining the serum levels of the bioactive $1,25(OH)_2D$, VDBP could impact vitamin D levels under different physiologic and pathologic conditions,[11,12] contributing jointly or independently to a variety of adverse health outcomes.[13]

Vitamin D binding protein is encoded by the *GC* (group-specific component) gene located at 4q11-13.[14] The two most common single nucleotide polymorphisms (SNP) associated with approximately 80% of the variation in levels of VDBP are rs7041 and rs4588, which have been identified in the coding region of exon 11 of this gene.[15] These variants have been associated with both circulating vitamin D levels and their function[16,17] and show different allele frequencies based on ethnic variations.[18]

Vitamin D exerts most of its biological activities by binding to a specific high-affinity receptor, the vitamin D receptor (VDR). This receptor binds target DNA sequences as a heterodimer with retinoid X receptor alpha (RXRA) to regulate transcription.[19] This heterodimer receptor belongs to the superfamily of nuclear receptors for steroid hormones and regulates gene expression by acting as a ligand-dependent transcription factor.[19,20] VDR activation and expression are necessary for the effects of vitamin D, in which several SNPs have been identified.[21]

The vitamin D metabolic axis could be implicated in many aspects of bone mineral density (BMD) in β thalassemia. To our knowledge, the association of VDR/RXR expression and *VDBP* variants with BMD in β-thalassemia children has not been studied before. In this sense, the current study aimed to evaluate the association between *VDR/RXR* expression levels, as well as *VDBP* polymorphisms (rs7041 and rs4588) with BMD in a sample of Egyptian pediatric β-thalassemia on vitamin D supplementation.

Patients and Methods.

Study participants. A total of forty-four children with beta-thalassemia and forty age- and sex-matched healthy controls were enrolled in the study. All cases were prepubertal children aged 2-12 years who were followed up in the Hematology clinic, Suez Canal University Hospital, Ismailia, Egypt. All thalassemic children were receiving the daily requirement of vitamin D_2. None of them had ever been on Vitamin D_3 therapy, while only 70% of the controls were on vitamin D_2 supplements. Healthy children who were attending the pediatric clinics for general check-up were assigned as controls. Children with chronic renal or liver disease, clinically diagnosed rickets, or using medications influencing bone mineral metabolism (as glucocorticoids or antiepileptic drugs), were excluded. The study was approved by the Suez Canal University Ethical Committee (Approval no. 3125). Written informed consent was taken from all participants' parents.

Clinical assessment of patients. All participants were subjected to history taking, thorough examination, and data collection by screening the hospital medical records, including socio-demographic data and course of thalassemia (age at diagnosis, transfusion therapy, drug therapy, presence of complications). Weight and height were plotted on the Center for Disease Control and Prevention (CDC) curves, and puberty staging was assessed using Tanner staging.

Blood biochemical profile. The following laboratory workup was performed on all participants: (a) Complete blood picture using fully automated hematology analyzer (HORIBA ABX Micros 60, France) with blood film examination; (b) Serum calcium, phosphorus, alkaline phosphatase, liver enzymes using commercially available kits (Cobas 6000 analyzer, USA); (c) Serum ferritin using electrochemiluminescence technology on immunoassay analyzer Cobas 411 (Roche Diagnostics, Japan); (d) Parathyroid hormone assay immunoassay analyzer Cobas 411 (Roche Diagnostics, Japan).

Serum vitamin D level quantification. Total 25 (OH) Vitamin D was assessed for all participants by a commercially available ELISA kit (EIA-5396, DRG International Inc., USA). The procedure and the quality control measurements were performed according to the manufacturer's instructions. The detection limit was 3.2-120 ng/mL, the interassay coefficient of variation (CV) was around 3.7%, and the interassay CV was 7.1%.

Vitamin D status was defined sufficient at a level of ≥ 20 ng/mL, insufficient between 10 and 19 ng/mL, and deficient <10 ng/mL.[22]

Dual Energy X-ray absorptiometry (DEXA). Dual-energy X-ray absorptiometry (DXA) is the most widely used method for evaluating bone mineral content and BMD in patients of all ages.[23] BMD was measured using a DEXA densitometer (GE Lunar DPX NT, USA) with dedicated pediatric software (GE enCORE, USA) at the lumbar spine (L1–L4) in the AP projection. The instrument was calibrated daily according to the manufacturer's instructions. Reproducibility was calculated as a CV obtained by weekly measurements of a standard phantom on the instrument. The CV of the current instrument was 0.5% with the standard phantom, and the *in vivo* precision of the BMD measurement at the L1–L4 region was 1.2%. BMD data were expressed as g/cm^2 and as Z- scores after being compared with BMD values of healthy subjects of the same age.

The results were expressed as absolute values with a Z- Score (difference in SD of healthy age and sex-matched subjects) (Figure S1). BMD Z-score ≤ -2.0 was considered as osteoporosis, according to the International Society for Clinical Densitometry (Official Position 2013 available at https://www.iscd.org/official-positions/2013-iscd-official-positions-pediatric/).

Expression profiling. RNA extraction was carried out from the separated peripheral mononuclear cells (PMNCs) by Ficoll-Paque as a density-gradient medium using ABIOpure Total RNA (AllianceBio, Catalog no. M541RP50-B) following the protocol supplied by the manufacturer. Nucleic acid concentration and purity at the "absorbance ratio 260/280 nm" were determined by the NanoDrop ND-1000 spectrophotometer (NanoDrop Tech., USA). High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, P/N 4368814) was used to convert RNA into cDNA. RT was carried out in T-Professional Basic, Biometra PCR System (Biometra, Goettingen, Germany). Gene expression of *RXRA* and *VDR* genes were quantified in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines using SYBR Green qPCR analysis and compared to *GAPDH* using the following primers (**Table 1**). The reaction mixture and PCR thermal conditions were applied in StepOne™ Real-Time PCR System (Applied Biosystems) with an annealing temperature of 58°C for *GAPDH*, 62°C for *RXRA*, and 70°C for *VDR*. Melting curve analysis confirmed the specificity of the amplicons, using appropriate negative controls; the fold change was calculated using the delta threshold cycle equation.[24]

SNP identification. Genomic DNA was isolated from whole blood using ABIOpureTM Total DNA (AllianceBio, Catalog no. M501DP100) following the instructions supplied with the kits. DNA assessment was executed using NanoDrop ND-1000 (NanoDrop Tech., Inc. Wilmington, DE, USA). Samples were genotyped for VDBP polymorphisms (rs7041 and rs4588) using Real-Time polymerase chain reaction allelic discrimination technology. PCR reaction was carried out in a 25-µL reaction volume containing 12.5 µL 2x Taqman® genotyping Master Mix and 1.25 µL TaqMan® SNP Genotyping Assay Mix (Applied Biosystems) with 40 ng genomic DNA. Appropriate controls were used. PCR amplification was performed on StepOne™ Real-Time PCR System (Applied Biosystems, USA) in duplicates with 100% concordance using the conditions as described in an earlier publication.[25]

Statistical analysis. Statistical analysis was managed using the R software version 3.3.2, GraphPad prism 7.0, and "Statistical Package for the Social Sciences (SPSS) for Windows" software, version 23. Online software, (http://www.oege.org/software/hwe-mr-calc.shtml) was used for calculating Hardy–Weinberg equilibrium. Chi-square, Fisher's exact, Student's t-, Mann-Whitney U

Table 1. The designed primers using Primer3 and UCSC genome browser.

Gene	Strand	Primers	Product length
RXRA	Forward	AGATGGACAAGACGGAGCTG	120 bp
	Reverse	CCAAGGACGCATAGACCTTC	
VDR	Forward	GGAAGTGCAGAGGAAGCGGGAGATG	380 bp
	Reverse	AGTGCTGGGACAGCTCTAGGGTCAC	
GAPDH	Forward	CGGATTTGGTCGTATTGGG	208 bp
	Reverse	CTGGAAGATGGTGATGGGATT	

RXRA: retinoid X receptor alpha; *VDR*: vitamin D receptor; *GAPDH*: Glyceraldehyde 3-phosphate dehydrogenase, bp: base pairing. For checking *in silico* PCR amplification, UCSC genome browser (hosted by the University of California, Santa Cruz) was used (https://genome.ucsc.edu/).

Table 2. The baseline characteristics and biochemical profile of thalassemia children and controls.

Variables	Thalassemia Children (n=44)	Controls (n=40)	*P* values

Sex Males (%) Females (%)	 24 (54.5%) 20 (45.5%)	 19 (47.5%) 21 (52.5%)	0.519[α]
Age (mean ± SD) years	7.3±2.7	7.6±1.8	0.488[¥]
Height (Z score)	-.056±1.16	.063±.79	**.005**[≠]
BMI (Z score)	.198±1.13	.217±.8	.929[≠]
Vitamin D level (ng/ml)	41.6±30.1	18.7±5.8	**<0.001**[*¥]
Deficient Vitamin D (%)	6 (13.6%)	4 (10.0%)	**<0.001**[α]
Insufficient Vitamin D (%)	10 (22.7%)	33 (82.5%)	
Sufficient Vitamin D (%)	28 (63.7%)	3 (7.5%)	
Serum Calcium (mg/dl)	9.0±0.7	8.2±0.6	**<0.01**[≠]
Serum Phosphorus (mg/dl)	4.8±0.8	4.5±0.5	0.083[≠]
Alkaline phosphatase (IU/l)	158.6±57.9	126.8±29.8	**0.003**[¥]
Parathyroid hormone (pg/ml)	22.2±13.1	38.4±21.2	**0.001**[¥]

[≠] Student-t test; [¥] Mann-Whitney test; [α] Chi -square test; Bold values are statistically significant at *P*-value < 0.05.

(MW), and Kruskal-Wallis (KW) tests were used. Genotype and allele frequencies were estimated for each group to calculate the odds ratios (ORs) and 95% confidence intervals (CIs) for multiple genetic association models.[26] Logistic regression was employed to adjust confounder parameters. A two-tailed *p* $P < 0.05$ was considered statistically significant.

Results.

Characteristics and biochemical profile of the study groups. **Table 2** demonstrates the baseline characteristics and biochemical profile of thalassemia children and controls. Although the height Z score was significantly reduced in patients with thalassemia compared to controls ($P = 0.005$), BMI Z score was also reduced in the patient group compared to controls, but not reach statistical significance ($P = 0.929$). Thalassemia patients exhibited higher levels of serum 25 (OH) vitamin D (41.6 ± 30.1 versus 18.7 ± 5.8, $P < 0.001$), serum calcium (9 ± 0.7 and 8.2 ± 0.6, $P < 0.01$), and alkaline phosphatase (158.6 ± 57.9 versus 126.8 ± 29.8, $P = 0.003$), and lower levels of parathyroid hormone (22.2 ± 13.1 versus 38.4 ± 21.2, $P < 0.001$) compared with controls. Serum ferritin among thalassemia children was 1000 ± 241µg/L, and iron overload was not correlated with 25 (OH) vitamin D level or bone density ($P = 0.143$, and 0.211, respectively).

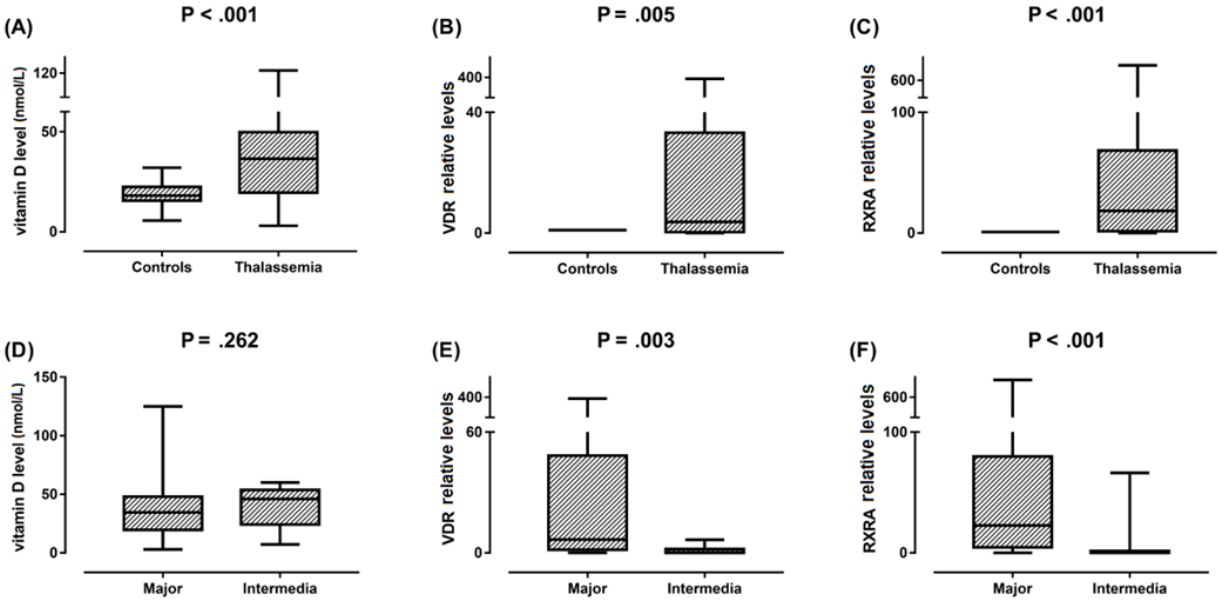

Figure 1. Expression profile of *VDR* and *RXRA* in β-thalassemia patients and controls. Values are presented as medians. The box defines upper and lower quartiles (25% and 75%, respectively) and the error bars indicate upper and lower adjacent limits. Vitamin D was measured by ELISA, while gene expression was quantified using Real-Time PCR. Fold-change was normalized to *GAPDH* and calculated using the delta-delta CT method [$= 2^{(-\Delta\Delta CT)}$] compared to controls with relative expression at 1.0. Mann-Whitney U test was used. Statistically significant at *P* value < 0.05.

Gene expression profiling. VDR and *RXRA* mRNAs were significantly higher in thalassemia children compared to controls ($P = 0.001$ and < 0.001) (**Figure 1**). Additionally, significantly higher expression values

of both transcripts were observed in thalassemia major cases compared to thalassemia intermedia children (P = 0.003 and < 0.001, respectively). Expression levels of *VDR* and *RXRA* genes were not associated with sex (P = 0.786 and 0.548) or bone density (P = 0.208 and 0.176, respectively).

Genotype analysis of VDBP polymorphisms. Genotype frequencies in both patients and controls were found in accordance with those expected by the Hardy Weinberg equilibrium. *VDBP* rs4701 GG shows borderline association with thalassemia under recessive model [OR 95% CI: 3.62 (0.9-14.2); P = 0.053]. Otherwise, the genotyping of both variants revealed no significant difference between patients and controls under all genetic association models (**Table 3**). The frequency of T*rs7041 was 0.70 in patients, and 0.66 in the controls and that of C*rs4588 was 0.74 in patients, and 0.78 in the controls, being these alleles the most common in our population.

Association of clinical and biochemical features with VDBP polymorphisms. Disease characteristics of patients, according to *VDBP* rs4701 and rs4588 genotypes, are demonstrated in **Table 4**. The heterozygote form of the rs4701 variant was associated with higher weight in thalassemia patients (P = 0.031). The same TG genotype showed a higher frequency of osteoporosis among thalassemia patients (P = 0.023), while the homozygote state (GG) was associated with lower BMD than other genotypes (TT and TG) (P = 0.021).

Discussion. Recent evidence supports the prevalence of low BMD in β-thalassemia pediatric patients despite vitamin D supplementation.[27] Osteoporosis had been observed among adult and pediatric thalassemia,[28] and vitamin D deficiency has been reported by many previous studies. [8,28-30] However, El-Edel et al.[31] could not find a significant difference in 25-hydroxy vitamin D level between pediatric thalassemia and healthy children.

The present study revealed that vitamin D status and mineral concentrations were normal in β-thalassemia children and controls. Apart from that, the included patients were on continuous vitamin D

Table 3. The genotype analysis of *VDBP* polymorphisms.

Genetic model	Genotype	Controls (n=40)	Patients (n=44)	*P* value	OR (95% CI)
***VDBP* rs7041**					
***P* HWE**		0.651	0.055		
Co-dominant model [A]	**TT**	19 (47.5)	19 (43.2)	0.145	*Reference*
	TG	18 (45.0)	15 (34.1)		0.83 (0.32-2.12)
	GG	3 (7.5)	10 (22.7)		3.33 (0.79-14.0)
Dominant model	**TT**	19 (47.5)	19 (43.2)	0.526	*Reference*
	TG+GG	21 (52.5)	25 (56.8)		1.19 (0.50-2.81)
Recessive model	**TT+TG**	37 (92.5)	34 (77.3)	0.053	*Reference*
	GG	3 (7.5)	10 (22.7)		3.62 (0.9-14.2)
Allelic model	**T**	56 (70.0)	53 (66.3)	0.185	*Reference*
	G	24 (300)	35 (43.7)		1.53 (0.80-2.94)
***VDBP* rs4588**					
***P* HWE**		0.563	0.117		
Co-dominant model [A]	**CC**	21 (52.5)	24 (54.5)	0.316	*Reference*
	CA	17 (42.5)	14 (31.8)		0.72 (0.28-1.80)
	AA	2 (5.0)	6 (13.6)		2.62 (0.47-14.4)
Dominant model	**CC**	21 (52.5)	24 (54.5)	0.851	*Reference*
	CA+AA	19 (47.5)	20 (45.5)		0.92 (0.39-2.17)
Recessive model	**CC+CA**	38 (98.0)	38 (86.4)	0.178	*Reference*
	AA	2 (5.0)	6 (13.6)		3.0 (0.56-15.8)
Allelic model	**C**	59 (73.7)	62 (77.5)	0.634	*Reference*
	A	21 (26.3)	26 (32.5)		1.17 (0.59-2.33)

VDBP: vitamin D binding protein. Values are shown as number (%). HWE P; P value of Hardy-Weinberg equilibrium. Chi square (χ^2) or Fisher's exact tests were used. OR (95% CI), odds ratio and confidence interval. ([A]) represented both heterozygote and homozygote comparison models. Statistically significant results were set at P-value < 0.05.

Table 4. Association of *VDBP* variants with clinical data in β-thalassemia patients.

Variables	*VDBP* rs7041			*P*-value	*VDBP* rs4588			*P*-value
	TT	TG	GG		CC	CA	AA	
Total number	19	15	10		24	14	6	
Demographic data								
Age (years)	6.7±2.3	8.3±3.1	6.7±2.5	0.270	7.2±2.7	7.1±2.8	7.6±2.7	0.875
Sex								
Female	9 (45.0)	8 (40.0)	3 (15.0)	0.505	12 (60.0)	60 (30.0)	2 (10.0)	0.743
Male	10 (41.7)	7 (29.2)	7 (29.2)		12 (50.0)	8 (33.3)	4 (16.7)	
Weight (Kg)	21.4±49	28.3±10.4	21.3±7.8	**0.031**	23.9±9.7	23.6±6.6	23.5±6.4	0.958
Height (cm)	115±13	126±16	113±17	0.077	118±18	117±14	121±12	0.768
Disease characteristics								
Clinical type								
Intermedia	4 (50.0)	3 (37.5)	1 (12.5)	0.745	3 (37.5)	4 (50.0)	1 (12.5)	0.462
Major	15 (41.7)	12 (33.3)	9 (25.0)		21 (58.3)	10 (27.8)	5 (13.9)	
Transfusion (mo)	1.3±0.6	1.8±0.9	1.4±0.8	0.236	1.6±0.8	1.2±0.6	1.3±0.8	0.407
Bone density								
Normal	4 (33.3)	6 (50.0)	2 (16.7)	**0.023**	6 (50.0)	4 (33.3)	2 (16.7)	0.581
Osteopenia	12 (52.2)	3 (13.0)	8 (34.8)		11 (47.8)	9 (39.1)	3 (13.0)	
Osteoporosis	3 (33.3)	6 (66.7)	0 (0.0)		7 (77.8)	1 (11.1)	1 (11.1)	
BMD								
BMD-L2	0.5±0.1	0.5±0.07	0.5±0.04	0.074	0.5±0.06	0.5±0.1	0.5±0.08	0.778
BMD-L3	0.5±0.1	0.5±0.07	0.5±0.04	0.083	0.5±0.07	0.5±0.1	0.5±0.09	0.984
BMD-L4	0.5±0.1	0.5±0.13	0.4±0.07	**0.021**	0.5±0.11	0.4±0.1	0.5±0.13	0.982
ZS								
ZS-L2	-1.8±1.3	-1.6±1.2	-1.4±0.4	0.822	-1.4±0.9	-1.8±1.0	-2.2±1.8	0.206
ZS-L3	-1.5±1.6	-1.5±1.7	-1.3±0.6	0.821	-1.2±1.1	-1.7±1.7	-2.0±1.9	0.735
ZS-L4	-1.5±1.3	-1.9±1.8	-1.3±0.4	0.861	-1.5±1.1	-2.1±2.0	-1.1±0.6	0.293

Data are presented as number (percentage) or mean ± standard deviation. Chi-square and one-way ANOVA tests were used. Bold values indicate statistically significant *P*-values at < 0.05. VDBP= vitamin D binding protein. BMD= bone mineral density as; ZS = Z score; L2, 3, and 4 = lumbar 2, 3, and 4 regions. BMD and Z scores are presented as standard deviations.

supplementation; it is worth noting that they were also on deferasirox chelation therapy for at least being2 years with adequate control of iron overload. A previous study similarly concluded a significant improvement of BMD after long term deferasirox chelation therapy.[32]

The controversial outcomes observed in the studies mentioned above, including the present one, could be related to the multifactorial etiology of bone disorders in thalassemia; probably due to defective liver hydroxylation, iron overload, the use of iron chelation therapy, and the contribution of different genetic elements in this context.[32-35]

The active vitamin D exerts most of its biological activities by binding to a high-affinity receptor; VDR that forms a heterodimer with the RXRA receptor, with subsequent interaction with several vitamin D response elements, initiating a transcriptional signal on multiple effector RNAs.[36,37] By this way, VDR/RXRA activation could be implicated in transcriptional control of hundreds of genes related to the diversity of vitamin D effects,[20,38] including regulation of the intestinal calcium uptake,[34] cytokine signaling, immune cells function, hematopoietic cells differentiation and proliferation,[39] and the final stages of monocyte and granulocyte colony-forming lines differentiation,[40] among others (reviewed in details previously).[41] The results of the present study have revealed a significant increase in peripheral *VDR* and *RXRA* expression in thalassemic children compared to controls. To the best of the authors' knowledge, the expression level of these receptors has not been tested previously in thalassemia. However, the authors cannot exclude the effect of the exogenous supplementation of vitamin D on the circulating receptor upregulation as confirmed by previous experimental studies that reported increased B cells VDR mRNA expression on exposure to the biologically active vitamin D compared to cells in the resting state.[42,43] In this sense, further expression studies in newly diagnosed cases of β-thalassemia with no history of receiving any type of medications are warranted to validate this

finding.

Accumulating evidence has suggested various factors could affect circulating vitamin D levels with subsequent bone mineral metabolism (e.g., ethnicity, gender, binding proteins, several variants in VDR and VDBP, and other pharmacogenetic factors in vitamin D metabolic pathways).[44,45] As VDR variants have been extensively studied in β-thalassemia,[21,46,47] and up to the authors' knowledge, no study uncovered the association of VDBP polymorphisms with BMD in β-thalassemia, the authors were interested in exploring for the first time the impact of two most common variants of *VDBP* gene; rs7041 and rs4588 in the coding region of exon 11, on BMD in pediatric β-thalassemia cases. These variants have been reported to be associated with approximately 80% of the VDBP level variations.[48] In addition, they have been associated with vitamin D function,[16] and show different allele frequencies based on ethnic variations.[18]

Our in silico analysis revealed that the exonic rs7041 and rs4588 variants are located in the forward strand of the chromosome 4, positions: 71762617 and 71752606, respectively. The former variant consists of two alleles, T and G, where T is the ancestral form. This single-nucleotide variation is a missense one that leads to the substitution of Aspartate by Glutamate at amino acid number 432. The later one included three alleles C, A, and T, where the ancestral allele is C, and the minor allele is A/T. Its missense variation changes Threonine to Lysine/Methionine at amino acid number 436.[45]

Currently, both study variants showed comparable frequencies in β-thalassemia children and controls. Interestingly, rs4701 GG and TG genotypes showed significant associations with lower BMD at level-L4 and a higher frequency of osteoporosis ($P = 0.021$ and 0.023, respectively) (**Table 4**). It is worth noting that the reflected phenotypic presentation of the combined effect of both study variants will change VDBP availability and affinity to vitamin D with subsequent impact on BMD.[49] The three phenotypic variations from these variants include "GC1F, GC1S, and GC2", which are sorted by their different VDBP levels in homozygote states and affinity for 25-hydroxy vitamin D[48] with some controversy for these associations remain.[50] As the GG genotype of the rs4701 variant represents the GC1S phenotype, which is known by its intermediate affinity to vitamin D, this could, in part, explain the observed association of this genotype with a high frequency of osteoporosis in the present pediatric thalassemia cases.

Several previous studies confirmed the association of vitamin D status and BMD, according to *VDBP* genotypes.[17,51,52] Johnsen et al.,[51] also, have reported that the correlations of the bio-available forms of 25-hydroxy vitamin D with bone density were stronger after adjusting for the study variants. Similarly, other studies found that the specified variants could be associated with either VDBP lower plasma concentration or lower affinity to the total serum levels of 25-hydroxy vitamin D and 1,25 dihydroxy vitamin D in cases of GC2 for rs4588, or GC1F for rs7041, respectively.[53-56] However, Sinotte et al.[54] confirmed that *VDBP* variants could explain only 2% or less of the variation in circulating vitamin D levels, similar to the amount explained by vitamin D intake. The latter finding can support the previously emerged conclusion by Bhan[57] in that "the genetic variant could impact the non-vitamin D binding activities of VDBP, including potential effects on macrophage and osteoclast activation, so the effects on vitamin D biology may not be the only relevant factor to explain the changes in BMD".

It is worth noting that our findings with that of Abbassy et al.,[27] who found associations of some VDR genetic variants (i.e., *BsmI* bb, *FokI* Ff, and ff) with BMD changes and occurrence of osteoporosis in the same type of population, confirm and support vitamin D metabolic-axis genetic variants implication in BMD of pediatric Egyptian β-thalassemia patients.

Although the present study could be limited by the small sample size and including β-thalassemia children on vitamin D supplementation that warrant further large-scale studies on newly diagnosed β-thalassemia cases in different ethnicities, an essential element of the potential reliability of our study is its agreement with HWE in both study groups, particularly the controls which ensures population representation, excluding any guided sample selection by the authors. Also, as explained previously, the external intake of vitamin D could explain ≤ 2% of circulating vitamin D levels, which supports the significant implications of other factors.

Conclusions. the present study has reported an increase of circulating *VDR* and *RXRA* expressions in pediatric well-chelated β-thalassemia patients on vitamin D supplementation, and a significant association of *VDBP* rs4701 variant with BMD-L4 and a higher frequency of osteoporosis in the study population. These findings suggest that the genetic background of pediatric β-thalassemia could be potentially implied in BMD pathogenesis in β-thalassemia, but it is worth noting that the simultaneous testing of multiple variants may be optimal for determining the contribution of the genetic background on BMD, at least in some populations. Further large-scale studies are warranted as stated above to verify the current conclusions for future improvement in the management of osteoporosis in this devastating disorder.

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References:

1. Weatherall DJ. The inherited diseases of hemoglobin are an emerging global health burden. Blood. 2010;115(22):4331-6. Epub 2010/03/18. doi: 10.1182/blood-2010-01-251348. PubMed PMID: 20233970; PubMed Central PMCID: PMCPMC2881491.
https://doi.org/10.1182/blood-2010-01-251348
PMid:20233970 PMCid:PMC2881491
2. El-Beshlawy A, Youssry I. Prevention of hemoglobinopathies in Egypt. Hemoglobin. 2009;33 Suppl 1:S14-20. Epub 2009/12/17. doi: 10.3109/03630260903346395. PubMed PMID: 20001619.
https://doi.org/10.3109/03630260903346395
PMid:20001619
3. Shawky RM, Kamal TM. Thalassemia intermedia: An overview. Egyptian Journal of Medical Human Genetics. 2012;13(3):245-55. doi: 10.1016/j.ejmhg.2012.03.006.
https://doi.org/10.1016/j.ejmhg.2012.03.006
4. Soliman AT. Vitamin D Status in Thalassemia Major: An Update. Mediterranean Journal of Hematology and Infectious Diseases. 2013;5(1). doi: 10.4084/mjhid.2013.057.
https://doi.org/10.4084/mjhid.2013.057
PMid:24106607 PMCid:PMC3787712
5. Fung EB, Aguilar C, Micaily I, Haines D, Lal A. Treatment of vitamin D deficiency in transfusion-dependent thalassemia. American Journal of Hematology. 2011;86(10):871-3. doi: 10.1002/ajh.22117.
https://doi.org/10.1002/ajh.22117
PMid:21818763
6. Singh K, Kumar R, Shukla A, Phadke SR, Agarwal S. Status of 25-hydroxyvitamin D deficiency and effect of vitamin D receptor gene polymorphisms on bone mineral density in thalassemia patients of North India. Hematology. 2013;17(5):291-6. doi: 10.1179/1607845412y.0000000017.
https://doi.org/10.1179/1607845412Y.0000000017
PMid:22971535
7. Nakavachara P, Viprakasit V. Children with hemoglobin E/beta-thalassemia have a high risk of being vitamin D deficient even if they get abundant sun exposure: a study from Thailand. Pediatr Blood Cancer. 2013;60(10):1683-8. Epub 2013/06/05. doi: 10.1002/pbc.24614. PubMed PMID: 23733667.
https://doi.org/10.1002/pbc.24614
PMid:23733667
8. Fahim FM, Saad K, Askar EA, Eldin EN, Thabet AF. Growth Parameters and Vitamin D status in Children with Thalassemia Major in Upper Egypt. Int J Hematol Oncol Stem Cell Res. 2013;7(4):10-4. Epub 2014/02/08. PubMed PMID: 24505537; PubMed Central PMCID: PMCPMC3915427.
9. Root A. Disorders of calcium metabolism in the child and adolescent. Pediatric endocrinology. 2002.
10. Christakos S, Ajibade DV, Dhawan P, Fechner AJ, Mady LJ. Vitamin D: metabolism. Endocrinol Metab Clin North Am. 2010;39(2):243-53, table of contents. Epub 2010/06/01. doi: 10.1016/j.ecl.2010.02.002. PubMed PMID: 20511049; PubMed Central PMCID: PMCPMC2879391.
https://doi.org/10.1016/j.ecl.2010.02.002
PMid:20511049 PMCid:PMC2879391
11. Yousefzadeh P, Shapses SA, Wang X. Vitamin D binding protein impact on 25-hydroxyvitamin D levels under different physiologic and pathologic conditions. International journal of endocrinology. 2014;2014.
https://doi.org/10.1155/2014/981581
PMid:24868205 PMCid:PMC4020458
12. Chun RF, Peercy BE, Orwoll ES, Nielson CM, Adams JS, Hewison M. Vitamin D and DBP: the free hormone hypothesis revisited. J Steroid Biochem Mol Biol. 2014;144 Pt A:132-7. Epub 2013/10/08. doi: 10.1016/j.jsbmb.2013.09.012. PubMed PMID: 24095930; PubMed Central PMCID: PMCPMC3976473.
https://doi.org/10.1016/j.jsbmb.2013.09.012
PMid:24095930 PMCid:PMC3976473
13. Malik S, Fu L, Juras DJ, Karmali M, Wong BY, Gozdzik A, et al. Common variants of the vitamin D binding protein gene and adverse health outcomes. Crit Rev Clin Lab Sci. 2013;50(1):1-22. Epub 2013/02/23. doi: 10.3109/10408363.2012.750262. PubMed PMID: 23427793; PubMed Central PMCID: PMCPMC3613945.
https://doi.org/10.3109/10408363.2012.750262
PMid:23427793 PMCid:PMC3613945
14. Cooke NE, Willard HF, David EV, George DL. Direct regional assignment of the gene for vitamin D binding protein (Gc-globulin) to human chromosome 4q11-q13 and identification of an associated DNA polymorphism. Hum Genet. 1986;73(3):225-9. Epub 1986/07/01. doi: 10.1007/bf00401232. PubMed PMID: 3015768.
https://doi.org/10.1007/BF00401232
PMid:3015768
15. Speeckaert M, Huang G, Delanghe JR, Taes YE. Biological and clinical aspects of the vitamin D binding protein (Gc-globulin) and its polymorphism. Clin Chim Acta. 2006;372(1-2):33-42. Epub 2006/05/16. doi: 10.1016/j.cca.2006.03.011. PubMed PMID: 16697362.
https://doi.org/10.1016/j.cca.2006.03.011
PMid:16697362
16. Agnello L, Scazzone C, Lo Sasso B, Bellia C, Bivona G, Realmuto S, et al. VDBP, CYP27B1, and 25-Hydroxyvitamin D Gene Polymorphism Analyses in a Group of Sicilian Multiple Sclerosis Patients. Biochem Genet. 2017;55(2):183-92. Epub 2016/12/03. doi: 10.1007/s10528-016-9783-4. PubMed PMID: 27904983.
https://doi.org/10.1007/s10528-016-9783-4
PMid:27904983
17. Carpenter TO, Zhang JH, Parra E, Ellis BK, Simpson C, Lee WM, et al. Vitamin D binding protein is a key determinant of 25-hydroxyvitamin D levels in infants and toddlers. J Bone Miner Res. 2013;28(1):213-21. Epub 2012/08/14. doi: 10.1002/jbmr.1735. PubMed PMID: 22887780; PubMed Central PMCID: PMCPMC3511814.
https://doi.org/10.1002/jbmr.1735
PMid:22887780 PMCid:PMC3511814
18. Fu L, Yun F, Oczak M, Wong BY, Vieth R, Cole DE. Common genetic variants of the vitamin D binding protein (DBP) predict differences in response of serum 25-hydroxyvitamin D [25(OH)D] to vitamin D supplementation. Clin Biochem. 2009;42(10-11):1174-7. Epub 2009/03/24. doi: 10.1016/j.clinbiochem.2009.03.008. PubMed PMID: 19302999.
https://doi.org/10.1016/j.clinbiochem.2009.03.008
PMid:19302999
19. Barsony J, Prufer K. Vitamin D receptor and retinoid X receptor interactions in motion. Vitam Horm. 2002;65:345-76. Epub 2002/12/17. doi: 10.1016/s0083-6729(02)65071-x. PubMed PMID: 12481554.
https://doi.org/10.1016/S0083-6729(02)65071-X
20. Huang P, Chandra V, Rastinejad F. Structural overview of the nuclear receptor superfamily: insights into physiology and therapeutics. Annu Rev Physiol. 2010;72:247-72. Epub 2010/02/13. doi: 10.1146/annurev-physiol-021909-135917. PubMed PMID: 20148675; PubMed Central PMCID: PMCPMC3677810.
https://doi.org/10.1146/annurev-physiol-021909-135917
PMid:20148675 PMCid:PMC3677810
21. Elhoseiny SM, Morgan DS, Rabie AM, Bishay ST. Vitamin D Receptor (VDR) Gene Polymorphisms (FokI, BsmI) and their Relation to Vitamin D Status in Pediatrics betaeta Thalassemia Major. Indian J Hematol Blood Transfus. 2016;32(2):228-38. Epub 2016/04/12. doi: 10.1007/s12288-015-0552-z. PubMed PMID: 27065588; PubMed Central PMCID: PMCPMC4789011.
https://doi.org/10.1007/s12288-015-0552-z
PMid:27065588 PMCid:PMC4789011
22. Braegger C, Campoy C, Colomb V, Decsi T, Domellof M, Fewtrell M, et al. Vitamin D in the healthy European paediatric population. J Pediatr Gastroenterol Nutr. 2013;56(6):692-701. Epub 2013/05/28. doi: 10.1097/MPG.0b013e31828f3c05. PubMed PMID: 23708639.
https://doi.org/10.1097/MPG.0b013e31828f3c05
PMid:23708639
23. Bianchi ML, Baim S, Bishop NJ, Gordon CM, Hans DB, Langman CB, et al. Official positions of the International Society for Clinical Densitometry (ISCD) on DXA evaluation in children and adolescents. Pediatr Nephrol. 2010;25(1):37-47. Epub 2009/07/16. doi: 10.1007/s00467-009-1249-z. PubMed PMID: 19603190.
https://doi.org/10.1007/s00467-009-1249-z
PMid:19603190
24. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8. Epub 2002/02/16. doi: 10.1006/meth.2001.1262. PubMed PMID: 11846609.
https://doi.org/10.1006/meth.2001.1262
PMid:11846609
25. Toraih EA, Fawzy MS, Mohammed EA, Hussein MH, El-Labban MM. MicroRNA-196a2 Biomarker and Targetome Network Analysis in Solid Tumors. Mol Diagn Ther. 2016;20(6):559-77. Epub 2016/06/28. doi: 10.1007/s40291-016-0223-2. PubMed PMID: 27342110.
https://doi.org/10.1007/s40291-016-0223-2
PMid:27342110
26. Hussein MH, Sobhy KE, Sabry IM, El Serafi AT, Toraih EA. Beta2-adrenergic receptor gene haplotypes and bronchodilator response in Egyptian patients with chronic obstructive pulmonary disease. Adv Med

Sci. 2017;62(1):193-201. Epub 2017/03/23. doi: 10.1016/j.advms.2016.07.008. PubMed PMID: 28327457.
https://doi.org/10.1016/j.advms.2016.07.008
PMid:28327457
27. Abbassy HA, Elwafa RAA, Omar OM. Bone Mineral Density and Vitamin D Receptor Genetic Variants in Egyptian Children with Beta Thalassemia Major on Vitamin D Supplementation. Mediterr J Hematol Infect Dis. 2019;11(1):e2019013. Epub 2019/01/24. doi: 10.4084/MJHID.2019.013. PubMed PMID: 30671219; PubMed Central PMCID: PMCPMC6328042.
https://doi.org/10.4084/mjhid.2019.013
PMid:30671219 PMCid:PMC6328042
28. Mirhosseini NZ, Shahar S, Ghayour-Mobarhan M, Banihashem A, Kamaruddin NA, Hatef MR, et al. Bone-related complications of transfusion-dependent beta thalassemia among children and adolescents. Journal of Bone and Mineral Metabolism. 2013;31(4):468-76. doi: 10.1007/s00774-013-0433-1.
https://doi.org/10.1007/s00774-013-0433-1
PMid:23475127
29. Sultan S, Irfan SM, Ahmed SI. Biochemical Markers of Bone Turnover in Patients with beta-Thalassemia Major: A Single Center Study from Southern Pakistan. Adv Hematol. 2016;2016:5437609. Epub 2016/03/24. doi: 10.1155/2016/5437609. PubMed PMID: 27006658; PubMed Central PMCID: PMCPMC4783526.
https://doi.org/10.1155/2016/5437609
PMid:27006658 PMCid:PMC4783526
30. Isik P, Yarali N, Tavil B, Demirel F, Karacam GB, Sac RU, et al. Endocrinopathies in Turkish Children with Beta Thalassemia Major: Results from a Single Center Study. Pediatric Hematology and Oncology. 2014;31(7):607-15. doi: 10.3109/08880018.2014.898724.
https://doi.org/10.3109/08880018.2014.898724
PMid:24854890
31. El-Edel RH, Ghonaim MM, Abo-Salem OM, El-Nemr FM. Bone mineral density and vitamin D receptor polymorphism in beta-thalassemia major. Pak J Pharm Sci. 2010;23(1):89-96. Epub 2010/01/14. PubMed PMID: 20067873.
32. Voskaridou E, Terpos E. New insights into the pathophysiology and management of osteoporosis in patients with beta thalassaemia. British Journal of Haematology. 2004;127(2):127-39. doi: 10.1111/j.1365-2141.2004.05143.x.
https://doi.org/10.1111/j.1365-2141.2004.05143.x
PMid:15461618
33. Casale M, Citarella S, Filosa A, De Michele E, Palmieri F, Ragozzino A, et al. Endocrine function and bone disease during long-term chelation therapy with deferasirox in patients with beta-thalassemia major. Am J Hematol. 2014;89(12):1102-6. Epub 2014/09/10. doi: 10.1002/ajh.23844. PubMed PMID: 25197009.
https://doi.org/10.1002/ajh.23844
PMid:25197009
34. Tantawy AA, El Kholy M, Moustafa T, Elsedfy HH. Bone mineral density and calcium metabolism in adolescents with beta-thalassemia major. Pediatr Endocrinol Rev. 2008;6 Suppl 1:132-5. Epub 2009/04/11. PubMed PMID: 19337166.
35. Gaudio A, Morabito N, Xourafa A, Curro M, Caccamo D, Ferlazzo N, et al. Role of genetic pattern on bone mineral density in thalassemic patients. Clin Biochem. 2010;43(10-11):805-7. Epub 2010/05/07. doi: 10.1016/j.clinbiochem.2010.04.070. PubMed PMID: 20444423.
https://doi.org/10.1016/j.clinbiochem.2010.04.070
PMid:20444423
36. Bunce C, Brown G, Hewison M. Vitamin D and hematopoiesis. Trends in Endocrinology and Metabolism. 1997;8(6):245-51. doi: 10.1016/s1043-2760(97)00066-0.
https://doi.org/10.1016/S1043-2760(97)00066-0
37. O'Kelly J, Hisatake J, Hisatake Y, Bishop J, Norman A, Koeffler HP. Normal myelopoiesis but abnormal T lymphocyte responses in vitamin D receptor knockout mice. Journal of Clinical Investigation. 2002;109(8):1091-9. doi: 10.1172/jci0212392.
https://doi.org/10.1172/JCI0212392
PMid:11956247
38. Ryan JW, Anderson PH, Morris HA. Pleiotropic Activities of Vitamin D Receptors - Adequate Activation for Multiple Health Outcomes. Clin Biochem Rev. 2015;36(2):53-61. Epub 2015/08/01. PubMed PMID: 26224895; PubMed Central PMCID: PMCPMC4504155.
39. Studzinski GP, Harrison JS, Wang X, Sarkar S, Kalia V, Danilenko M. Vitamin D Control of Hematopoietic Cell Differentiation and Leukemia. J Cell Biochem. 2015;116(8):1500-12. Epub 2015/02/20. doi: 10.1002/jcb.25104. PubMed PMID: 25694395.
https://doi.org/10.1002/jcb.25104
PMid:25694395
40. Taschner S, Koesters C, Platzer B, Jorgl A, Ellmeier W, Benesch T, et al. Down-regulation of RXRalpha expression is essential for neutrophil development from granulocyte/monocyte progenitors. Blood. 2007;109(3):971-9. Epub 2006/10/05. doi: 10.1182/blood-2006-04-020552. PubMed PMID: 17018855.
https://doi.org/10.1182/blood-2006-04-020552
PMid:17018855
41. Medrano M, Carrillo-Cruz E, Montero I, Perez-Simon JA. Vitamin D: Effect on Haematopoiesis and Immune System and Clinical Applications. Int J Mol Sci. 2018;19(9). Epub 2018/09/13. doi: 10.3390/ijms19092663. PubMed PMID: 30205552; PubMed Central PMCID: PMCPMC6164750.
https://doi.org/10.3390/ijms19092663
PMid:30205552 PMCid:PMC6164750
42. Morgan JW, Kouttab N, Ford D, Maizel AL. Vitamin D-mediated gene regulation in phenotypically defined human B cell subpopulations. Endocrinology. 2000;141(9):3225-34. Epub 2000/08/31. doi: 10.1210/endo.141.9.7666. PubMed PMID: 10965893.
https://doi.org/10.1210/endo.141.9.7666
PMid:10965893
43. Chen S, Sims GP, Chen XX, Gu YY, Chen S, Lipsky PE. Modulatory effects of 1,25-dihydroxyvitamin D3 on human B cell differentiation. J Immunol. 2007;179(3):1634-47. Epub 2007/07/21. doi: 10.4049/jimmunol.179.3.1634. PubMed PMID: 17641030.
https://doi.org/10.4049/jimmunol.179.3.1634
PMid:17641030
44. Fawzy MS, Beladi FIA. Association of Circulating Vitamin D, VDBP, and Vitamin D Receptor Expression with Severity of Diabetic Nephropathy in a Group of Saudi Type 2 Diabetes Mellitus Patients. Clin Lab. 2018;64(10):1623-33. Epub 2018/10/20. doi: 10.7754/Clin.Lab.2018.180401. PubMed PMID: 30336516.
https://doi.org/10.7754/Clin.Lab.2018.180401
45. Fawzy MS, Elgazzaz MG, Ibrahim A, Hussein MH, Khashana MS, Toraih EA. Association of group-specific component exon 11 polymorphisms with bronchial asthma in children and adolescents. Scand J Immunol. 2019;89(3):e12740. Epub 2018/12/15. doi: 10.1111/sji.12740. PubMed PMID: 30548492.
https://doi.org/10.1111/sji.12740
PMid:30548492
46. Tayel SI, Soliman SE, Elsayed HM. Vitamin D deficiency and vitamin D receptor variants in mothers and their neonates are risk factors for neonatal sepsis. Steroids. 2018;134:37-42. Epub 2018/03/14. doi: 10.1016/j.steroids.2018.03.003. PubMed PMID: 29530503.
https://doi.org/10.1016/j.steroids.2018.03.003
PMid:29530503
47. Dimitriadou M, Christoforidis A, Fidani L, Economou M, Perifanis V, Tsatra I, et al. Fok-I gene polymorphism of vitamin D receptor in patients with beta-thalassemia major and its effect on vitamin D status. Hematology. 2011;16(1):54-8. Epub 2011/01/29. doi: 10.1179/102453311X12902908411878. PubMed PMID: 21269569.
https://doi.org/10.1179/102453311X12902908411878
PMid:21269569
48. Powe CE, Evans MK, Wenger J, Zonderman AB, Berg AH, Nalls M, et al. Vitamin D-binding protein and vitamin D status of black Americans and white Americans. N Engl J Med. 2013;369(21):1991-2000. Epub 2013/11/22. doi: 10.1056/NEJMoa1306357. PubMed PMID: 24256378; PubMed Central PMCID: PMCPMC4030388.
https://doi.org/10.1056/NEJMoa1306357
PMid:24256378 PMCid:PMC4030388
49. Bhan I. Vitamin d binding protein and bone health. Int J Endocrinol. 2014;2014:561214. Epub 2014/07/06. doi: 10.1155/2014/561214. PubMed PMID: 24987416; PubMed Central PMCID: PMCPMC4058579.
50. Lauridsen AL, Vestergaard P, Nexo E. Mean serum concentration of vitamin D-binding protein (Gc globulin) is related to the Gc phenotype in women. Clin Chem. 2001;47(4):753-6. Epub 2001/03/29. PubMed PMID: 11274031.
https://doi.org/10.1093/clinchem/47.4.753
PMid:11274031
51. Johnsen MS, Grimnes G, Figenschau Y, Torjesen PA, Almas B, Jorde R. Serum free and bio-available 25-hydroxyvitamin D correlate better with bone density than serum total 25-hydroxyvitamin D. Scand J Clin Lab Invest. 2014;74(3):177-83. Epub 2014/01/05. doi: 10.3109/00365513.2013.869701. PubMed PMID: 24383929.
https://doi.org/10.3109/00365513.2013.869701
PMid:24383929
52. Nimitphong H, Sritara C, Chailurkit LO, Chanprasertyothin S, Ratanachaiwong W, Sritara P, et al. Relationship of vitamin D status and bone mass according to vitamin D-binding protein genotypes. Nutr J.

2015;14:29. Epub 2015/04/19. doi: 10.1186/s12937-015-0016-1. PubMed PMID: 25890042; PubMed Central PMCID: PMCPMC4389666. https://doi.org/10.1186/s12937-015-0016-1
PMid:25890042 PMCid:PMC4389666

53. Lauridsen AL, Vestergaard P, Hermann AP, Brot C, Heickendorff L, Mosekilde L, et al. Plasma concentrations of 25-hydroxy-vitamin D and 1,25-dihydroxy-vitamin D are related to the phenotype of Gc (vitamin D-binding protein): a cross-sectional study on 595 early postmenopausal women. Calcif Tissue Int. 2005;77(1):15-22. Epub 2005/05/04. doi: 10.1007/s00223-004-0227-5. PubMed PMID: 15868280. https://doi.org/10.1007/s00223-004-0227-5
PMid:15868280
54. Sinotte M, Diorio C, Berube S, Pollak M, Brisson J. Genetic polymorphisms of the vitamin D binding protein and plasma concentrations of 25-hydroxyvitamin D in premenopausal women. Am J Clin Nutr. 2009;89(2):634-40. Epub 2009/01/01. doi: 10.3945/ajcn.2008.26445. PubMed PMID: 19116321. https://doi.org/10.3945/ajcn.2008.26445
PMid:19116321
55. Lauridsen AL, Vestergaard P, Nexo E. Mean serum concentration of vitamin D-binding protein (Gc globulin) is related to the Gc phenotype in women. Clinical chemistry. 2001;47(4):753-6. https://doi.org/10.1093/clinchem/47.4.753
PMid:11274031
56. Wang TJ, Zhang F, Richards JB, Kestenbaum B, van Meurs JB, Berry D, et al. Common genetic determinants of vitamin D insufficiency: a genome-wide association study. The Lancet. 2010;376(9736):180-8. doi: 10.1016/s0140-6736(10)60588-0. https://doi.org/10.1016/S0140-6736(10)60588-0
57. Bhan I. Vitamin D Binding Protein and Bone Health. International Journal of Endocrinology. 2014;2014:1-5. doi: 10.1155/2014/561214. https://doi.org/10.1155/2014/561214
PMid:24987416 PMCid:PMC4058579

Supplementary Files

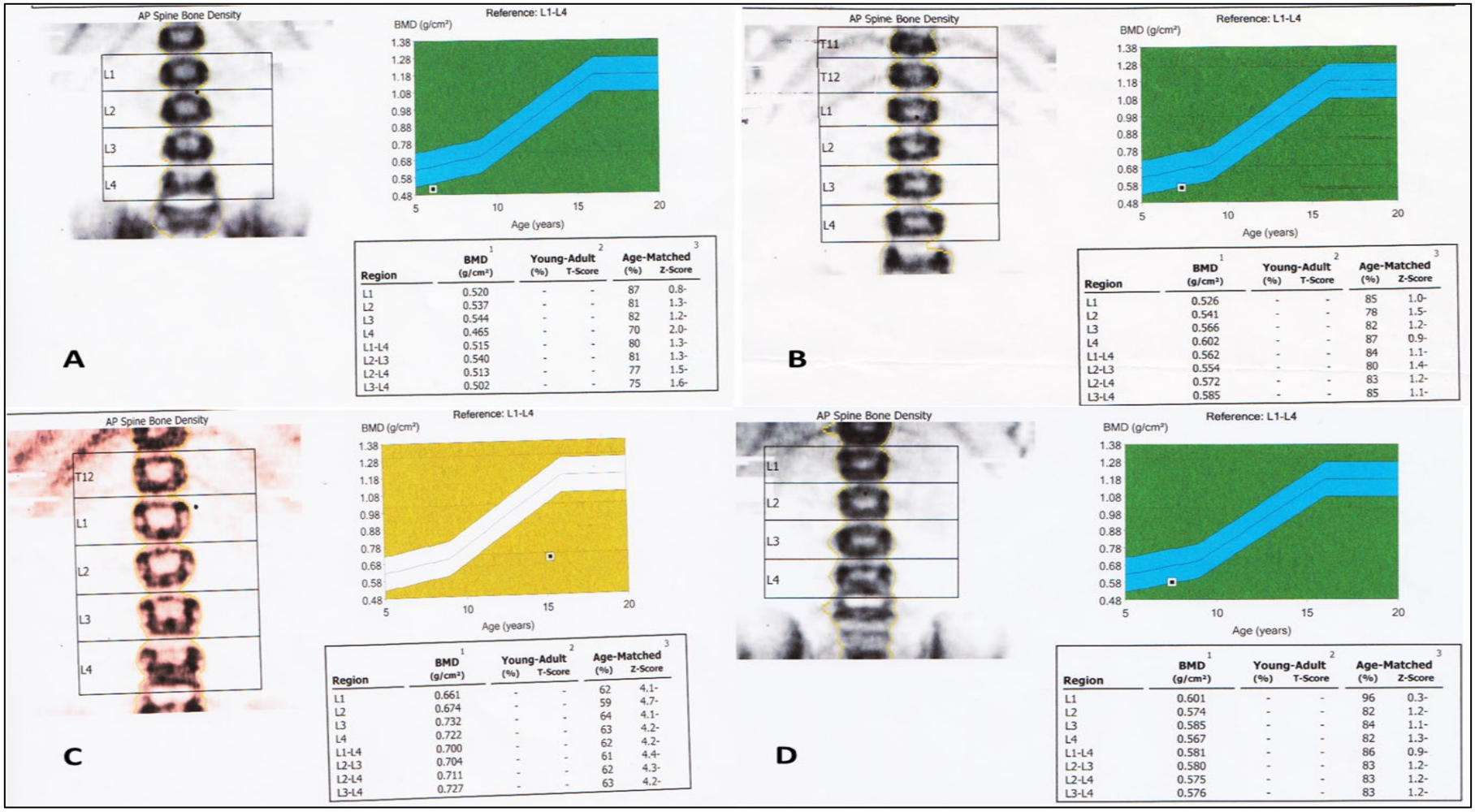

Figure S1 Some results of lumbar spine Dual-energy X-ray absorptiometry (DEXA) for study pediatric β-thalassemia cases. A patient no. 5 (male; 6 year-old), **B** patient no. 17 (male; 7 year-old), **C** patient no. 28 (female; 15 3/12 year-old), **D** patient no. 34 (female; 8 year-old).

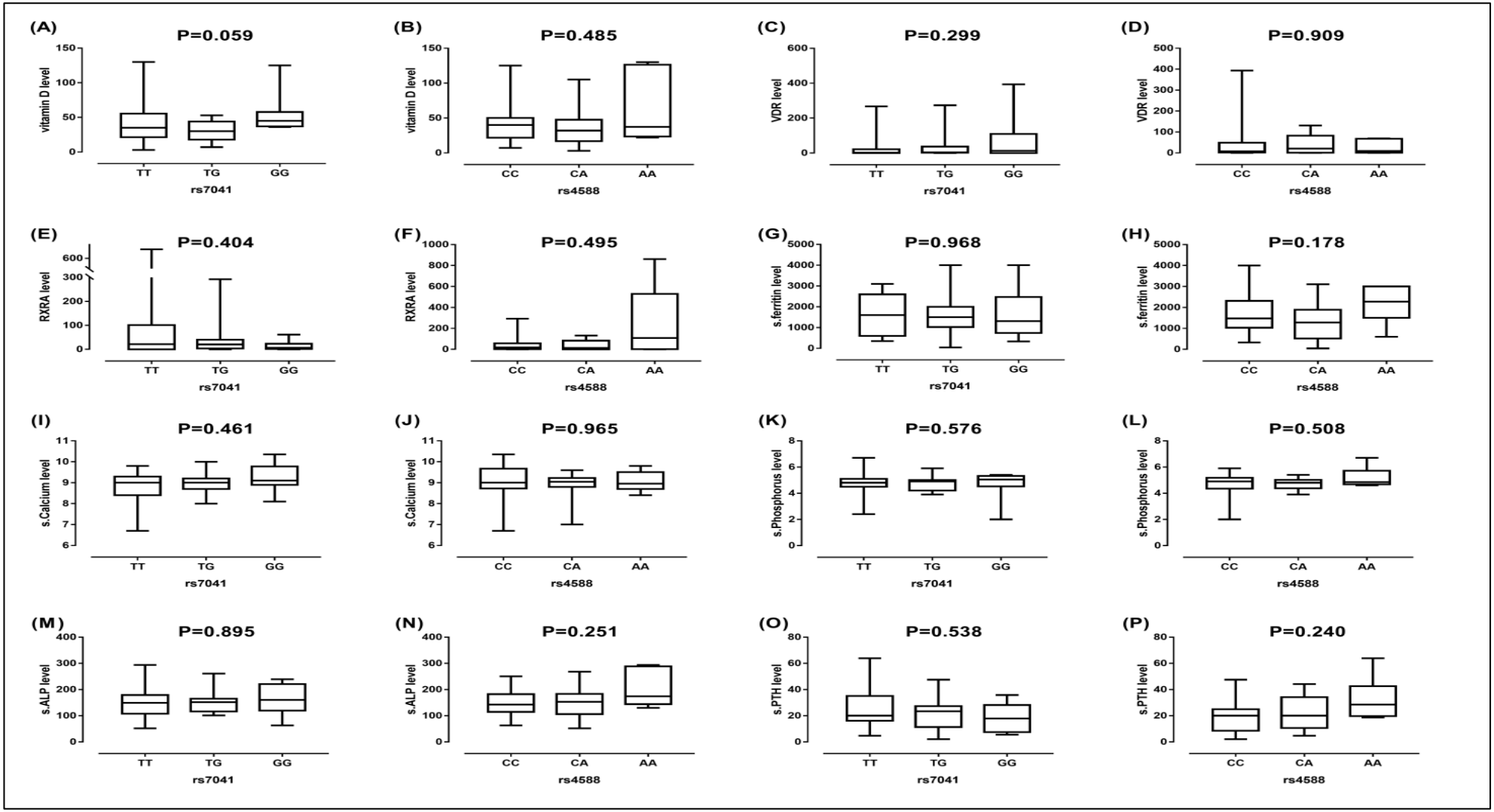

FIGURE S2. Association of *VDBP* polymorphisms (rs 7041 and rs4588) with biochemical data in pediatric β-Thalassemia cases. Kruskal Wallis test followed by Dunn's multiple comparison tests were used.