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

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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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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. In Egypt, β-thalassemia is considered the most common monogenic disorder with a carrier rate of almost 5.3 to 9%, representing the most common genetically determined chronic hemolytic anemia (85.1%).

Vitamin D deficiency has been reported to be prevalent among children and adolescents with thalassemia in several countries, including upper Egypt. 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. The main carrier protein which supports the bioavailability of circulating vitamin D and its metabolites is vitamin D binding protein (VDBP). By maintaining the serum levels of the bioactive 1,25(OH)2D, VDBP could impact vitamin D levels under different physiologic and pathologic conditions, contributing jointly or independently to a variety of adverse health outcomes.

Vitamin D binding protein is encoded by the GC (group-specific component) gene located at 4q11-13. 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. These variants have been associated with both circulating vitamin D levels and their function and show different allele frequencies based on ethnic variations.

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. 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. VDR activation and expression are necessary for the effects of vitamin D, in which several SNPs have been identified.

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. Allthalassemic children were receiving the daily requirement of vitamin D2. None of them had ever been on Vitamin D3 therapy, while only 70% of the controls were on vitamin D2 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%.

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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/cm² 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/](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.oeg.org/software/hwe-mr-calc.shtml](http://www.oeg.org/software/hwe-mr-calc.shtml)) was used for calculating Hardy–Weinberg equilibrium. Chi-square, Fisher's exact, Student's t-, Mann-Whitney U

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**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| GGAAGTGCAGAGGAACCGGGAGATG       | 380 bp         |
|        | Reverse| AGTGCCTGGACACGGCTTAGGGTAC       |                |
| GAPDH  | Forward| CGGATTTGCTGCTATTTGGG            | 208 bp         |
|        | Reverse| CTGGAAGATGTTGATGGATT            |                |

**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/](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 |
|-----------|-----------------------------|-----------------|----------|

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Sex
Males (%) 24 (54.5%) 19 (47.5%) 0.519
Females (%) 20 (45.5%) 21 (52.5%)

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
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 (-ΔΔCT)] 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 \((P = 0.003 \text{ and } < 0.001, \text{ respectively})\). Expression levels of \(VDR\) and \(RXRA\) genes were not associated with sex \((P = 0.786 \text{ and } 0.548, \text{ respectively})\).

**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 \([\text{OR (95\% CI): 3.62 (0.9-14.2); } P = 0.053]\). 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 |            |                |                |             |             |
| Co-dominant model | TT | 0.651 | 0.055 | | |
| | TG | 19 (47.5) | 19 (43.2) | 0.145 | Reference |
| | GG | 18 (45.0) | 15 (34.1) | 0.83 (0.32-2.12) | |
| Dominant model | TT | 3 (7.5) | 10 (22.7) | 3.33 (0.79-14.0) | |
| | TG+GG | 21 (52.5) | 25 (56.8) | 0.526 | Reference |
| Recessive model | TT+TG | 37 (92.5) | 34 (77.3) | 0.053 | Reference |
| | GG | 2 (5.0) | 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 |            |                |                |             |             |
| Co-dominant model | CC | 0.563 | 0.117 | | |
| | CA | 21 (52.5) | 24 (54.5) | 0.316 | Reference |
| | AA | 17 (42.5) | 14 (31.8) | 0.72 (0.28-1.80) | |
| Dominant model | CC | 2 (5.0) | 6 (13.6) | 2.62 (0.47-14.4) | |
| | 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 (%). \(P\) value of Hardy-Weinberg equilibrium. Chi square (\(\chi^2\)) or Fisher's exact tests were used. OR (95% CI), odds ratio and confidence interval. (*) 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                      | VDRP rs7041 |         |         | VDBP rs4588 |         |         |
|-------------------------------|-------------|---------|---------|-------------|---------|---------|
|                               | TT          | TG      | GG      | P-value     | CC      | CA      | AA      | P-value     |
| 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                 |             |         |         |             |         |         |         |             |
| Intermédia                    | 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)|             | 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 being 2 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 effectors 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). As VDR variants have been extensively studied in β-thalassemia, 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. In addition, they have been associated with vitamin D function, and show different allele frequencies based on ethnic variations.

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.

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. 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 with some controversy for these associations remain. 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. Johnsen et al. 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. However, Sinotte et al. 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 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., 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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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.