Association of Vitamin D Receptor Gene Polymorphisms with Serum Vitamin D Levels in a Greek Rural Population (Velesino Study)

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Keywords

Nutrigenetics · Vitamin D receptor · Vitamin D receptor polymorphisms · Serum 25(OH)D₃ levels · Deficiency

Abstract

Background/Aim: An alarming increase in vitamin D deficiency even in sunny regions highlights the need for a better understanding of the genetic background of the vitamin D endocrine system and the molecular mechanisms of gene polymorphisms of the vitamin D receptor (VDR). In this study, the serum levels of 25(OH)D₃ were correlated with common VDR polymorphisms (*Apal*, *Bsml*, *FokI*, and *TaqI*) in 98 subjects of a Greek homogeneous rural population. **Methods:** 25(OH)D₃ concentration was measured by ultra-HPLC, and the VDR gene polymorphisms were identified by quantitative real-time PCR followed by amplicon high-resolution melting analysis. **Results:** Subjects carrying either the B *Bsml* (OR: 0.52, 95% CI: 0.27–0.99) or t *TaqI* (OR: 2.06, 95%: 1.06–3.99) allele presented twice the risk for developing vitamin

D deficiency compared to the reference allele. Moreover, subjects carrying 1, 2, or all 3 of these genotypes (BB/Bb, Tt/tt, and FF) demonstrated 2-fold (OR: 2.04, 95% CI: 0.42–9.92), 3.6-fold (OR: 3.62, 95% CI: 1.07–12.2), and 7-fold (OR: 6.92, 95% CI: 1.68–28.5) increased risk for low 25(OH)D₃ levels, respectively. **Conclusions:** Our findings reveal a cumulative effect of specific VDR gene polymorphisms that may regulate vitamin D concentrations explaining, in part, the paradox of vitamin D deficiency in sunny regions, with important implications for precision medicine.

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Introduction

For most Mediterranean regions, there is a common belief in general population as well as among healthcare professionals that vitamin D levels are adequate, due to the abundance of sunlight [1, 2]. However, the prevalence

of vitamin D deficiency has been dramatically increased due to lifestyle changes that have occurred over the past decades, such as lower exposure to sun irradiation (accounting for over 80% of vitamin D synthesis) and changes of dietary habits [3–6]. In particular, elderly people are at higher risk for vitamin D deficiency as concentrations of the provitamin form, 7-dehydrocholesterol, are progressively decreasing in their epidermis [7].

Vitamin D deficiency has been recently recognized as endemic to a myriad of health consequences, since new research studies revealed the importance of vitamin D in the endocrine system and its association not only with bone mineral density but also with certain cancers, autoimmune diseases, diabetes mellitus, cardiovascular disease, allergy, depression, pregnancy complications, infertility, and even frailty [8–10]. In elderly people, low vitamin D levels predict loss of mobility with disability in their daily basic activities and are linked to clinical progression of their chronic diseases [11].

Collective evidence from several studies has demonstrated that variability of vitamin D status depends on a number of environmental and genetic factors. The non-genetic determinants of vitamin D status include gender, age, skin pigmentation, exposure to sunlight, sunscreen use, season, latitude, altitude, air pollution, dietary habits, supplemental vitamin D intake, obesity, and physical exercise [12, 13]. However, investigation of the genetic background of vitamin D metabolism has highlighted the importance of several genes such as *CG*, *DHCR1*, *CYP2R1*, *CYP24A1*, and *VDR* [12].

The active form of vitamin D, $1,25(\text{OH})_2\text{D}_3$, the formation and catabolism of which are driven by several cytochromes, exerts transcriptional activation and repression of various target genes by binding to the vitamin D receptor (VDR) which belongs to the steroid/thyroid hormone receptor superfamily [14]. Several single-nucleotide polymorphisms (SNPs) have been identified in the *VDR* gene and were involved in a variety of physiological and pathological phenotypes in many populations [15]. The expression and function of VDR is likely influenced by VDR gene polymorphisms [16]. However, only few studies have examined the association between vitamin D-related gene polymorphisms and levels of $25(\text{OH})\text{D}_3$ and $1,25(\text{OH})_2\text{D}_3$ [17]. Four of the commonly reported SNPs in the *VDR* gene, *BsmI* B>b (rs1544410), *FokI* F>f (rs2228570), *Apal* A>a (rs7975232), and *TaqI* T>t (rs731236), were investigated in several Greek population subgroups [18–20]. *BsmI*, *Apal*, and *TaqI* polymorphisms are located near the 3' end of the *VDR* gene and may correlate with gene transcription [16]; the *BsmI* and *Apal*

SNPs are both located in intron 8, and *TaqI* is a silent SNP in exon 9 [19]. Allele F of the *FokI* SNP may lack the first start codon producing a VDR protein which is shortened by 3 amino acids. This protein variant was shown to modulate a more efficient transactivation of vitamin D-regulated target genes [16]. Also, the f allele was associated with a higher level of $25(\text{OH})\text{D}_3$ in a longitudinal population-based study [12].

In an effort to gain more insights into the VDR polymorphisms and vitamin D levels, in this observational study, we investigated possible associations between common VDR polymorphisms and serum vitamin D levels in a homogenous rural population of elderly residents from a central Greek town (Velesino). The clinical relevance of this investigation lays on the possibility to stratify individuals at high risk for deficiency of vitamin D based on the analysis of their genetic profiles (precision medicine).

Experimental Section

Sample

This investigation was performed on Velesinians who voluntarily attended at local Health Center of Velesino at Ahiolopouleio General Hospital of Volos during the 3rd phase of a health assessment study called the “Velesino study” on October 19–20, 2013. A description on study recruitment and inclusion criteria is published in detail before [21, 22]. The participants consisted of 98 elderly permanent inhabitants (67 women and 31 men) aged 72.8 ± 7.3 years who were asked to voluntarily participate in the study and provided a written informed consent; all participants were community dwelling and noninstitutionalized.

Anthropometric Measurements, Body Composition Assessment, and Clinical Characteristics

Bioelectrical impedance measurements were acquired using Tanita BC-420 MA [23]. This instrument consists of a stand-alone unit where the subject has to step on barefoot (standard mode). Information concerning the participants (age, gender, and height) is entered by the experimenter. Once body mass had been assessed by the scale, the participants had to take grips in both hands (alongside their body) during the impedance measure (hand to foot BIA). Segmental fat mass and fat-free mass values were indicated by the end of the analysis on the digital screen (trunk and left right arms and legs), as well as total body fat, fat-free mass, and water. An index of

visceral fat was generally used as an estimated measurement ranging from 1 to 55 (rating from 1 to 12 and 13 to 59 indicates healthy range and an excess level of visceral fat, respectively) [24].

History of previous diagnoses and use of medications were based on self-reported information, along with an in-depth review of medical records. Hypercholesterolemia was determined by previous diagnosis or low-density lipoprotein cholesterol levels ≥ 160 mg/dL (4.14 mmol/L) [25] and diabetes mellitus type 2 by medical history or fasting blood glucose levels ≥ 126 mg/dL (6.97 mmol/L) [26]. Heart disease was defined by history of coronary artery disease, cerebrovascular disease, heart failure, and atrial fibrillation.

Serum/Plasma Collection

Morning blood samples were collected by venipuncture [27] and obtained in appropriate vacutainer tubes by a nurse assistant for vitamin D quantification and DNA isolation.

Vitamin D Quantification in Serum Samples

The first blood sample (5 mL) was centrifuged (at 2,000 g for 10 min at room temperature), and the serum was stored at -20°C for vitamin D measurements. Measurement of the serum 25(OH) D_3 concentration was chosen because it is the accepted approach to evaluate a person's vitamin D status [28]. This biomarker has a long half-life in circulation, and its concentration is not under tight homeostatic regulation. Also, it reflects vitamin D supply and usage over a period of time [29, 30]. In this study, the 25(OH) D_3 concentration was determined by the UHPLC method with online solid-phase extraction of the metabolite from serum samples (intraday and inter-day precision is 7.9 and 10.6 CV%, respectively) [31]. In particular, after protein precipitation by acetonitrile 1:1 and centrifugation, 500 μL of supernatant was injected to the Ultimate 3000 UHPLC dual gradient system supplied with the online SPE concentration column coupled through a 6-port switching valve to the analytical column. After extraction of 25(OH) D_3 on the SolEx HRP SPE concentration column, the extract was transferred by back flashing to the Acclaim 120C18 2.2 μm 3 $\times$ 100 mm analytical column (Dionex Corporation, Sunnyvale, CA, USA) and analyzed. The currently accepted standards for defining vitamin D status in adults are 25(OH) D_3 ≥ 30 ng/mL as sufficient, 25(OH) D_3 between 21 and 29 ng/mL as insufficient, and 25(OH) D_3 ≤ 20 ng/mL as deficient [32, 33].

Analysis of the VDR Gene

The second blood sample (1.5 mL) was collected from each participant in EDTA-containing tubes and stored at -20°C until processed. Genomic DNA was extracted from whole blood using the commercial kit NucleoSpin Blood (Macherey-Nagel, Duren, Germany) following the manufacturer's instructions. The concentration and quality of the extracted DNA was assessed with an Eppendorf Biophotometer. DNA samples were stored at -20°C for further analysis.

In our study, we determined the 4 commonly reported VDR polymorphisms [18–20] using high-resolution melting (HRM) analysis on the real-time polymerase chain reaction (RT-PCR) platform Rotor-Gene 6000 5P Series (Corbett Research, Sydney, Australia). HRM is a simpler and more cost-effective way to characterize samples than probe-based genotyping assays, and unlike conventional methods, it is a closed assay system requiring no post-PCR processing [34, 35]. HRM results are comparable to more time-consuming and expensive conventional methods such as single-strand conformation polymorphism, denaturing high-pressure liquid chromatography, restriction fragment length polymorphism, and DNA sequencing [36].

Primers for each polymorphism were designed according to common HRM specifications (www.genequantification.de/hrm-protocol-cls.pdf). First, the genomic sequence of the human VDR (vitamin 1,25-dihydroxyvitamin D_3 receptor) gene (GenBank Accession No. J03258) was used as a template to design primers for PCR amplification which are specific, short (86–99 bp), and suitable for HRM fragments (see online suppl. Table 1; see www.karger.com/doi/10.1159/000514338 for all online suppl. material) using the open-source Primer3 software [37]. Primer specificity was ensured using Basic Local Alignment Search Tool (BLAST), NCBI, and assessed by RT-PCR and agarose electrophoresis of PCR products.

Genotypes were determined according to the restriction site. The lowercase allele (f, b, a, or t) represents the presence of the restriction site and the uppercase allele (F, B, A, or T) represents the absence of the restriction site. The *BsmI* b and *TaqI* t alleles correspond to a T $\rightarrow$ C transition in introns 8 and 9, respectively. The *FokI* f results in a C $\rightarrow$ T transition at junction of intron 1 and exon 2 and the *Apal* a in a T $\rightarrow$ G transition in intron 8.

RT-PCR followed by HRM analysis was initially performed in a part of our sample in order to detect and discriminate all 3 possible genotypes of each polymorphism (common homozygotes, heterozygotes, and rare homozygotes) in 3 distinct groups, using the Kapa HRM Fast

kit. Cycling conditions were optimized for each polymorphism (online suppl. Table 2). Samples from each different genotype group for each polymorphism were subjected to Sanger sequencing (ABI3730xl) using primers flanking the HRM product (PCR products 300–500 bp) in order to confirm the specificity of the products and their genotypes. These samples were later used as reference samples in order to determine the genotypes of the rest of the samples, as HRM analysis is based on the comparison of samples of the known genotype. Primer design was carried out as described above (online suppl. Table 3). PCR was carried out using the My Taq polymerase (Bioline) under the following conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturing at 95°C for 15 s, annealing at 58°C for 15 s, and chain extension at 72°C for 10 s, followed by a final extension step at 72°C for 10 min. PCR products were purified using the Nucleospin Gel and PCR clean-up kit (Macherey-Nagel, Duren, Germany) and assessed by agarose electrophoresis, before sequencing on an ABI3730xl sequence analyzer.

Statistical Analysis

The distribution of study variables by vitamin D levels (low serum vitamin D levels <30 ng/mL; normal vitamin D levels ≥30 ng/mL) and the agreement of genotype frequencies with Hardy-Weinberg equilibrium for each SNP were derived, and *p* values were subsequently calculated using one-way ANOVA or χ^2 as appropriate. Subsequently, we conducted logistic regression analysis to investigate correlations of VDR polymorphisms with the risk of low serum vitamin D levels. Our study estimated the effect of allele contrast, the contrast of homozygotes, and the contrasts for the dominant and recessive models. The defined model for *TaqI*, *Apal*, *BsmI*, and *FokI* is as follows, respectively: *TaqI*; dominant model (tt + Tt vs. TT), recessive model (tt vs. Tt + TT), and allelic model (t vs. T); *Apal*; dominant model (aa + Aa vs. AA), recessive model (aa vs. Aa + AA), and allelic model (a vs. A); *BsmI*; dominant model (bb + Bb vs. BB), recessive model (bb vs. Bb + BB), and allelic model (b vs. B); *FokI*; dominant model (ff + Ff vs. FF), recessive model (ff vs. Ff + FF), and allelic model (f vs. F). Besides univariate analyses, multi-variable logistic regression models were also calculated through incorporating age and sex, but also use of medication for osteoporosis, due to its direct impact on vitamin D levels. Because of the potential effect of locus-locus interactions of the polymorphisms on 25-hydroxyvitamin D level, the combined effect of the risk genotypes [38, 39], as identified from the former analysis (*p* values

<0.10), was also evaluated. The results were expressed as odds ratios (OR) and the corresponding 95% confidence intervals (CI). Statistical analysis was conducted using SAS statistical software (version 9.4; SAS Institute Inc., Cary, NC, USA).

Results

Population Characteristics

For the investigated cohort, the anthropometric parameters, clinical characteristics, and genotype frequencies of the VDR by the vitamin D level categories are summarized in Table 1. In total, 98 subjects were considered (72.8 ± 7.3 years; 66% females), among whom 69 (70.4%) had low serum vitamin D levels (<30 ng/mL). Concerning VDR polymorphisms, all SNPs were in Hardy-Weinberg equilibrium (online suppl. Table 4). No significant differences were noted between the 2 groups with regard to demographic variables, anthropometric characteristics, and history of common medical conditions. However, VDR genotype differences by vitamin D level status were noted; particularly, subjects with low vitamin D levels were less likely to carry the recessive homozygous tt *TaqI* genotype and bb *BsmI* genotype.

VDR Allele and Genotype Distributions and Association with Deficiency of Vitamin D

The results of the logistic regression genotyping and allelic analyses are presented in Table 2. Statistically significant associations between VDR polymorphisms and 25(OH)D₃ levels were identified for *TaqI* and *BsmI* genotypes. No effect was identified for *FokI* and *Apal*. The t allele (*TaqI* genotype) was associated with a 2-fold increase in the odds for low 25(OH)D₃ levels (OR: 2.06, 95% CI: 1.06–3.99), and individuals carrying the Tt (OR: 3.56, 95% CI: 1.27–9.97) *TaqI* genotype were at approximately 3.5-fold increased odds, when compared to TT carriers. Furthermore, the b allele (*BsmI* genotype) was associated with approximately half the odds of low 25(OH)D₃ levels (OR: 0.52, 95% CI: 0.27–0.99).

Association of VDR-Combined Genotypes with the Risk to Develop Deficiency of Vitamin D

The potential combined effect of risk genotypes, as identified from the aforementioned analyses, was also tested (Fig. 1). BB/Bb *BsmI*, Tt/tt *TaqI*, and FF *FokI* were considered as risk genotypes. After adjusting for age, gender, and medication for osteoporosis, the multivariable logistic regression analysis revealed a cumulative

Table 1. Anthropometric parameters, clinical characteristics, and genotype frequencies of VDR of study participants ($N = 98$)

Variables ^a	Vitamin D <30 ng/mL ($n = 69$)	Vitamin D ≥30 ng/mL ($n = 29$)	p value ^b
Age, years	73.5±8.0	71.1±5.3	0.15
Female sex	48 (69.6)	19 (65.5)	0.69
Vitamin D levels, ng/mL	19.49±6.59	40.68±15.7	
BMI, kg/m ²	30.1±5.5	30.5±5.0	0.71
Fat mass, %	35.8±8.9	36.1±8.4	0.88
Muscle mass, %	60.9±8.5	60.7±8.1	0.83
Bone mass, %	3.3±0.5	3.2±0.4	0.79
Visceral fat, %	13.2±2.9	13.3±3.5	0.86
Waist circumference, cm	107.5±11.4	104.9±9.6	0.30
Fat-free mass, %	64.2±8.9	63.9±8.6	0.89
Systolic blood pressure, mm Hg	138.4±20.7	140.0±23.4	0.78
Diastolic blood pressure, mm Hg	71.8±11.1	71.0±9.9	0.79
Hypertension	53 (76.8)	23 (79.3)	0.79
Diabetes mellitus type II	17 (24.6)	7 (24.1)	0.96
Hypercholesterolemia	35 (50.7)	18 (62.1)	0.30
Heart disease ^c	21 (30.4)	8 (31)	0.78
Medication for osteoporosis	14 (20.3)	8 (27.6)	0.43
VDR genotypes			
TaqI genotype			
TT	16 (23.2)	14 (48.3)	0.048*
Tt	40 (58.0)	11 (37.9)	
tt	13 (18.8)	4 (13.8)	
ApaI genotype			
AA	20 (29.0)	10 (34.5)	0.24
Aa	38 (55.1)	11 (37.9)	
aa	11 (15.9)	8 (27.6)	
BsmI genotype			
BB	15 (21.7)	4 (13.8)	0.04**
Bb	40 (58.0)	12 (41.4)	
bb	14 (20.3)	13 (44.8)	
FokI genotype			
FF	30 (43.5)	10 (34.5)	0.45
Ff	31 (44.9)	13 (44.8)	
ff	8 (11.6)	6 (20.7)	

VDR, vitamin D receptor. * Statistically significant differences ($p < 0.05$) in the comparisons Tt versus TT and tt versus TT genotypes. ** Statistically significant difference ($p < 0.05$) in the comparison bb versus BB genotype.

^a Results are expressed as mean value ± standard deviation in case of continuous variables or n (%) in case of categorical variables. ^b p values are derived from the χ^2 test for categorical variables and the t test for continuous variables. ^c Heart disease includes coronary artery disease, cerebrovascular disease, heart failure, and atrial fibrillation.

dose-response effect of these different genotypes. Subjects carrying 1, 2, or all 3 of these genotypes were at 2-fold (OR: 2.04, 95% CI: 0.42–9.92), 3.6-fold (OR: 3.62, 95% CI: 1.07–12.2), and 7-fold (OR: 6.92, 95% CI: 1.68–28.5) increased risk for low 25(OH)D₃ levels, respectively (Fig. 1).

Discussion

The importance of the genetic background in maintaining vitamin D adequacy is likely to be most significant in regions where UVR exposure is limited or is seasonally limited. Nevertheless, accumulating evidence suggests that the genetic contribution to vitamin

Table 2. Multivariable logistic regression analysis in the total group

VDR polymorphisms	Multivariable analysis ^a			
	%	OR	95% CI	<i>p</i> value
TaqI genotypes				
TT	30.1	Ref		
Tt	50.5	3.56	1.27–9.97	0.01
tt	19.4	3.26	0.81–13.17	0.09
TT/Tt	80.6	Ref		
tt	19.4	2.20	1.08–4.49	0.03
TaqI alleles				
T	55.3	Ref		
t	44.7	2.06	1.06–3.99	0.03
ApalI genotypes				
AA	32.0	Ref		
Aa	48.6	1.77	0.62–5.04	0.28
aa	19.4	0.63	0.19–2.10	0.45
AA/Aa	80.6	Ref		
aa	19.4	0.87	0.46–1.63	0.65
ApalI alleles				
A	56.3	Ref		
a	43.7	0.87	0.46–1.62	0.65
BsmI genotypes				
BB	21.2	Ref		
Bb	51.9	0.88	0.23–3.44	0.86
bb	26.9	0.25	0.06–1.03	0.06
BB/Bb	73.1	Ref		
bb	26.9	0.44	0.22–0.89	0.02
BsmI alleles				
B	47.6	Ref		
b	52.4	0.52	0.27–0.99	0.05
FokI genotypes				
FF	41.7	Ref		
Ff	43.7	0.55	0.19–1.61	0.27
ff	14.6	0.31	0.08–1.21	0.09
FF/Ff	85.4	Ref		
ff	14.6	0.56	0.29–1.10	0.09
FokI alleles				
F	63.6	Ref		
f	36.4	0.56	0.29–1.09	0.09

VDR, vitamin D receptor. ^a The multivariable analysis is adjusted for age (1-year increment), gender, and medication for osteoporosis.

D status warrants further investigation even in regions with abundant sunlight exposure, such as Greece, where vitamin D deficiency is reaching alarming levels, posing high health risks to over 50% of the general population and 80% of the elderly [1, 40]. A better understanding of the association between VDR gene polymorphisms and vitamin D status may explain why some individuals are more susceptible to low 25(OH)D₃ concentrations [41].

Fig. 1. Risk of vitamin D deficiency by number of present risk genotypes. OR and 95% CI are derived from multivariable logistic regression analysis adjusted for age (1-year increment), gender, and presence of osteoporosis. BB/Bb *BsmI*, Tt/tt *TaqI*, and FF *FokI* are considered as risk genotypes. OR, odds ratio; CI, confidence interval.

In the present study, elderly residents from a homogeneous rural population in Central Greece were characterized by a high prevalence of 25(OH)D₃ deficiency associated with VDR polymorphisms in a cumulative effect of 3 different genotypes, *BsmI*, *TaqI*, and *FokI*. The genotype distribution of these VDR polymorphisms did not differ considerably from that of European populations according to the HapMap project [42]. Interestingly, we observed that subjects carrying either B or t allele seemed to have 2 times higher risk of developing vitamin D deficiency compared to the reference allele, as the b or T allele seemed to be associated with higher levels of 25(OH)D₃ in serum. Also, the f allele seemed to be associated with higher 25(OH)D₃ levels in a dose-dependent way, although the results were borderline. Moreover, the cumulative effect of the risk genotypes (BB/Bb *BsmI*, Tt/tt *TaqI*, and FF *FokI*) escalated the increased risk for low 25(OH)D₃ levels from 2- to 3.6- and to 7-fold for subjects carrying 1, 2, or all 3 of these genotypes, respectively. These findings support that VDR polymorphisms are significantly correlated with serum vitamin D levels. To our knowledge, this is the first study examining a possible relation of VDR polymorphism to 25(OH)D₃ levels in a sample of homogeneous Greek population.

No considerable differences were noted between the 2 groups with regard to demographic variables, anthropometric characteristics, history of common medical conditions, and *ApaI* polymorphism, although the sample size was rather small to reveal statistically significant associations. Also, sunlight and UV exposure in the sample were not measured, and these may have affected the study results.

Many publications have reviewed the correlation of vitamin D status or VDR locus and common diseases [41, 42]. The VDR locus has been studied for association with susceptibility to several diseases, but findings have often been inconsistent among different populations worldwide. This inconsistency might be a result of ethnic differences or of interactions with diverse genetic or environmental factors. VDR polymorphism itself may not be a disease-affecting locus, but acts as a marker for functional variants that affect expression levels of VDR [43]. Studies concluded that VDR polymorphism has shown that the VDR gene may be a significant determinant of the amount of VDR mRNA, VDR protein, and subsequent downstream vitamin D-mediated effect [44–47]. Allele *f* of the *FokI* SNP leads to a longer VDR protein by directly introducing a start codon and may influence the activity of the VDR protein and result in a less effective transcriptional activator. Also, the *f* allele was associated with a higher level of 25(OH)D₃ in a longitudinal population-based study [12]. While the *BsmI*, *ApaI*, and *TaqI* polymorphisms are located near the 3' end of the VDR gene and may correlate with gene transcription, especially through regulation of mRNA stability [16, 45], only few studies have investigated the degree of genetic contribution of VDR polymorphisms to levels of 25(OH)D₃ and 1,25(OH)₂D₃.

Subsequent studies had investigated whether VDR polymorphisms influence serum 25(OH)D₃ levels in vitamin D deficiency diseases, and they yielded conflicting findings. A study in Egypt indicated that there was no association between VDR genotype and 25(OH)D₃ levels in patients with tuberculosis [47]. A study in Bangladesh showed that the serum 25(OH)D₃ concentration did not vary with VDR polymorphisms [44]. On the other hand, a study from India reported that *FokI* polymorphism had an effect on serum 25(OH)D₃ levels and may be affected by *FokI* polymorphisms in children with autism spectrum disorder [48] and in meta-analysis with many neuropsychiatric diseases [49]. Another study indicated a significant role for VDR polymorphisms (*BsmI/ApaI/TaqI* haplotype) in the susceptibility of childhood epilepsy which is highly associated with vitamin D deficiency [50]. In

thalassemic patients, it was reported that *FokI* [51] and *BsmI* polymorphisms influence vitamin D levels [52]. In a study by Ragia et al. [53], the CC genotype of *FokI* (corresponding to the FF genotype) was associated with the lowest concentration of vitamin D in patients with obstructive sleep apnea syndrome, compared to those bearing the CT (Ff) or TT (ff) genotype, and the interaction of vitamin D with VDR *FokI* polymorphism was significantly associated with obstructive sleep apnea syndrome. In another study examining the role of VDR polymorphisms, the mean level of 25(OH)D₃ was significantly lower in preeclamptic patients. Moreover, the haplotype *FokI* C, *TaqI* C, and *BsmI* A (CCA) compared with haplotype CTG increased the risk of preeclampsia by 1.4-fold [54]. This haplotype corresponds to the risk alleles Ftb of our study.

Similar to our study, the *ApaI* polymorphism recessive *aa* and *TaqI* polymorphism dominant TT genotypes are found to be associated with higher levels of vitamin D in serum of children with low energy fractures [55]. In an elderly population in Northern China, increased levels of 25(OH)D₃ and having T allele (corresponding to the *f* allele) of *FokI* and the *bb* genotype of *BsmI* were significantly associated with more handgrip strength in men [56]. Also, AA, FF, and TT of *ApaI*, *FokI*, and *TaqI*, respectively, showed significant higher values of vitamin D in Egyptian obese women [57]. In Egyptian women with polycystic ovarian syndrome, there was a significant decrease of 25(OH)D₃ levels in carriers of haplotype *t* of *TaqI* and *a* of *ApaI* [58]. In a study with healthy children and adolescent girls, the B, A, and T alleles of *BsmI*, *ApaI*, and *TaqI*, respectively, were associated with lower 25(OH)D₃ levels [38]. Normal vitamin D levels with the presence of the *bb* genotype were associated with lower total and LDL cholesterol in a study with elderly subjects [59].

Although the precise mechanism for the association between VDR polymorphisms and vitamin D levels remains unclear, our findings point toward an upstream modulation of 25(OH)D₃ levels by VDR-related genetic variations. Apparently, vitamin D status is a phenotype influenced by interindividual variations in the rate-limiting enzymes involved in vitamin D synthesis and catabolism, including its hydroxylation enzymes, which are dictated by complex genetic variations [45, 60].

It is well known that VDR functions downstream of the circulating active form of vitamin D as the 1 α ,25(OH)₂D₃-mediated transcriptional regulation involves activation and/or repression of target genes by binding to the VDR. Epigenetic modifications of VDR regulate the biological function of VDR to allow tran-

scription of genes or the negative-feedback control of $1\alpha,25(\text{OH})_2\text{D}_3$ biosynthesis [14]. The functionality of VDR, which is affected by genetic variations, might define these epigenetic modifications and regulate the conversion of vitamin D into its metabolites through the P450 cytochrome.

The successive hydroxylations (25-, 1α -, and 24-) of vitamin D are all performed by cytochrome P450 mixed-function oxidases (CYPs). These enzymes are located either in the ER (e.g., CYP2R1) or in the mitochondria (e.g., CYP27A1, CYP27B1, and CYP24A1). The most important 25-hydroxylase of cholecalciferol is CYP2R1, but there are also others such as mitochondrial CYP27A1 [61]. Recent studies show that human mitochondrial CYP27A1 can also act as 1α -hydroxylase catalyzing 1α -hydroxylation of $25(\text{OH})\text{D}_3$ to $1\alpha,25(\text{OH})_2\text{D}_3$ [62]. The fact that there are other 25-hydroxylases is suggested by double knock out of CYP2R1 and CYP27A1 studies. These studies show a reduction of the $25(\text{OH})\text{D}_3$ level but not to zero and actually has little impact on blood levels of calcium and phosphate. Studies with deletion of CYP2R1 or CYP27A1 show that CYP2R1 has the major impact in blood levels of $25(\text{OH})\text{D}_3$ [61].

Several studies have showed that the activity of CYP27B1 and CYP24A1 is predominantly controlled by levels of $1\alpha,25(\text{OH})_2\text{D}_3$, serum calcium, and parathyroid hormone. In addition, CYP27B1 is negatively regulated by the concerted action of fibroblast growth factor 23 and klotho, a process that closely links vitamin D metabolism to phosphate homeostasis [63]. Interestingly, the influence of antiepileptic drugs on vitamin D status can be explained by the fact that antiepileptic drugs can induce the catabolizing cytochrome P450 enzymes and accelerate the conversion of vitamin D into its inactive metabolites [50]. Additional evidence explains that antiepileptic drugs can increase the expression of the CYP24A1 gene increasing the catabolism of $25(\text{OH})\text{D}_3$ and $1\alpha,25(\text{OH})_2\text{D}_3$ and leading to lower concentration of active vitamin D [64].

Conclusions

Up until now, there has been much investigation on environmental influences into the regulation of vitamin D metabolite concentrations, whereas only few research studies have been focused on a possible genetic contribution on that. In addition to this, the majority of genotyping studies are focused on VDR disease associations, and most of them do not relate the contribution of VDR gene

polymorphisms in the regulation of vitamin D metabolite concentrations. Our study confirmed that deficiency of vitamin D still exists in a Greek region with abundance of sunlight and also showed significantly a linkage between low $25(\text{OH})\text{D}_3$ levels with the presence of B and t alleles of the *BsmI* and *TaqI* polymorphisms of the VDR gene, respectively. Also, the presence of more adverse polymorphisms in the same person has cumulative effect in a dose-dependent way. A better understanding of the genetic involvement of VDR gene polymorphisms in the regulation of vitamin D metabolite concentrations from further studies may have important implications in the use of the genetic profile to identify individuals who may be at risk for deficiency of vitamin D and recommend them to daily preventively intake vitamin D supplements. Therefore, an assessment of the VDR genetic profile along with measurements of $25(\text{OH})\text{D}_3$ concentrations could be a helpful additional part of a complete physical examination in order to prevent vitamin D deficiency diseases.

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Statement of Ethics

The study conforms to the principles outlined and conducted ethically in accordance with the World Medical Association Declaration of Helsinki. All participants provided a written informed consent. Approval for this study was obtained by the Ethics Committee of the Athens University Medical School and the Peripheral General Hospital of Volos with permission number 10161/01-07/2013.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest associated with this manuscript.

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Author Contributions

N.D. contributed to the data collection, design and conduct of the study, analysis of the experimental data, data interpretation, and manuscript writing; D.K. contributed to the data collection, data interpretation, and manuscript writing; G.P. contributed to the statistical analysis; E.S., A.A., and A.T. contributed to the design and conduct of the study and analysis of the experimental data; K.P.P. contributed to the final manuscript;

M.G. contributed to the statistical analysis and manuscript writing; E.P. contributed to the Velestino study design and data collection.

Availability of Data and Material

Supplementary tables can be found online at <https://karger.figshare.com> or from the corresponding author.

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