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The role of vitamin D receptor gene polymorphisms in the bone mineral density of Greek postmenopausal women with low calcium intake

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Abstract

The aim of this study was to investigate the effect of common vitamin D receptor (VDR) gene polymorphisms on the bone mineral density (BMD) of Greek postmenopausal women. Healthy postmenopausal women (n=578) were recruited for the study. The BMD of the lumbar spine and hip was measured using dual-energy X-ray absorptiometry with the Lunar DPX-MD device. Assessment of dietary calcium intake was performed with multiple 24-h recalls. Genotyping was performed for the BsmI, TaqI and Cdx-2 polymorphisms of the VDR gene. The selected polymorphisms were not associated with BMD, osteoporosis or osteoporotic fractures. Stratification by calcium intake revealed that in the low calcium intake group (<680 mg/day), all polymorphisms were associated with the BMD of the lumbar spine (P<.05). After adjustment for potential covariates, BsmI and TaqI polymorphisms were associated with the presence of osteoporosis (P<.05), while the presence of the minor A allele of Cdx-2 polymorphism was associated with a lower spine BMD (P=.025). In the higher calcium intake group (>680 mg/day), no significant differences were observed within the genotypes for all polymorphisms. The VDR gene is shown to affect BMD in women with low calcium intake, while its effect is masked in women with higher calcium intake. This result underlines the significance of adequate calcium intake in postmenopausal women, given that it exerts a positive effect on BMD even in the presence of negative genetic predisposition.

Keywords: VDR polymorphisms; Bone mineral density; Calcium intake; Postmenopausal women

1. Introduction

Vitamin D is a steroid hormone that plays a crucial role in calcium homeostasis and skeletal metabolism. The vitamin D receptor (VDR) mediates the action of its ligand and results in normal bone mineralization and remodelling. Therefore, the gene that encodes for the VDR is considered a candidate gene for osteoporosis [1]. It is the first gene that actually initiated the study of the molecular genetics of osteoporosis [2].

The first report of a significant association between VDR polymorphisms and bone mineral density (BMD) was extremely promising, as Morrison et al. [3] found that one polymorphism in the VDR gene accounted for up to 75% of the total genetic basis of BMD in healthy Caucasians. Although this initial finding has been partially withdrawn, in the following years, a large number of studies investigated the potential associations of VDR polymorphisms with BMD in different ethnic populations [4–10], as well as with other parameters such as bone loss [11,12], fractures [11,13,14] and bone turnover [14–16]. However, these studies have provided controversial and inconclusive results due to differences in sample size, ethnic background and age group [17,18]. Also, possible gene–environment [19–22] and gene–gene [23–25] interactions have been shown to modify the role of VDR polymorphisms in BMD and to enhance discrepancy between studies. Several meta-analyses have been performed in order to provide estimations of the population-wide effects of VDR [10,26–30]. Again, the results were inconclusive since while four studies found a modest association of VDR polymorphism with BMD [10,26,27] and osteoporosis [28], two studies showed no association of VDR polymorphism with BMD [29] and osteoporosis [30].

Apart from the effect of genetics on the pathogenesis of osteoporosis, the significant role of nutrition in bone health has also been well established. Calcium owns a special position among nutrients that affect bone health, since it is one of the main bone-forming minerals, and an appropriate supply to the bone is essential at all stages of life [31]. Association studies have shown that calcium intake influences the relationship between VDR genotype and BMD; but again, the results have been inconsistent [20,32,33]. The controversy may be attributed to the different levels of calcium intake being examined in each population [20], suggesting that

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assessment of the effect of calcium intake on VDR genotypes should be performed in each population based on the particular intake levels of this nutrient.

Researchers suggest that the use of a large ethnically homogenous sample, as well the assessment of environmental factors that affect BMD and modify the effect of polymorphisms, are highly important in investigating the role of VDR polymorphisms in BMD [2,12]. Therefore, the purposes of the present study were to investigate the effects of BsmI, TaqI and Cdx-2 VDR gene polymorphisms on BMD, fractures of the peripheral skeleton and the presence of osteoporosis in a group of Greek postmenopausal women, and to assess the role of the population's calcium intake in genetic effects. The first two single-nucleotide polymorphisms (SNPs) have been previously studied in three small Greek studies [34–36], while Cdx-2 has not been assessed before.

2. Materials and methods

2.1. Subjects

Six hundred unrelated women were recruited for this cross-sectional study from December 2006 to January 2008. The participants were consecutive unselected postmenopausal Caucasian women of Greek origin who were asked to voluntarily participate in the study through advertisements in four randomly selected Centers of Open Protection for the Elderly in the Athens region (community centers that aimed for primary health prevention and social support of elderly persons). The women were prospectively recruited and included in the study regardless of their bone density values. Exclusion criteria included the following: the presence of any known bone disease or any other disease that affects bone metabolism (e.g., Paget disease, thyroid dysfunction, parathyroid dysfunction, hepatic disease or renal insufficiency), use of any medication that is known to affect bone metabolism (e.g., corticosteroids) and early menopause. None of them has ever been treated for osteoporosis. After all exclusions, 22 women were excluded, and the final sample consisted of 578 women. The study protocol was approved by the Bioethics Committee of the Harokopio University of Athens, and all subjects signed a volunteer consent form. Data on medical history, medication use, fractures of the peripheral skeleton, smoking, and family medical and fracture history were obtained.

2.2. Dietary assessment

Dietary information was collected by applying two nonconsecutive 24-h recalls. Both dietary calcium intake (including enriched products) and supplement calcium intake were assessed (using separate questions on regular or occasional use of dietary supplements, duration of supplementation, and the dose, type and name of the supplements). The 24-h recall data were analyzed using Nutritionist Pro software, version 2.2 (Axxya Systems, Stafford, TX, USA). Macronutrient intake and calcium intake were estimated in terms of absolute amounts (g/day and mg/day).

2.3. BMD and anthropometric measurements

The BMD of the lumbar spine (L_2–L_4) and hip was measured using dual-energy X-ray absorptiometry with the Lunar DPX-MD device. Weight and height were measured to the nearest 0.5 kg and 0.005 m, respectively, and body mass index (BMI) was calculated as weight (kg) divided by height (m) squared.

2.4. Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using standard methods. Genotyping was performed using the iPLEX Gold assay (Sequenom, Inc.). Assays for all SNPs were designed using the eXTEND suite and MassARRAY Assay Design software, version 3.1 (Sequenom, Inc.). Amplification was performed in a total volume of 5 μL containing ~10–20 ng of genomic DNA, 100 nM of each PCR primer, 500 nM of each dNTP, 1.25× PCR buffer (Qiagen), 1.625 mM $MgCl_2$ and 0.2 U of HotStar Taq (Qiagen). Reactions were denatured at 95°C for 15 min, followed by 45 cycles at 94°C for 20 s, 56°C for 30 s and 72°C for 60 s, and a final extension at 72°C for 3 min. Unincorporated dNTPs were SAP digested prior to iPLEX-Gold-allele-specific extension with mass-modified ddNTPs using an iPLEX Gold reagent kit (Sequenom, Inc.). SAP digestion and extension were performed in accordance with the manufacturer's instructions, with reaction extension primer concentrations adjusted to between 0.731 and 2.193 μM, depending on primer mass. Extension products were desalted and dispensed onto a SpectroCHIP using a MassARRAY Nanodispenser prior to analysis with a MassARRAY Analyzer Compact mass spectrometer. Genotypes were automatically assigned and manually confirmed using MassArray TyperAnalyzer software, version 4.0 (Sequenom, Inc.).

Table 1
Unadjusted means (±S.D.) and percentages of anthropometric, clinical and nutrient intake parameters (n=578), by VDR polymorphisms

	BsmI			P^*	TaqI			P^*	Cdx-2			P^*
	BB	Bb	bb		TT	Tt	tt		AA	AG	GG	
Age (years)	61.35 (±10.46)	61.83 (±9.36)	62.70 (±9.87)	.494	62.31 (±9.99)	61.70 (±9.34)	61.83 (±10.43)	.799	64.03 (±11.65)	61.80 (±9.20)	62.00 (±9.93)	.516
Weight (kg)	66.78 (±11.82)	66.26 (±10.63)	68.88 (±11.80)	.051	68.58 (±11.98)	65.95 (±10.53)	66.91 (±11.95)	.048	60.92 (±9.07)	68.33 (±11.68)	66.94 (±11.13)	.005
Height (m)	1.55 (±0.07)	1.56 (±0.06)	1.56 (±0.06)	.698	1.56 (±0.06)	1.56 (±0.06)	1.55 (±0.07)	.305	1.54 (±0.07)	1.55 (±0.06)	1.56 (±0.07)	.311
BMI (kg/m^2)	27.63 (±4.79)	27.05 (±4.39)	28.33 (±4.80)	.016	28.12 (±4.91)	26.93 (±4.34)	27.80 (±4.71)	.019	25.53 (±3.50)	28.12 (±4.96)	27.38 (±4.47)	.014
Spine BMD (g/cm^2)	0.962 (±0.173)	0.956 (±0.181)	0.966 (±0.182)	.851	0.967 (±0.178)	0.954 (±0.179)	0.959 (±0.176)	.758	0.933 (±0.184)	0.946 (±0.178)	0.969 (±0.180)	.322
Neck BMD (g/cm^2)	0.755 (±0.114)	0.767 (±0.120)	0.764 (±0.107)	.808	0.775 (±0.115)	0.757 (±0.116)	0.753 (±0.114)	.352	0.720 (±0.145)	0.758 (±0.119)	0.773 (±0.111)	.298
Trochanter BMD (g/cm^2)	0.699 (±0.144)	0.703 (±0.134)	0.721 (±0.132)	.534	0.721 (±0.131)	0.694 (±0.134)	0.702 (±0.146)	.286	0.641 (±0.180)	0.701 (±0.141)	0.715 (±0.129)	.180
Total hip BMD (g/cm^2)	0.912 (±0.129)	0.919 (±0.151)	0.914 (±0.119)	.958	0.918 (±0.126)	0.910 (±0.150)	0.912 (±0.129)	.922	0.875 (±0.169)	0.931 (±0.131)	0.907 (±0.136)	.399
Calcium intake (mg/day)	789.39 (±411.5)	741.20 (±377.8)	757.47 (±377.5)	.694	767.61 (±371.43)	735.19 (±386.9)	787.01 (±384.8)	.602	817.41 (±427.4)	771.88 (±386.1)	754.27 (±385.1)	.788
Smokers (%)	20.4	19.6	22.3	.869	21.2	19.3	22.4	.871	26.7	19.8	20.4	.826
Osteopenic (%)	52.8	39.7	58.2	.951	56.8	39.3	52.2	.887	33.3	47.1	48.3	.508
Osteoporotic (%)	33.3	36.1	29.8		29.7	37.4	33.3		45.8	31.9	35.1	
Presence of at least one peripheral fracture (%)	27.3	27.8	26.7	.971	26.7	28.8	23.2	.610	24.0	30.3	25.3	.480

One-way analysis of variance was used for differences in continuous variables (age, weight, height, BMI and calcium intake), and chi-square test was used for differences in categorical variables (smoking). Associations between genotypes and BMD (continuous variable) were assessed using unadjusted linear regression models, and associations with the presence of osteoporosis and peripheral fractures (categorical variable) were assessed using unadjusted logistic regression models.

* Statistically significant differences at P<.05.

Table 2
Frequencies of selected VDR SNPs in the study population (n=578)

SNP	Allele	Frequency (%)	HWE (P)*
Cdx-2	A/G	GG (61.3)	.823
		GA (33.5)	
		AA (5.2)	
BsmI	A/G	bb (31.8)	.613
		Bb (51.3)	
		BB (17.0)	
TaqI	C/T	TT (34.5)	.752
		Tt (49.7)	
		tt (15.7)	

* P<.05 (not consistent with HWE).

2.5. Statistical analysis

Continuous variables are presented as means [±standard deviation (S.D.)], while categorical variables are presented as frequencies. Hardy–Weinberg equilibrium (HWE) was tested using standard chi-square test comparing expected and actual allele frequencies. Differences in continuous variables (age, weight, height, BMI and calcium intake) between genotypes were assessed using one-way analysis of variance, and differences in categorical variables (smoking) were assessed using chi-square test. These analyses were performed with SPSS, version 13.

PLINK 1.2 software (http://pngu.mgh.harvard.edu/purcell/plink) [37] was used for assessing the associations between VDR and BMD, the presence of osteoporosis and fractures. In the total sample, linear regression analysis was performed in order to assess the effect of VDR genotypes on BMD (continuous dependent variable) before and after controlling for potential covariates (age, weight, height, calcium intake and calcium intake–VDR genotype interactions). Logistic regression was used for assessing the associations between genotypes and the presence of osteoporosis and fractures (categorical dependent variables) before and after controlling for the same potential covariates (age, weight, height, calcium intake and calcium intake–VDR genotype interactions). In addition, the possible interactions between calcium intake and VDR genotypes were tested using the abovementioned model. Also, in order to assess the impact of calcium intake on VDR genotypes, we dichotomized the population according to calcium intake using the median value (680 mg/day) as cutoff point. Selection of this cutoff point is a common methodological practice in this type of study [38,39], as it is a value derived from the calcium intake of a particular population and results in two subgroups of similar size. In this way, the effect of calcium intake on genotypes is examined within the calcium intake levels of the study sample. In the two subgroups of calcium intake, the same analyses were performed (however, calcium intake and calcium intake–VDR genotype interactions were not used as covariates, since stratification was based on calcium intake values). All analyses were performed under the assumption of an additive model. Power calculation was performed using Quanto, version 1.2 [40,41]. Statistical significance was assessed at a two-tailed P=.05 level.

3. Results

The characteristics of the subjects are given in Table 1. None of the participants was using calcium supplements; therefore, calcium intake was derived only from dietary sources. According to the World Health Organization definition of osteoporosis [42], the sample included 17.6% normal women, 47.4% osteopenic women and 35% osteoporotic women. Among them, 71.3% had never smoked, 20.9% were smokers and 7.8% were former smokers, while 26.8% reported at least one fracture of the peripheral skeleton and 73.2% had no fractures. Only body weight and BMI were significantly different between VDR genotypes. Therefore, body weight was used as covariate in the regression models in order to assess the effect of the polymorphisms on bone phenotypes independently of the possible indirect effect of the polymorphisms through weight (which is known to directly affect BMD).

Information on selected SNPs of the VDR gene is presented in Table 2. All SNPs were in HWE (Table 2). None of the polymorphisms was associated with the presence of osteoporosis and the presence of fractures. VDR polymorphisms were not associated with unadjusted BMD in the lumbar spine and hip (Table 1). Furthermore, no associations between any of the polymorphisms and BMD at any skeletal site, fractures and the presence of osteoporosis were found when adjustment for age, weight, height and calcium intake was performed. Also, when the covariate of calcium intake–VDR genotype interaction was added in the regression models, the results were not altered, and both genotypes and their interactions with calcium intake were not significantly associated with BMD, fractures and the presence of osteoporosis (P>.05).

In order to evaluate whether the genetic effect on BMD differs by calcium intake, we stratified our analysis according to calcium intake [above median to high intake (>680 mg/day) and below median to low intake (<680 mg/day)]. In the lower calcium intake group, all polymorphisms were associated with lumbar spine BMD (Table 3), while TaqI and BsmI were also associated with the presence of osteoporosis [odds ratio (OR)=2.141, 95% confidence interval (95% CI)=1.098–4.174, P=.025, and OR=2.216, 95% CI=1.141–4.306, P=.019, respectively]. After adjustment for age, weight and height, TaqI (t allele) and BsmI (B allele) polymorphisms remained associated with the presence of osteoporosis (OR=2.321, 95% CI=1.131–4.765, P=.021, and OR=2.180, 95% CI=1.084–4.387, P=.028, respectively), while only Cdx-2 polymorphism was significantly associated with lumbar spine BMD (P=.025) (Fig. 1). In particular, subjects with A allele had lower spine BMD compared to subjects with the common G allele (Table 4). BsmI and TaqI polymorphisms were also associated with adjusted spine BMD, but these results did not reach statistical significance (P=.074 and P=.080, respectively). In the higher calcium intake group, none of the polymorphisms studied was associated with any of the assessed phenotypes.

4. Discussion

This cross-sectional association study aimed to analyze the contribution of VDR polymorphisms to variations in lumbar spine and hip BMD in a sample of Greek postmenopausal women. Our results confirm the association of VDR gene with BMD only in subjects with low calcium intake, thus verifying the concept that the effect of this gene is modified by calcium intake.

The frequencies of the selected SNPs were similar to those in previous studies in Greek populations [34,36], as well as to those in other studies in Caucasian populations [5,43]. We found significant associations of VDR polymorphisms with body weight and BMI. The bb and TT genotypes of BsmI and TaqI polymorphisms, respectively, and the G allele of Cdx-2 polymorphisms were associated with higher weight and BMI. These findings have also been shown in previous studies in women [44] and men [45]. The pathophysiological

Table 3
Unadjusted means (±S.D.) of lumbar spine BMD, by VDR polymorphisms in the lower calcium intake group (<680 mg/day)

Variable	BsmI			P*	TaqI			P*	Cdx-2			P*
	BB	Bb	bb		TT	Tt	tt		AA	AG	GG	
Spine BMD (g/cm^2)	0.870 (±0.164)	0.918 (±0.177)	0.965 (±0.191)	.022	0.957 (±0.193)	0.917 (±0.169)	0.865 (±0.176)	.032	0.878 (±0.228)	0.873 (±0.143)	0.954 (±0.189)	.008

An unadjusted linear regression model using spine BMD (continuous variable) as dependent variable was applied.
* Statistically significant differences at P<.05.

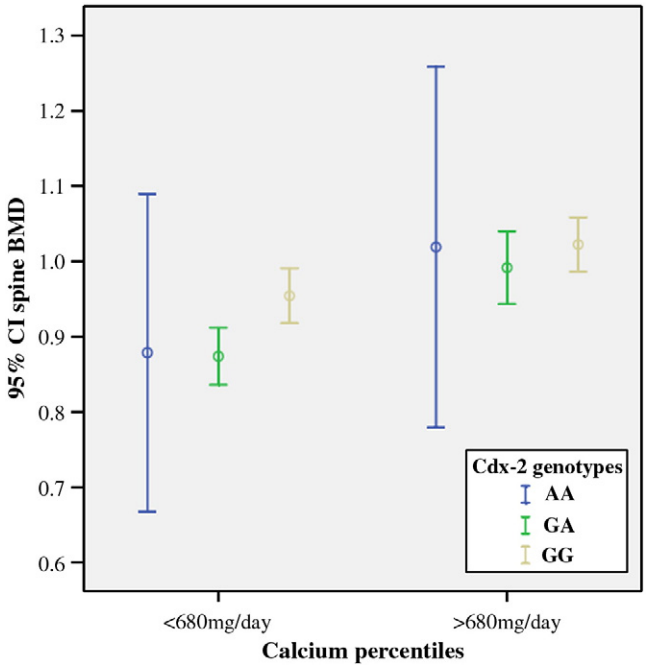

Fig. 1. The effect of Cdx-2 genotypes on spine BMD, by calcium intake.

mechanisms of these associations still remain unexplained. A direct effect of vitamin D on adipocyte differentiation and metabolism is a possible mechanism [46]. Some researchers also suggested that the effect of VDR polymorphisms on BMD is mediated through an effect on body weight [5]. However, since the main purpose of this study was the assessment of the direct associations of VDR polymorphisms with BMD, body weight was used as covariate in the regression models. Therefore, the results given in all the adjusted models describe the direct effects of the polymorphisms on BMD independently of their effect on body weight.

We found that VDR polymorphisms were not associated with BMD, fractures of the peripheral skeleton or the presence of osteoporosis in the total sample. These findings are in accordance with previous Greek studies [35,36], which also did not detect significant associations of BsmI and TaqI polymorphisms with BMD in postmenopausal women. Also, Cdx-2 polymorphism was not associated with BMD in a large Caucasian population in the study of Fang et al. [43].

However, we did observe significant associations of VDR variants with BMD and the presence of osteoporosis when analysis was performed in the lower calcium intake group (<680 mg/day). It has been suggested that significant BMD differences between VDR polymorphisms might be detectable mainly when calcium intake is in the low to medium range, while higher calcium intake would improve BMD in all genotypes and, therefore, the genetic effect would be masked [2,19,20,40]. In this perspective, we performed a separate analysis for higher and lower calcium intake groups. Our results confirmed that the effect of VDR polymorphisms is significant only in subjects with lower calcium intake (<680 mg/day).

More precisely, we revealed that in the lower calcium intake group, the presence of the B allele of BsmI polymorphism increased the risk of osteoporosis by 118%, and the presence of the t allele of TaqI polymorphism increased the risk by 132%. This finding is stable even after adjustment for age, weight and height. Also, these polymorphisms were associated with spine BMD, with the minor allele exhibiting a negative effect. These results, however, did not reach significance following adjustment. Our results are in accordance with previous findings. The meta-analysis of Thakkinstian et al. [26] also showed that the B allele of BsmI polymorphism was associated with lower spine BMD in postmenopausal women. Smaller studies in Caucasian samples of postmenopausal women also confirm our results regarding the effect of both BsmI and TaqI polymorphisms [14,47].

Concerning the effect of Cdx-2 polymorphism, the results were also significant only in the lower calcium intake group. The A allele was associated with lower spine BMD compared to G allele. This polymorphism is a G/A substitution in the Cdx-2 site, which is a functional binding site for the intestine-specific transcription factor Cdx-2 in the 1a promoter region of the VDR gene [48]. Also, Arai et al. [49] demonstrated that there are functional differences between the two alleles, whereby the A allele had increased binding to Cdx-2 *in vitro*. However, epidemiological and functional data provide independent lines of evidence [29]. The A allele was also found to be associated with increased BMD in a small sample of Japanese postmenopausal women (a population with low calcium intake due to different food patterns) [49]. This result was verified in Spanish postmenopausal women [50]. A large study in a Caucasian population found a trend of association towards this direction only in the low calcium intake group, but the result did not reach statistical significance [43], while a meta-analysis did not verify the association of Cdx-2 polymorphism with BMD [29]. However, in this study, no adjustments for calcium intake were performed. The study of Macdonald et al. [5] showed an association of the G allele of Cdx-2 polymorphism with decreased femoral neck BMD in the low calcium intake group, which, however, was not statistically significant when adjusted for body weight. In the present study, we found an opposite association of Cdx-2 polymorphism with BMD, where the risk allele is the minor allele A. Nevertheless, this is a common finding when studying VDR polymorphisms. A characteristic example is the case of BsmI polymorphisms, where the risk allele is either B or b when studies are performed in different ethnic populations and under different study designs and methodologies [2]. Furthermore, the study has an 81% power to detect the effect of Cdx-2 polymorphism on the lumbar spine BMD of subjects with lower calcium intake (posterior analysis).

The significance of the abovementioned results is important. Subjects with low calcium intake are known to be at risk for low BMD. The genetic effect of VDR polymorphisms exposes subjects to a greater risk of osteoporosis. Therefore, this high-risk population should be encouraged to improve its daily calcium intake in order to overcome the negative influence of genetic background. In line with this, VDR polymorphisms did not seem to have any negative effect on BMD or osteoporosis risk in the higher calcium intake group, a finding that implies that high calcium intake has a protective role against the genetic impact of VDR polymorphisms. Therefore, it appears that calcium intake can modify the genetic effect of VDR polymorphisms on BMD in postmenopausal women. However, a direct interaction of calcium intake with VDR polymorphisms was shown to be statistically insignificant in the total sample; thus, an indirect effect of calcium intake could be speculated. Nevertheless, an elucidation of

Table 4
Results of the multiple linear regression model in the lower calcium intake group (<680 mg/day) using lumbar spine BMD (continuous variable) as dependent variable

Parameters	Core model[a]	
	Standardized *B*	*P**
Cdx-2 genotypes	−0.050	**.025**
Age (years)	0.001	.442
Weight (kg)	0.006	<**.001**
Height (m)	−0.310	.190

[a] Linear regression analysis using age, weight and height as covariates.
* Statistically significant differences at $P<.05$.

biological mechanisms underlying this observation cannot be made due to the observational design of the study.

Among the strengths of this study is the use of a homogenous study population, which was composed of postmenopausal women of Greek origin only. This guarantees against false-positive results due to population admixture. Also, we assessed and included in the analysis most of the potential important covariates (age, weight, height and calcium intake). Finally, all BMD measurements were performed both at the lumbar spine and at the hip by the same operator and with the same device. Sample size is usually a limitation when studying the genetic bases of complex diseases, especially when the sample must be subdivided into groups (e.g., of different calcium intakes) for analysis. However, the study has satisfactory power (81%) to detect the effect of Cdx-2 polymorphism on lumbar spine BMD in the low calcium intake group. Limitations are as follows: (a) only a small number of VDR variants were analyzed; (b) the effect of other SNPs on the same gene could not be assessed; and (c) the study used an observational design, which does not allow the elucidation of biological mechanisms.

In conclusion, this study showed that VDR polymorphisms play a significant role in osteoporosis risk and spine BMD determination in Greek postmenopausal women with low calcium intake. BsmI and TaqI polymorphisms are associated with the presence of osteoporosis, while Cdx-2 polymorphism is associated with spine BMD. Increased calcium intake in our population seems to overcome the genetic effect of these polymorphisms. Therefore, high calcium intake and dietary counseling are considered important for subjects carrying the risk alleles of VDR polymorphisms.

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