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ORIGINAL ARTICLE

CYP2R1 polymorphisms are important modulators of circulating 25-hydroxyvitamin D levels in elderly females with vitamin insufficiency, but not of the response to vitamin D supplementation

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Abstract

Summary We studied the association between *CYP2R1* genetic polymorphisms and circulating 25-hydroxyvitamin D [25(OH)D] before and after supplementation with vitamin D3 in 218 elderly. We found differences between 3 and 8 ng/ml in circulating levels at baseline in women but not in the response after 1 year of supplementation.

Introduction This study evaluated the association between polymorphisms in four single nucleotide polymorphisms (SNPs) of the *CYP2R1* gene and 25(OH)D levels before and 1 year after supplementation with two different doses of vitamin D3 (600 IU daily or a dose equivalent to 3750 IU daily), in a cohort of 218 (96 men and 122 women) Lebanese elderly overweight subjects.

Methods Genotyping was performed for rs12794714, rs10741657, rs1562902, and rs10766197 SNPs using real-time PCR. The 25(OH)D levels were measured by liquid chromatography tandem mass spectrometry.

Results At baseline, the mean ± SD age was 71.0 ± 4.7 years, BMI 30.3 ± 4.6 kg/m^2, and 25(OH)D level was 20.5 ± 7.6 ng/ml. There were significant differences in mean 25(OH)D levels between genotypes in women, but not in men. After adjustment for age, season, and BMI, the homozygous for the low frequency gene variant (HLV) of rs1562902 and rs10741657 SNPs had the highest mean 25(OH)D levels with difference of 7.6 ng/ml for rs1562902 SNP ($p < 0.01$) and of 5.9 ng/ml for rs10741657 ($p = 0.05$) compared to the homozygous for the major polymorphisms (HMPs). Conversely, for rs10766197 and rs12794714 SNPs, HMP had the highest mean 25(OH)D levels with difference of 6 ng/ml for rs10766197 ($p = 0.003$) and of 4.8 ng/ml ($p = 0.02$) for rs12794714, compared to the HLV. *CYP2R1* genetic polymorphisms explained 4.8 to 9.8 % of variability in 25(OH)D in

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women. After 1 year, there was no difference in the response to vitamin D3 supplementation between genotypes in either gender.

Conclusion This study showed a difference in 25(OH)D levels between *CYP2R1* genotypes that equates a daily supplementation of 400–800 IU vitamin D, depending on genotype. It underscores possible important genetic contributions for the high prevalence of hypovitaminosis D in the Middle East.

Keywords 25(OH) vitamin D · CYP2R1 · Genetic polymorphisms · Metabolic pathway · Vitamin D supplementation

Introduction

Over the last decade, an emerging body of evidence suggests that vitamin D (VitD) may not be limited to musculoskeletal health in humans [1]. Although the importance of VitD on mineral metabolism is well recognized, the optimal circulating 25-hydroxyvitamin D [25(OH)D] level has been a matter of great debate lately, as illustrated in the discrepancy between the Institute of Medicine 2011 recommendations and the Endocrine Society 25(OH)D guidelines [2, 3]. This also translates into differences in the recommended dose of VitD needed to reach such recommended desirable target levels.

VitD is in large part obtained from skin synthesis under ultraviolet B radiation exposure, and smaller amount is obtained from diet. VitD from diet (D2 and D3) or skin (D3) is biologically inactive, and is converted in the liver, to 25(OH)D, the major circulating form of inactive VitD [4]. Activation of VitD at the liver is carried out by the hepatic vitamin D3 25-hydroxylase. Six cytochrome P450 enzymes have been reported as the hepatic VitD 25-hydroxylase. These include CYP2C11, CYP2D25, CYP2J3, CYP3A4, CYP27A1, and CYP2R1 [5, 6]. The first three of these cytochromes have been reported as the hepatic VitD 25-hydroxylase in animals. CYP3A4 is a human hepatic P450 with 25-hydroxylation activity, but its activity has been demonstrated toward D2, but not D3. The contribution of CYP27A1 to VitD activation in humans has been questioned and, in a study comparing VitD metabolism by CYP2R1 with human mitochondrial CYP27A1, it has been shown that CYP2R1 hydroxylation activity toward D3 was 26-fold higher than that of CYP27A1, suggesting that CYP2R1 plays the major role in VitD hydroxylation in humans [7]. Nevertheless, it is important to note that, although recent in vivo experiments on Cyp2R1 and Cyp27A1 knockout mice strongly supported this conclusion, they indicated that CYP2R1 is a major, but not exclusive contributor to 25(OH)D production and that an additional as-yet unknown enzyme contributes to this step [8].

Determinants of circulating 25(OH)D concentrations include sun exposure and diet, but heritability and genes involved in VitD metabolism, transport, degradation, and downstream pathways also play an important role [9–12]. The most consistent evidence has been the positive association of *CYP2R1* genetic polymorphisms and increased risk of VitD insufficiency, and two common SNPs, rs10766197 and rs10741657, located in the promoter region of *CYP2R1*, were consistent predictors of 25(OH)D levels [9, 11–18]. However, most of these studies were conducted in individuals suffering from diseases associated with VitD deficiency [14, 15, 17, 19], and applicability of findings from these studies to all races and age groups remain to be elucidated. Genes may also have an impact on the response to VitD supplementation, but studies assessing this effect are scarce and limited to western populations [20–22].

Although Middle Eastern countries enjoy plenty of sunshine most days of the year, subjects from this region have some of the lowest levels of 25(OH)D worldwide [23–28]. The basis for such low levels has not been completely elucidated; it includes gender, season, clothing style, socioeconomic status, and lifestyle [23–26]. However, the possibility for an underlying genetic modulation remains largely unexplored. In this study, we investigated the association between four *CYP2R1* genetic polymorphisms and baseline circulating 25(OH)D levels, as well as the response to VitD3 supplementation in 218 Lebanese elderly patients.

Methods

Study protocol

This is a randomized, double blind-placebo controlled trial evaluating the impact of two doses of VitD (600 IU per day or a dose equivalent to 3750 IU daily) on musculoskeletal and nonmusculoskeletal parameters in overweight elderly subjects. The trial protocol is registered on the NIH Clinical Trial website, ClinicalTrials.gov, trial no. NCT01315366. The current analyses were prespecified under secondary outcomes. The study was approved by the Institutional Review Board of the American University of Beirut. All subjects signed an informed consent before participation.

Study participants

Two hundred fifty-seven subjects participated in the trial, 221 recently completed the 1-year study, and DNA was available for genotyping for 218 subjects (122 women and 96 men). The study participants were recruited between January 2011 and July 2013 in collaboration with three academic institutions in Lebanon: the American University of Beirut Medical Center (AUBMC), the medical center of the Saint Joseph University (Hotel Dieu de France Hospital), and the

medical center of the Lebanese University (Rafic Hariri University Hospital), or were referred from community health dispensaries of the Ministry of Social Affairs from various regions in the Greater Beirut Area. A simple randomization procedure was implemented by the senior pharmacist, allocation was concealed, and all study team members and subjects were blinded to treatment until study completion. Randomization and all study visits after screening took place at a single site (AUBMC). Subjects were enrolled in the study if they were ≥65 years and overweight (BMI ≥ 25 kg/m^2). Exclusion criteria included rickets, osteomalacia, history of kidney stones, chronic diseases or major organ failure, intake of medications known to affect bone or glucose metabolism, and a history of diabetes or HbA1c ≥ 6.5 % at screening. Other exclusion criteria were a history of fragility fracture, a 10-year estimated risk for major osteoporotic fractures calculated using FRAX Lebanon ≥10 % (http://www.shef.ac.uk/FRAX/tool.aspx?country=21), a serum Ca >10.6 mg/dl, or a baseline 25(OH)D level less than 10 ng/ml.

Assessments

Clinical assessment

Height (cm) was measured in triplicate using a wall stadiometer, and the average of the three measurements was used in the analyses. Weight (kg) was measured using a regular balance. BMI (kg/m^2) was calculated.

Laboratory studies

Blood was drawn at baseline and at 12 months for serum calcium, phosphate, creatinine, and 25(OH) D. Calcium and phosphate were measured on fresh serum the same day using the Roche Modular Auto-analyzer (Roche Diagnostics GmBH, Mannheim, Germany). Serum creatinine level was also measured the same day using the kinetic colorimetric assay. Serum was stored at −70 °C, and 25(OH)D levels were measured by liquid chromatography tandem mass spectrometry (LCMS/MS) at the Mayo Clinic Laboratories (Mayo Clinic Foundation, Rochester, MN. ThermoFisher Scientific, Franklin, MA 02038, and Applied Biosystems-MDS Sciex, Foster City, CA 94404). Intra-assay coefficient of variations (CVs) are 3.8, 2.4, and 4.7 % at 25, 54, and 140 ng/ml, respectively. Inter-assay CVs are 6.4, 6.8, and 5.0 % at 25, 54, and 140 ng/ml, respectively.

Genetic studies

Two milliliters of whole blood were collected in EDTA tubes. DNA was isolated using a DNA isolation kit from Qiagen (Germantown, MD, USA) according to the manufacturer's guidelines and stored at −20 °C until analysis. Four *CYP2R1* SNPs (rs12794714, rs10741657, rs1562902, and rs10766197) were screened using TaqMan® allele discrimination assays on a CFX36 real-time PCR from BioRad. Ten percent of samples were genotyped twice, and results showed 100 % reproducibility.

Statistical analyses

Analyses were carried out using SPSS software, version 22.0 (SPSS, Inc., Chicago, IL) and SigmaPlot 12.0 (Systat Software Inc., San Jose, CA). p Values <0.05 were considered statistically significant.

The allele frequencies of each SNP were assessed for Hardy-Weinberg equilibrium (HWE) using chi-squared and compared to the frequencies reported from Caucasian and Arab populations.

At baseline

Mean 25(OH)D levels were compared for each *CYP2R1* SNP using the one-way analyses of variance (ANOVAs), and post hoc tests were performed using the least significant difference (LSD) test to assess the paired difference between two genotypes within each SNP.

Multivariate linear regression analyses were conducted to study the relationship between 25(OH)D levels and *CYP2R1* genotypes. Regression models were created for each SNP separately. Possible confounders such as gender, age, BMI, and seasonal variation were included into the model using the Enter method. The predictors were entered by blocks where the first block included age and gender, the second block included BMI, the third block included season (after creating dummy variable, considering fall season as a reference), and the fourth block included *CYP2R1* genotypes, after creating dummy variables whereby the homozygous for the major polymorphisms (HMP) was considered the reference for all SNPs. The last block included the interaction between *CYP2R1* genotypes and gender. We then reproduced the linear regression analyses stratified by gender.

Multivariate logistic regression was also conducted to study the factors contributing to 25(OH)D levels ≥20 ng/ml.

At 1 year

We checked for interaction between treatment arm and *CYP2R1* genetic polymorphisms by creating linear regression models with the absolute changes in 25(OH)D levels as outcome and the *CYP2R1* genotypes, treatment dose, and interaction term (treatment dose × *CYP2R1* genotypes) as predictors. Because there were no significant interactions at any SNP, we merged the high and low dose in the 1-year analyses.

The absolute changes in serum 25(OH)D in response to vitamin D supplementation were compared between different genotypes for each SNP by ANOVA. The tests were performed in the overall group and within each gender.

Multivariate analyses were also conducted to adjust for potential confounders, where linear regression models were created with the changes in 25(OH)D at 1 year as outcome, and the *CYP2R1* genotypes, age, gender, season at enrollment, BMI, and baseline 25(OH)D levels were entered as predictors.

Results

Baseline analyses

The baseline characteristics are shown in Table 1. Study subjects were recruited throughout the four seasons of the year. Their mean age was 71 ± 4.7 years, BMI 30.3 ± 4.6 kg/m^2. Men were older than women, but women had higher BMI, tended to have a higher serum 25(OH)D level, and were more likely to be on calcium and low-dose VitD supplementation (17 and 1 %, respectively). Half of the study subjects had 25(OH)D levels below 20 ng/ml at baseline, with similar rates of VitD insufficiency between genders. Allele frequencies were in HWE, and the minor allele frequencies were similar to those observed in Caucasian populations for all four SNPs, and also to those reported in Arab populations for rs12794714 and rs10741657 SNPs (Supplemental Table 1).

Relationship between baseline 25(OH)D and potential predictors in bivariate analyses

There was a seasonal variability in 25(OH)D levels. In the overall population, the lowest levels were noted in winter with a mean ± SD of 18.6 ± 5.9 ng/ml and the highest levels were observed in summer (22.7 ± 6.8 ng/ml). They were in between in the spring (18.8 ± 7.9 ng/ml) and in the fall (20.8 ± 8.2 ng/ml) ($p = 0.04$ by ANOVA between seasons). However, female subjects did not exhibit seasonal variability ($p = 0.16$), possibly because of their intake of VitD supplementation at study entry. 54.5 % of the women were veiled at randomization (55.6 % of women in the high-dose group and 53.3 % of the women in the low-dose group), without difference in baseline 25(OH)D levels between veiled and nonveiled women

Table 1 Baseline demographics and clinical characteristics of the study cohort, in the overall group, by gender, and by *CYP2R1* genotype

	Overall, $N = 218$	Men, $N = 96$	Women, $N = 122$
Age (years)*	71.0 ± 4.7	72.5 ± 5.5	69.8 ± 3.6
BMI (kg/m^2)*	30.3 ± 4.6	28.7 ± 3.1	31.5 ± 5.1
Season, *N* (%)			
Fall	32 (14.7)	18 (18.8)	14 (11.5)
Winter	48 (22.0)	20 (20.8)	28 (23.0)
Spring	87 (39.7)	36 (37.5)	51 (41.8)
Summer	51 (23.4)	22 (22.9)	29 (23.7)
Serum creatinine (mg/dl)*	0.8 ± 0.2	0.9 ± 0.2	0.7 ± 0.1
Estimated GFR (ml/min)*[a]	81.1 ± 13	78.9 ± 13	83 ± 12.8
Serum calcium (mg/dl)*	9.5 ± 0.4	9.4 ± 0.4	9.5 ± 0.4
Serum phosphate (mg/dl)*	3.4 ± 0.5	3.2 ± 0.4	3.6 ± 0.4
Total protein (g/l)	71.7 ± 4.2	71.5 ± 4.2	71.8 ± 4.3
Serum 25(OH)D (ng/ml)	20.5 ± 7.6	19.5 ± 6.5	21.3 ± 8.3
25(OH)D ≥ 20 ng/ml N (%)	109 (50)	43 (44.8)	66 (54.1)
25(OH)D < 20 ng/ml N (%)	109 (50)	53 (55.2)	56 (45.9)
CYP2R1 genotype	25(OH)D (ng/ml)		
rs1562902 SNP**			
HMP	19.1 ± 7.3	16.0 ± 5.3	18.4 ± 6.8
Heterozygous	20.8 ± 7.6	17.0 ± 6.2	22.5 ± 8.5
HLV	22.5 ± 8	19.3 ± 8.9	26.5 ± 8.4
rs10741657 SNP**			
HMP	19.7 ± 7.3	16.2 ± 5.5	18.5 ± 7.8
Heterozygous	20.9 ± 7.2	16.9 ± 6.3	19.9 ± 8.0
HLV	22.1 ± 10	21.0 ± 9.5	23.3 ± 11.3
rs10766197 SNP**			
HMP	22.1 ± 8.2	18.4 ± 7.6	24.8 ± 9.3
Heterozygous	20.1 ± 7.6	16.9 ± 6.3	20.9 ± 8.0
HLV	19.1 ± 6.4	16.0 ± 5.7	18.3 ± 6.4
rs12794714 SNP**			
HMP	21.9 ± 8.4	18.8 ± 7.7	23.6 ± 9.8
Heterozygous	20.2 ± 7.4	16.7 ± 6.4	21.5 ± 7.1
HLV	19.2 ± 6.5	15.9 ± 5.1	18.5 ± 6.5

HMP homozygous for the major polymorphism, *HLV* homozygous for the low frequency gene variant

*p Value <0.01 for difference between males and females by t test or chi-squared, as applicable; **significant differences between genotypes in women only ($p < 0.05$ for all SNPs by ANOVA)

[a] Estimated GFR calculated using the CKD-EPI equation

(21.7 ± 7.8 ng/ml in nonveiled versus 19.9 ± 8.4 ng/ml in veiled). The lack of baseline seasonal variability in women persisted after excluding veiled women.

Relationship between baseline 25(OH)D and CYP2R1 polymorphisms in bivariate analyses

There was no significant difference in 25(OH)D levels between *CYP2R1* genotypes with any of the four SNPs studied in the overall population (Table 1).

However, when the analyses were repeated separately for each gender, there was a significant difference in 25(OH)D levels between genotypes in women, with the highest difference for the four SNPS being for rs1562902 where the mean 25(OH)D level for homozygous for the low frequency gene variant (HLV) was 26.5 ± 8.4 ng/ml, 22.5 ± 8.5 ng/ml in heterozygous, and 18.4 ± 6.8 ng/ml in HMP (p = 0.001). The differences between genotypes were in the same direction for rs10741657 SNP (Fig. 1 and Table 1). Similar differences between the genotypes of rs10766197 SNP and rs12794714 SNP were noted, but HMP had the highest levels (Fig. 1 and Table 1). Such trends were not observed in males (Table 1 and Supplemental Fig. 1).

Multivariate analyses

Because of the lack of association between *CYP2R1* polymorphisms and 25(OH)D levels in bivariate analyses in the overall

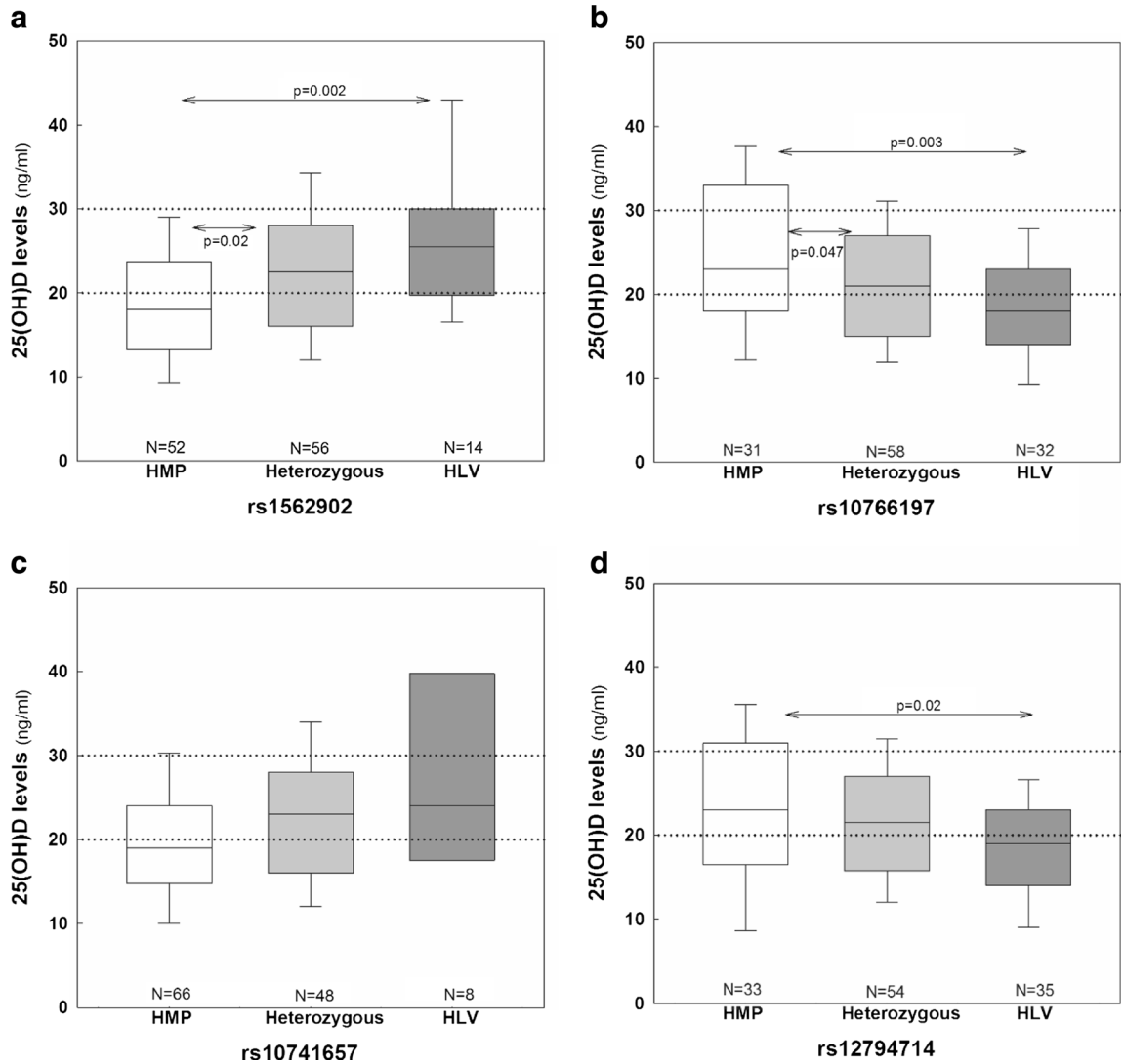

Fig. 1 Boxplots showing the median and interquartile levels (25th and 75th) for 25(OH)D levels in female group (N = 122) according to *CYP2R1* genotypes, using **a** rs1562902, **b** rs10766197, **c** rs10741657, and **d** rs12794714 SNPs. *Solid black lines* represent the median 25(OH)D levels whereas the *dotted ones* represent 20 and 30 ng/ml levels. There was a significant difference in 5(OH)D levels between genotypes in rs1562902 (p = 0.001), rs10766197 (p = 0.007), and rs12794714 (p = 0.03) by ANOVA. Post hoc p values are shown in the body of the figure. The highest levels were observed in females subjects who were homozygous for the low frequency gene variant (HLV) for rs1562902 SNPs and in female subjects who were homozygous for the major polymorphism (HMP) for rs10766197 and rs12794714 SNPs

population, and because there was a significant interaction between *CYP2R1* polymorphisms and gender in the linear regression models for three out of four SNPs (Supplemental Table 2), we performed the multivariate analyses by gender.

Table 2 Linear regression showing the association between *CYP2R1* genetic polymorphisms and baseline 25(OH)D levels, after adjustment for age, BMI, and season in women, according to the SNP used

		β (SE)	*p* value	Delta R^{2a} (*p* value)	R^2 of model
rs1562902 SNP					
Block 1	Age	0.03 (0.2)	0.89	0.00 (0.96)	
Block 2	BMI	−0.10 (0.1)	0.49	0.012 (0.23)	
Block 3	Fall[b]				
	Winter	−3.93 (2.6)	0.14	0.036 (0.23)	
	Spring	4.35 (2.4)	0.08		
	Summer	4.96 (2.6)	0.06		
Block 4	HMP[c]				
	Heterozygous	4.12 (1.5)	0.008	0.098 (0.002)	
	HLV	7.63 (2.4)	0.002		0.146
rs10741657 SNP					
Block 1	Age	0.02 (0.2)	0.94	0.00 (0.96)	
Block 2	BMI	−0.13 (0.1)	0.39	0.012 (0.23)	
Block 3	Fall[b]				
	Winter	−3.36 (2.7)	0.22	0.036 (0.23)	
	Spring	4.42 (2.5)	0.08		
	Summer	5.31 (2.7)	0.06		
Block 4	HMP[c]				
	Heterozygous	2.88 (1.5)	0.07	0.048 (0.055)	
	HLV	5.93 (3.0)	0.05		0.096
rs10766197 SNP					
Block 1	Age	−0.05 (0.2)	0.82	0.00 (0.96)	
Block 2	BMI	−0.11 (0.1)	0.45	0.012 (0.23)	
Block 3	Fall[b]				
	Winter	−3.47 (2.7)	0.20	0.036 (0.23)	
	Spring	4.02 (2.4)	0.11		
	Summer	4.82 (2.6)	0.07		
Block 4	HMP[c]				
	Heterozygous	−3.71 (1.7)	0.04	0.07 (0.012)	
	HLV	−6.06 (2.0)	0.003		0.119
rs12794714 SNP					
Block 1	Age	−0.04 (0.2)	0.87	0.00 (0.96)	
Block 2	BMI	−0.13 (0.1)	0.40	0.012 (0.23)	
Block 3	Fall[b]				
	Winter	−3.12 (2.7)	0.26	0.036 (0.23)	
	Spring	4.27 (2.5)	0.09		
	Summer	4.82 (2.7)	0.08		
Block 4	HMP[c]				
	Heterozygous	−2.28 (1.8)	0.21	0.048 (0.054)	
	HLV	−4.83 (1.9)	0.02		0.096

β estimate represents mean difference in serum 25(OH)D between genotypes compared to HMP

HMP homozygous for the major polymorphism, *HLV* homozygous for the low frequency gene variant

[a] Delta R^2: provides proportion of variance in 25(OH)D levels that is explained by the specific contribution of each block as a predictor

[b] Reference is fall

[c] Reference is HPM

In female subjects, a significant difference in 25(OH)D levels was observed for most SNPs. After adjustment for age, BMI, and season, *CYP2R1* polymorphisms explained between 4.8 and 9.8 % of variability (R^2) in 25(OH)D levels, depending on the specific SNP studied (Table 2).

In females, HLV for rs1562902 SNP had the highest 25(OH)D levels with differences of 7.6 ng/ml as compared to HMP and of 4.1 ng/l as compared to the heterozygous ($p < 0.01$). A similar trend was observed with rs10741657 SNP ($p = 0.05$). Conversely, for the rs10766197 and rs12794714 SNPs, HMP had the highest levels with differences of 6.0 ng/ml ($p = 0.003$) and of 4.8 ng/ml ($p = 0.02$) as compared to HLV for the rs10766197 and rs12794714 SNPs, respectively (Table 2).

In contrast, no such trends were observed in male subjects, and the variance in 25(OH)D levels explained by *CYP2R1* genetic polymorphisms was very minimal in male subjects, with values up to a maximum of 1.4 % (Supplemental Table 3).

In female subjects, additional logistic regression analyses revealed that, after adjusting for age, BMI, and seasonal variations, the likelihood of having a 25(OH)D level ≥20 mg/dl increased between 2.3- and 5.6-fold in rs1562902 and rs10741657, depending on the SNPs and zygocity (Table 3). Conversely, HLV of rs10766197 and rs12794714 SNPs were one fifth to one third less likely to have 25(OH)D levels ≥20 mg/dl (Table 3). Such association was not significant in males (Supplemental Table 4).

Table 3 Multivariate logistic regression models showing the association between the different *CYP2R1* genetic polymorphisms and the likelihood of having baseline 25(OH)D levels ≥20 mg/dl, adjusted for potential confounders in women Ω

SNP	Genotype[a]	OR (95 % CI)	*p* value
rs1562902	HMP		
	Heterozygous	3.35 (1.47–7.63)	0.004
	HLV	5.68 (1.34–23.99)	0.02
rs10741657	HMP		
	Heterozygous	2.30 (1.02–5.16)	0.04
	HLV	1.92 (0.41–9.05)	0.41
rs10766197	HMP		
	Heterozygous	0.51 (0.19–1.37)	0.18
	HLV	0.23 (0.08–0.70)	0.009
rs12794714	HMP		
	Heterozygous	0.21 (0.21–1.42)	0.21
	HLV	0.33 (0.12–0.92)	0.04

HMP homozygous for the major polymorphism, *HLV* homozygous for the low frequency gene variant

[a] HMP was considered reference for all SNPs

Ω Adjusted for confounders as age, BMI, and season

One-year data

There was no interaction between treatment dose and *CYP2R1* genetic polymorphisms in any of the four SNPs used. We therefore merged the low dose and high dose in the 1-year analyses.

The serum 25(OH)D levels at 1 year and the delta values in response to vitamin D supplementation in the overall population, according to *CYP2R1* genotypes, age group, gender, BMI, baseline 25(OH)D levels, and vitamin D dose, are shown in Table 4. In summary, 25(OH)D levels increased by 15.0 ± 10.1 ng/ml in the high-dose group and by 5.8 ± 7.7 ng/ml in the low-dose group ($p < 0.05$ for difference between groups). There was no significant difference in the delta 25(OH)D from baseline or in the 25(OH)D levels achieved at 1 year between genotypes with any of the four SNPs used (Table 4). The lack of significant relationship persisted after adjustment for age, gender, BMI, season at enrollment, and baseline 25(OH)D levels (data not shown). Similarly, there was no significant difference in the 25(OH)D levels achieved or in the delta values after 1 year within each gender (Table 5). Similar results were obtained when the percent changes in serum 25(OH)D levels were used as outcome (data not shown).

Discussion

The present study showed an association between baseline VitD status and *CYP2R1* genetic polymorphisms in Lebanese elderly overweight women. The likelihood of having sufficient 25(OH)D levels was 5.6 times higher in some genotypes compared to others. Moreover, after adjustment for age, BMI, and seasonal variability, there were differences ranging between 3.7 and 8 ng/ml in mean 25(OH)D levels between genotypes. Genetic polymorphisms explained between 4.8 and 9.8 % of variability in 25(OH)D levels in these women. We could not detect any effect of *CY2R1* genetic polymorphisms on changes of 25(OH)D levels after supplementation.

Genetic determinants of VitD status have been described in association studies of individuals of European [9, 29], Hispanic and African American [30, 31], and Asian descent [16, 32–35]. Twin and family-based studies have also previously confirmed that heritable factors have a considerable influence on 25(OH)D concentrations in Chinese subjects [32]. In previous studies looking at potential determinants of 25(OH)D in 203 elderly subjects and 336 children and adolescents, our group did not find any relationship between 25(OH)D levels and VitD receptor (VDR) gene polymorphisms assessed with three restriction enzymes [36, 37]. However, VDR mediates the action and not the synthesis of

Table 4 Serum 25 OHD at baseline, 12 months and delta values in relation to SNP genotypes, age, gender, BMI, baseline 25(OH)D levels, and vitamin D dose

Variables	Number	Baseline 25(OH)D (ng/ml)	12 months 25(OH)D (ng/ml)	Delta 25(OH)D (ng/ml)
SNP genotype				
rs1562902				
HMP	82	19.1 ± 7.3	30.3 ± 9.0	10.8 ± 9.6
Heterozygous	103	20.8 ± 7.6	30.4 ± 10.5	9.6 ± 9.9
HLV	33	22.5 ± 8	30.9 ± 9.7	11.2 ± 8.2
rs10741657				
HMP	108	19.7 ± 7.3	29.5 ± 8.7	9.4 ± 9.6
Heterozygous	89	20.9 ± 7.2	31.7 ± 10.6	10.9 ± 11.0
HLV	21	22.1 ± 10	34.3 ± 9.9	12.2 ± 7.2
rs10766197				
HMP	60	22.1 ± 8.2	32.2 ± 10.0	10.1 ± 10.3
Heterozygous	105	20.1 ± 7.6	30.8 ± 10.1	10.5 ± 10.3
HLV	52	19.1 ± 6.4	29.6 ± 8.7	10.3 ± 9.1
rs12794714				
HMP	62	21.9 ± 8.4	32.5 ± 10.2	10.7 ± 9.7
Heterozygous	104	20.2 ± 7.4	30.8 ± 9.9	10.4 ± 10.7
HLV	52	19.2 ± 6.5	29.1 ± 8.6	10.5 ± 8.9
Gender				
Women	122	21.1 ± 8.1	31.5 ± 10.3	10.2 ± 10.9
Men	96	19.4 ± 6.2	30.1 ± 9.0	10.5 ± 8.9
Age group				
65–75 years	184	20.5 ± 7.5	31.0 ± 10.0	10.2 ± 9.9
> 75 years	34	19.4 ± 6.8	30.1 ± 8.3	10.8 ± 10.4
BMI				
25–29.9 kg/m^2	182	20.5 ± 7.1	30.6 ± 10.2	9.7 ± 10.0
30–34.9 kg/m^2	36	20.5 ± 7.7	31.1 ± 9.1	10.8 ± 10.2
> 34.9 kg/m^2		19.3 ± 7.7	31.3 ± 9.3	11.6 ± 9.7
Baseline 25 OHD				
10–19.9 ng/ml	109	14.4 ± 3.3*	29.2 ± 9.2*	14.6 ± 9.2*
> 19.9 ng/ml	109	26.3 ± 5.2	32.6 ± 10.0	6.0 ± 9.0
Vitamin D dose				
600 IU daily	109	20.1 ± 6.1	25.9 ± 6.9*	5.8 ± 7.6*
3750 IU weekly	109	20.6 ± 7.8	36.0 ± 9.7	15.0 ± 10.1

HMP homozygous for the major polymorphism, *HLV* homozygous for the low frequency gene variant

*Significant difference between groups by independent *t* test ($p < 0.001$)

VitD, thus the need to study polymorphisms in genes involved in the hydroxylation of VitD.

We selected the CYP2R1 enzyme in this study because it has been shown to have the most potent hydroxylation activity toward VitD3 [6–8, 38], and because studies including two GWAS and others using few candidate genes showed an association between polymorphisms in *CYP2R1* and VitD insufficiency in Western populations [9, 11, 12]. Moreover, polymorphisms in *CYP2R1* gene have been associated with rickets in several studies in Asia, the Middle East, and Africa [25–28, 30–32, 36–42], and a recent report of two adolescent siblings from a Saudi family presenting with short stature and rickets revealed two new mutations in this gene [43]. The choice of the four candidate SNPs was based on the fact that they were the most studied with promising results. As shown in Supplemental Table 1, neither is associated with amino acid substitution; it is hence possible that they are located near genetic variants that are functionally important for the regulation of 25(OH)D levels. Interestingly and similarly to previous studies in Caucasians [11] and Asians [33], linkage disequilibrium (LD) analysis using Haploview 4.2 (Cambridge, MA) showed that all four SNPs were in strong LD (Supplemental Fig. 2).

Table 5 Serum 25(OH)D levels in ng/ml at 1 year and delta values by genotypes (according to the SNP used), in men and women

SNP	Genotype	One-year 25(OH)D levels (ng/ml)		Delta 25(OH)D (ng/ml)	
		Men	Women	Men	Women
rs1562902	HMP	29.7 ± 7.8	30.7 ± 9.8	9.5 ± 7.7	11.7 ± 10.7
	Heterozygous	29.3 ± 10.0	31.4 ± 10.9	10.0 ± 9.6	9.2 ± 11.9
	HLV	30.1 ± 9.0	34.8 ± 10.3	13.6 ± 8.8	8.2 ± 6.5
rs10741657	HMP	28.5 ± 7.5	30.2 ± 9.5	8.3 ± 8.2	10.2 ± 10.5
	Heterozygous	31.0 ± 102	32.4 ± 11.1	11.9 ± 9.4	10.1 ± 11.2
	HLV	32.8 ± 9.0	36.5 ± 11.1	13.4 ± 8.5	10.3 ± 4.6
rs10766197	HMP	31.8 ± 8.6	32.6 ± 11.3	12.0 ± 10.5	8.3 ± 10.0
	Heterozygous	30.0 ± 9.9	31.5 ± 10.3	10.7 ± 8.4	10.3 ± 10.8
	HLV	28.1 ± 7.1	30.7 ± 9.7	7.9 ± 7.6	11.0 ± 9.8
rs12794714	HMP	32.8 ± 9.2	32.2 ± 11.0	13.1 ± 8.9	8.6 ± 10.1
	Heterozygous	29.2 ± 9.2	32.4 ± 10.5	10.1 ± 9.1	10.8 ± 12.2
	HLV	28.4 ± 7.5	29.5 ± 9.2	7.8 ± 7.1	10.6 ± 9.4

There was no interaction between treatment arm (vitamin D supplementation dose) and genetic polymorphisms. Thus, the data are presented for low dose (600 IU per day) and high dose (3500 IU per day) combined. No statistically significant difference was found between genotypes with any of the four SNPs, in either gender

HMP homozygous for the major polymorphism, *HLV* homozygous for the low frequency gene variant

In the current study, genetic polymorphisms explained up to 9 % of variability in 25(OH)D levels in women after adjustment for potential predictors. This is higher than the difference shown in a GWAS of 30,000 European subjects, in which Wang et al. showed that polymorphisms in the *GC*, *DHCR7/NADSYN1*, and *CYP2R1*genes explained up to 1–4 % of the variation in 25(OH)D concentrations [9]. In that GWAS, multiple SNPs in the *CYP2R1* gene, including rs12794714 and rs10741657, were significantly associated with 25(OH)D levels [9].

In the current study, HLV (AA) for rs10741657 and (CC) for rs1562902 SNPs, had the highest 25(OH)D levels compared to HMP (GG) for rs10741657 and (TT) for rs1562902 SNPs, with a difference reaching 7.6 ng/ml between genotypes [9]. This finding is in line with previous studies in healthy subjects from variable ethnic backgrounds [11, 13, 20], as well as in patients with autoimmune disorders such as type 1 diabetes and multiple sclerosis (MS) [14, 15]. Elkum et al. showed a similar association with rs10741657 SNP in 489 South Asian people living in Kuwait with a difference up to 2.8 ng/ml between genotypes [13]. Barry et al. showed a difference of 3.3 % (for rs1562902) and of 4.9 % (for rs10741657) per variant allele compared to the major polymorphism in non-Hispanic white US population, after adjustment for age, gender, and season [20]. Ramos-Lopez et al. also showed an association between rs10741657 SNP and 25(OH)D levels in 133 type 1 diabetes patients where subjects carrying the AA genotype of the rs10741657 polymorphism had higher levels of 25(OH)D compared to those with the GG and GA genotypes, with a difference up to 14 ng/ml between genotypes [14]. Similarly, Simon et al. found that, in women with MS, each additional A allele of *CYP2R1* rs10741657 was associated with a 2.1 ng/ml increase in 25(OH)D level [15]. On the other hand, in their nine candidate gene studies in healthy Caucasian subjects, Bu et al. showed an association between 25(OH)D levels and rs1562902 SNP in the discovery cohort, although this was not confirmed in the replication cohort [11].

Conversely to the findings with rs10741657 and rs1562902 SNPs, the HMP (GG) for rs12794714 and rs10766197 SNPs had the highest levels of 25(OH)D with a difference up to 6.1 ng/ml between genotypes. This is also consistent with others [11, 13, 20, 44]. In their candidate gene study, Bu et al. showed that the average serum 25(OH)D after pooling the discovery ($n = 156$) and replication ($n = 340$) cohorts were higher for the HMP (GG) as compared to other genotypes for rs12794714 SNP [11]. Similarly, Barry et al. showed that the HLV for these two SNPs had lower 25(OH)D levels with an estimated percentage difference of 3.8 % (for rs12794714) and 4.7 % (for rs10766197) per variant allele compared to the HMP, after adjustment for sex, season, and age [20]. Elkum et al. showed a similar association for rs12794714 SNP in 907 Arab subjects living in Kuwait state with a difference of 2.4 ng/ml between genotypes [13]. Similar results were shown by Batai et al. who found minor alleles of rs12794714 and rs10741657 to be associated with lower and higher baseline levels of 25(OH)D, respectively [44]. The effect of rs10743657 was also confirmed in a sample of Jordanese [42]. On the other hand, Zhang et al. screened 15 key genes within the VitD metabolic pathway in 2897 unrelated healthy Chinese subjects. Variants and/or haplotypes in *GC*, *DHCR7/NADSYN1*, and *CYP2R1* were identified as

being associated with 25(OH)D levels [16]. For *CYP2R1* gene, the significant association was observed with rs10766197 polymorphism with the highest mean levels being observed in the HMP [16].

The absolute differences in adjusted mean 25OHD levels we note are quite large. To our knowledge, one of the largest differences in 25(OH) D levels due to polymorphism in the coding region of *DBP*, causing the classical polymorphism of GC1–2, is associated with a modest difference in serum 25(OH) D of 2–6 ng/ml [31], as opposed to environmental and lifestyle factors accounting for a larger variability in 25(OH)D levels, varying between 5 and 10 ng/ml and reaching 15 ng/ml in some studies [45]. Supplemental Table 5 summarizes reported differences in serum 25(OH)D levels, and these are for the most part, with the exception of the study by Hassanein et al. [46], much more modest than ours, and internally consistent between each other. In our study, the highest difference in mean serum 25(OH) levels for the rs1562902 amounts to 7.6 ng/ml for HLV vs. HMP, that is accounting for 30–43 % of the total mean 25(OH)D level depending on the mean 25(OH) level considered. These differences are quite similar to those observed by Hassanein et al. in an Egyptian population [46]. This observation raises the intriguing possibility for an important genetic contribution to the high prevalence of hypovitaminosis in populations from the Middle East.

We did not find a significant effect for *CYP2R1* genetic polymorphisms in the overall population, and we do not have an explanation for the discriminative effect of gender on the relationship between *CYP2R1* genetic polymorphisms and serum 25(OH)D levels in the current study except for the smaller sample size in men. However, previous studies included either women only or both genders, but did not evaluate the association within genders.

There was no effect of genetic polymorphisms on the response to vitamin D supplementation after 1 year. The lack of relationship was persistent in the overall group and within genders, and there was no interaction with vitamin D dose. A recent study by Nissen et al. [21] assessed for the association between 25 widely studied SNPs located in or near genes involved in synthesis, transport, activation, or degradation of vitamin D and the increase in 25(OH)D concentration after 6 months of artificial UVB irradiation in 92 healthy Caucasian subjects with a mean age of 38 years. They found that rs10741657 in *CYP2R1* modulated the response to UVB, and carriers of the two risk alleles of this SNP had the lowest baseline mean 25(OH)D concentration but also the smallest increase in 25(OH)D concentrations after UVB treatment [21]. Only three studies assessed the genetic effect on the response to vitamin D supplementation [20, 22, 47]. In a randomized controlled trial in 1787 healthy white participants aged 45–75 years, Barry et al. showed that the increase in 25(OH)D after 1 year of vitamin D3 supplementation at a daily dose of 1000 IU was modified by rs10766197 near *CYP2R1*, rs6013897 near *CYP24A1*, and rs7968585 near *VDR* [20]. Didriksen et al. pooled data from three randomized controlled trials (total of 568 subjects, 74 % women, aged 30–80 years), where vitamin D3 was given at a dose of 40,000 IU per week for 6 months. They found that baseline 25(OH)D levels were significantly related to SNPs in the *DBP* and *CYP2R1* genes. Those with SNPs associated with the lowest baseline 25(OH)D levels also had the smallest increase after supplementation [47]. More recently, Sollid et al. conducted a large randomized controlled trial on patients with prediabetes. They showed a significant association between three SNPs in three candidate genes and the levels of 25(OH)D after 12 months therapy with 20,000 IU of vitamin D3. Those with SNPs associated with the highest baseline 25(OH)D levels also had the highest increase after supplementation [22]. The reason for the difference between our findings and the findings of these studies could be related to the difference in 25(OH)D levels at baseline, the difference in the vitamin D3 dose, or maybe related to the smaller sample size in our population.

There are few limitations to our study. This is not a GWAS, and the data does not come from a population-based study, although it draws from a large proportion of the total population (Greater Beirut hosts 30 % of the Lebanese population at large) and included dispensaries from the Ministry of Health that attract subjects from the mountain areas. We nevertheless believe our study group to be illustrative of the Lebanese population. Indeed, using the most accurate assay method for VitD measurements, namely LC/MS, 50 % of the study subjects had serum 25(OH)D levels below 20 ng/ml. This finding is comparable to those of multiple studies that assessed VitD status among Lebanese over the past decade [23–27]. Similarly, although only overweight individuals were considered, because the primary outcome was insulin resistance, the overall BMI was also comparable to those reported in our population [48]. Only elderly subjects with baseline total of 25(OH)D ≥10 ng/ml were included, and this may have somewhat biased our results and underestimated the real impact of such polymorphisms on circulating 25(OH)D levels in the overall population. The study may be underpowered to address one of the post hoc questions this manuscript addresses, namely to investigate the impact of *CY2R1* polymorphisms on achieved 25(OH)D levels in response to therapy. However, the positive findings at baseline provide valuable information on modulators of serum 25(OH)D in subjects from Lebanon.

In conclusion, this is the first study in the MENA to systematically investigate a possible underlying genetic cause for the high prevalence of hypovitaminosis D in our sunny country, based on a large age segment of the population in whom VitD deficiency has a major impact on musculoskeletal health. It showed a difference in 25(OH)D levels between *CYP2R1*

genotypes that equates to a daily supplementation of 400–800 IU VitD. GWAS or additional candidate gene association studies are needed to identify additional polymorphisms that could potentially affect VitD status, and possibly the response to vitamin D supplementation in our region.

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Compliance with ethical standards The study was approved by the Institutional Review Board of the American University of Beirut. All subjects signed an informed consent before participation.

Conflicts of interest None.

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