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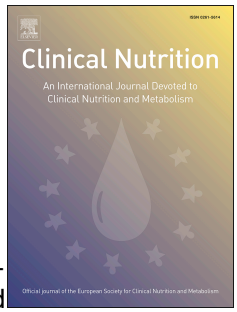

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A genetic variant in the Cytochrome P450 Family 2 Subfamily R Member 1 determines response to vitamin D supplementation

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Abstract

Background: Globally, about 1 billion people have inadequate levels of serum vitamin D and it is prevalent in all ethnicities and age groups. Few foods naturally contain sufficient vitamin D; therefore, most people get their requirements through supplementation. Hence vitamin D status is affected by genetic and environmental determinants including season of measurement, diet habitual, health status, body mass index and concurrent medication. Further studies are necessary to understand how genetic variation influences vitamin D metabolism. We aimed to explore the association between a potential vitamin D-related polymorphism (the rs10766197 polymorphism in the CYP2R1 gene) with the response to supplementation of vitamin D in 253 healthy Iranian girls.

Material and method: A total of 253 healthy subjects received 50000 IU of vitamin D3 weekly for 9 weeks. Serum 25(OH)D concentrations and metabolic profiles were measured at baseline and after 9 weeks of supplementation. The genotypes of the CYP2R1 variant (rs10766197) were identified using TaqMan genotyping assays.

Results: Serum 25(OH)D during the supplementation, increased in all individuals. Subjects with a AA major genotype at this locus had higher vitamin D concentrations after intervention (Changes (%) 448.4%±425% in AA vs 382.7%±301% in GG). This genetic variant modulated the response to supplementation (p <0.001 and p-value SNP=0.05). Regression analysis showed that the probability of affecting serum 25(OH)D, in individuals who had homozygous major allele GG was two-fold higher than carriers of the uncommon allele A (OR=2.1 (1-4.2); p = 0.03). Interestingly, the Hs-CRP was reduced in AA carries while was elevated in individuals with GG and AG genotypes, after high-dose vitamin D supplementation.

Conclusion: Changes in serum vitamin D and metabolic profile following high dose supplementation with vitamin D were associated with CYP2R1 polymorphism. Although carriers of the common G allele showed a greater response in the serum vitamin D.

Key words: 25(OH)D, Supplementation, CYP1R2, rs10766197

Introduction

Diet and other environmental factors such as the intake of vitamin D supplements and exposure to sunlight are known to influence serum vitamin D concentrations[1]. The assessment of serum 25-hydroxyvitamin D (25(OH)D) is the best biomarker of vitamin D status; however, the optimal serum concentration is unclear [2, 3]. A study in in the United States, has suggested that a serum 25(OH)D concentration of 50 nmol/L is sufficient for normal bone health in most individuals [4] whilst other studies have suggested that 60 nmol/L is necessary for reduction in the risks of falling and fractures risk [5, 6]. Vitamin D has functions other than bone health. It is involved in the regulation of more than 2000 genes. Vitamin D deficiency may be associated with several non-skeletal diseases, including cancer[7], obesity [8], asthma [9], diabetes [10], cardiovascular diseases (CVD)[11] and metabolic syndrome (MS) [12] and has been reported as a major public health concern, even in regions with high levels of sunlight [13], for example it is common in the Middle East, India, Africa, Australia and South America [14-16].

In line with this, there is increasing evidence for a high prevalence of vitamin D deficiency in Iran; with reports of deficiency in >80% of the adolescence in Tehran and Arak [17, 18], about 60% of school-age girls in Yazd [19] and >70% in newborn infants in Zanjan [20]. Few foods naturally contain enough vitamin D , the most natural way to get vitamin D is cutaneous production when skin is exposed to the sunlight [3]. Public concern about the high prevalence of vitamin D deficiency has caused increasing demand for supplementation and testing. Since individual responses to supplementation is variable, a more tailored approach to supplementation may be required. The variation in serum 25(OH)D level response after supplementation has been attributed to body mass [21], baseline serum 25(OH)D level [22], supplement dose [23], and the season [22]; however, there is also convincing evidence that vitamin D status is affected by genotype[24]. Several studies have

reported polymorphisms in candidate genes associated with serum vitamin D that include CYP24A1 and CYP2R1 [25, 26]. Each cytochrome P450 gene is known with CYP, implied that is part of the cytochrome P450 gene family. The common SNP, rs10766197, located in the promoter region of CYP2R1 gene, were reliable predictor of serum 25(OH)D levels[27].

The current study was carried out to examine whether treatment with high dose vitamin D supplementation is influenced by a variant in the CYP2R1 gene, using data obtained from a randomized controlled trial of vitamin D supplementation in healthy Iranian school-age girls of 12-18 years old; a group in which vitamin D deficiency is common.

Material and method

Study population

The 253 adolescent girls were recruited between January and April 2015 in Mashhad city, using a randomized cluster sampling method. Informed consent was collected from all participants using protocols approved by the Ethics Committee of the Mashhad University of Medical Sciences.

Participants with any chronic diseases history, or who were taking any kinds of dietary supplements and anti-depressant or psychotropic drugs were excluded from study.

Individuals with history of infectious disease, diabetes mellitus, family history of stroke, and myocardial infarction were excluded from study. Subjects received 50,000 IU vitamin D/week for 9 weeks. Serum 25(OH)D and metabolic profiles were measured at baseline and after 9 weeks.

Anthropometric and biochemical measurements

Anthropometric parameters (e.g., height, body weight, waist and hip circumference) were measured. BMI levels among teens expressed relative to other children of the same sex and age. Percentiles

were calculated using CDC growth charts (US Centers for Disease Control and Prevention (CDC) growth reference), which were based on national survey data collected from 1963-65 to 1988-94 [28]. Biochemical factors including serum serum calcium (Ca),and phosphate (P), , fasting blood glucose (FBG), creatinie, blood urea nitrogen (BUN) and lipid profile; total cholesterol (TC), triglyceride (TG), and high-density lipoprotein cholesterol (HDL-C), measured by using commercial kits (Pars Azmun, Karaj, Iran) and the BT-3000 auto-analyzer (Biotechnica, Rome, Italy). Low-density lipoprotein cholesterol (LDL-C) was estimated using Friedewald formula if serum TGs concentrations < 4.52 mmol/L [29-31]. High sensitivity C-reactive protein (Hs-CRP) was quantified using an immunoturbidimetry method, with limit of detection (LoD) 0.06 mg/L (Biosystems, Spain). Cut of value for Hs-CRP was < 1.90 mg/L in 5-18 years woman according to the manufacturer's instructions.

An electrochemiluminescence method (ECL, Roche, Basel, Switzerland) was performed to measure serum 25-OH vitamin D. The LoD for the 25-OH vitamin D assay was 10 nmol/L for the ECL (Roche) and intra- and inter-assay variation were 5.7% and 9.9%, respectively.

DNA extraction and genotyping

Genomic DNA was extracted from blood samples using QIAamp® DNA Mini-Kit (Qiagen, San Diego, CA) according to the manufacturer's instructions. The purity and concentration of DNA samples were determined using the NanoDrop®-1000-Detector (NanoDrop-Technologies, Wilmington, USA). Genotype analysis of CYP2R1-rs10766197 polymorphism was carried out using Taq-man®-probes-based assay; PCR reactions were performed in 12.5 ml total volume, using 20 ng of DNA in TaqMan®n Universal MasterMix with specific primers and probes (Applied Biosystems

Foster City, CA). To assess the allelic content. The ABIPRISM-7500 instrument equipped with the SDS version-2.0 software was used.

Statistics analysis

Data was analyzed using SPSS version 20, IBM (SPSS Inc., IL, USA). Variables are reported as mean ± standard deviation (SD). Continuous variables were analyzed for normality using the Kolmogorov–Smirnov test. Analysis of variance (ANOVA) was performed to compare changes in biomarkers after intervention in different genotype groups. Post hoc analysis was done using Tukey's test. A Chi square test with continuity correction was used to determine whether genotype frequencies followed the Hardy–Weinberg Equilibrium. Repeated measures analysis of covariance (ANCOVA) was performed to investigate the effect of the genotypes. Logistic regression was performed to study the probability of change in serum 25(OH) D in the genetic dominant model. Significance was set at $p < 0.05$.

Results

Influences of supplementation on circulation 25(OH)D in CYP2R1 variant

In the total population of 253 healthy school-age Iranian girls, 88.1% suffered from vitamin D deficiency at baseline and only 4% of the total had a desirable vitamin D level. However, after intervention, 59.7% of the subjects were at a desirable concentration of 25(OH)D. About 20.2% of the subjects remained vitamin D deficient (Fig. 1). To examine the influence of CYP2R1 variant on the circulation levels of vitamin D after intervention, subjects were categorized across rs10766197 genotype. The results revealed no significant trend in distribution of vitamin D status (desirable, sufficiency and deficiency) among different genotypes at baseline (P-trend = 0.4). However, supplementation for 9 weeks led to significant trend (P-trend =0.05) (Table 1), with a reduction in the percent of subjects with a low serum vitamin D. The serum 25 (OH) D responses was dependent

on the SNP in CYP1 (Fig. 2). During the supplementation, serum (OH) D increased in all groups, but carriers who had the common G allele, had higher vitamin D concentrations after 9 weeks of intervention. The SNP rs10766197 modulated response to vitamin D supplementation (*p*-value of intervention effect <0.001 and *p*-value SNP=0.05) (Fig. 2). Regression analysis also indicated that the probability of altering serum 25(OH)D, in individuals who had homozygous major allele GG was two-fold higher than carriers of the uncommon A allele (OR=2.1 (1-4.2); p value=0.03). The regression model also was significant using a dominant model (OR=1.8 (1-3.1); p value=0.05) (Table 3). Data was adjusted for potential confounders such as age and BMI percentile.

Influence of supplementation on metabolic profile in CYP2R1 variant

Further analysis showed that fasting blood glucose and triglyceride concentration reduced in all subjects but carriers of a GG genotype showed a greater reduction in FBG and carriers of AA genotype showed a greater reduction in serum TG (Table 2). Interestingly, Hs-CRP was also reduced in AA carriers whilst the individuals with GG and AG genotypes, inflammation increased after 9 week of vitamin D supplementation (Table 2). Change in levels of Ca, BUN, creatinine and P after supplementation was not statistically significant among different genetic models (Table 2).

Discussion

Influence of supplementation on circulation 25(OH)D in CYP2R1 variant

In the present study, we explored the association of rs10766197 of the CYP2R1 vitamin D-related gene with serum 25(OH)D concentrations and found that this polymorphism was significantly associated with the serum 25(OH)D concentrations after 9 weeks of vitamin supplementation and it appeared that carriers of dominant G allele were better responder to vitamin D in respect to elevation serum vitamin D. Animal and human studies have shown that different cytochrome P450 enzymes

2(CYP) including CYP2R1, CYP2D25, CYP3A4CYP27A1 are vitamin D 3 25-hydroxylases and cause 25-hydroxylation of vitamin D 3 and related metabolites[25]. Unlike others 25-hydroxylases, CYP2R1 hydroxylates both vitamin D 2 and vitamin D 3 [32]. Therefore, genetic variations including rs10766197, in the promoter region of this gene, may influence 25(OH)D synthesis. Our data indicated although this genotypic variant was not associated with baseline 25(OH)D level, it influenced on the response to the supplementation. It is possible that the regulation of 25(OH)D synthesized by skin might be different from supplementation. In agreement with our study, Nissen et al. examined variants in some vitamin D-related genes in 201 healthy Danish population. They reported a significant association between serum 25(OH)D and rs10766197. Similarly, in a study by Engelman et al. in a female population, all individuals who had no risk alleles of rs4588 and rs2060793, consuming about 670 IU/d vitamin D, the circulation level of 25(OH)D concentrations were at sufficient level (> 50 nmol/L). For carriers with 1 and more risk alleles whose intakes were at least 670 IU/d vitamin D, only more than 50% of subjects had serum 25(OH)D > 50 nmol/L [26]. Thacher et.al in the cohort study on ricketic Nigerian children, reported that individuals with a defective *CYP2R1* allele had a mild form of VDDR1B and produce less 25(OH)D after intervention with vitamin D_2 or vitamin D_3. While, subjects who are homozygous for *CYP2R1* mutations showed a severe form of VDDR1B and had minimal rise in serum 25(OH)D after administration of vitamin D, and improvement would be only with high doses of vitamin D [25, 33]. In the study of Bu et al. they found that rs10741657 and rs10766197 were significantly associated with serum 25(OH)D concentrations in 496 healthy Caucasian people [34]. Based on similar results obtained from several studies on different population [26, 35, 36] it appeared that variants in the CYP2R1 gene predict serum 25(OH) D concentrations.

Influence of supplementation on fasted lipid profile and Fasting blood glucose in CYP2R1 variant

We found that, an intake of 50000 IU/D vitamin D per week had beneficial effects not only on 25(OH)D concentrations in all genotype groups but also on glycemic and lipid profile. However, these effects were greater in the subjects who had GG and AG genotypes at the rs10766197 locus. Noticeable that although carriers of the uncommon allele A, showed an increase in vitamin D concentration that was less than for other genotypes, the reduction in TG was more considerable. It was suggested that vitamin D has both direct and indirect effects on modifying the lipid profile.

An underlying mechanism on improving lipid profile may be through regulatory action of vitamin D in the simulation of lipoprotein lipase [37] and reduced intestinal absorption and synthesis [38].

Cross-sectional studies have reported a negative relationship between circulation levels of 25(OH) D and serum Triglyceride. However, the influence of 25(OH) D on TG concentrations in interventional studies after supplementation with vitamin D is inconsistent [39]. Pittas et al. illustrated that in the individuals with impaired fasting glucose, administration of vitamin D and calcium might ameliorate insulin resistance [40]. Jorde *et al*. in a cross sectional studies examined 8018 non-smoking individuals, found a significant positive relationship between serum 25(OH)D and serum HDL-C, TC, and LDL-C and also a significant inverse associations between serum 25(OH)D with both LDL-C/HDL-C ratio and TG [41]. In an interventional study on 438 obese Norwegian, they found no statistical association between supplementation with vitamin D and lipid profile [42]. Similarly, Sieda et al. in a meta-analysis showed no significant improvement in glucose parameters[43]. These controversial illustrations might be partly attributed to the inherent limitations and heterogeneity of the studied cohorts. Some common factors may be influenced on both the high serum 25(OH)D levels and favorable lipid profile include exercise, diet habitual and genetic profile.

Influence of supplementation on inflammation in CYP2R1 variant

Our data revealed that inflammation increased in the carriers of dominant allele G after intervention by vitamin D supplementation while individuals who had AA genotype showed significant reduction in serum Hs-CRP after supplementation. It appeared that clinical outcome in response to vitamin D supplementation was genetic-related. Emerging evidence has reported a relationship between vitamin D supplementation and serum levels of proinflammatory and inflammatory markers such as cytokines and CRP. Some studies have reported a positive association with circulation levels of 25(OH)D and others showed an inverse association while some declared no relationship [44-47]. Vitamin D has been shown to suppress *in vitro* and *in vivo* the production of proinflammatory cytokines and modulate both the innate and adaptive immune systems [48, 49]. It is proposed that macrophages, dendritic cells and activated lymphocytes influence on vitamin D receptor, implying a crucial role of vitamin D in the immune system [14]. Furthermore, the enzyme 25-hydroxyvitamin is produced by the immune system [50]. On the other hand, the activated vitamin D down-regulates proinflammatory mediators, such as interleukin (IL)-6, IL-8 , tumor necrosis factor (TNF) α, and monocyte chemoattractant protein (MCP)-1 [51, 52]. However, it is suggested that while supplementation with vitamin D elevate the 25(OH)D, the conversion of inactive 25(OH)D to active 1,25(OH) D in the kidneys is not immediate, and may not be efficient. Both the inactive and active form of vitamin D bind to the vitamin D receptor (VDR), only the 1,25-D allow VDR to perform its functions beneficially and the 25(OH)D inhibits the VDR functions. Since VDR is the "gate-keeper" of the innate immune system and modulate by thousand genes so increased levels of 25-D might show immunosuppressive effects [53].

Generally, discrepancies in the different literature indicate the need for further studies both in healthy and disease population to find out more details about the potential association between serum levels of vitamin D and inflammation biomarkers. On the other hand, regulation of serum

vitamin D in human body is a complex process that varies with individual genetic profiles and their health status. Research in genetic epidemiology of vitamin D is in its infancy and further comprehensive studies would be needed to understand how genetic variations modulate clinical outcomes of vitamin D supplementation.

Conclusion

We have found that although individuals with a GG genotype of CYP2R1 variant had a greater response to vitamin D supplements, the inflammation status was worsened. However, carriers of AA genotype showed less increase in 25(OH)D than others, but inflammation status only improved in this group. We conclude that personalized advice and recommendations tailored to individual's genetics seems help to determine how different individuals with various genetic background respond to the supplementation. People may need different health recommendations based on their genetic profiles, in order to elevate their serum 25(OH)D concentrations thereby avoiding adverse health outcomes.

263

264

265

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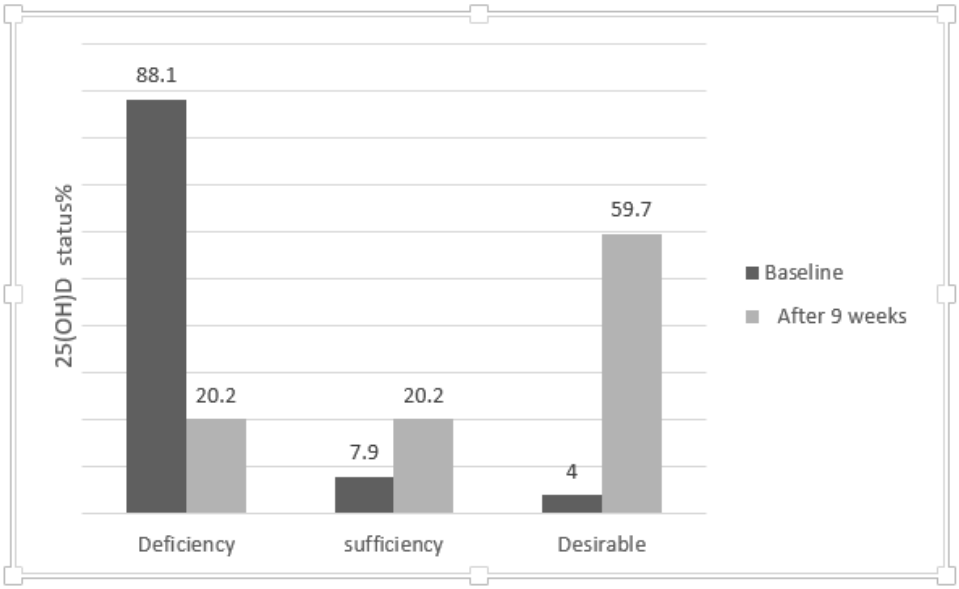

Figure 1. Comparison of the vitamin D status before and after 9 weeks of vitamin D supplementation. Deficiency: Serum 25(OH)D level<50nmol/L. Sufficiency: 50nmol/L<Serum 25(OH)D level<75nmol/L. Desirable≥75nmol/L[54].

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Table 1. Vitamin D status before and after 9 week of vitamin D supplementation according to CYP2R1 genotypes.

Vitamin D status (N=253)	GG (N=72)		AG (N=119)		AA (N=62)	
	Baseline	Follow-up	Baseline	Follow-up	Baseline	Follow-up
Desirable	4 (5.6)	50 (69.4)	2 (1.7)	70 (58.8)	4 (6.5)	31 (50)
Sufficiency	6 (8.3)	12 (16.7)	10 (8.4)	23 (19.3)	4 (6.5)	16 (25.8)
deficiency	62 (86.1)	10 (13.9)	107 (89.9)	26 (21.8)	54 (87.1)	15 (24.2)

Note: Σ2 test showed a $P_{trend}$of 0.4 at baseline; P_{trend} at 12-month follow-up is **0.05**. Data is presented as frequencies (%). Deficiency: Serum 25(OH)D level < 50 nmol/l. Sufficiency: Serum 25(OH) D level between 50 to 75 nmol/l. Desirable: Serum 25(OH)D level ≥ 75 nmol/l.

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Table 2. Comparisons of the variables before and after 9 weeks of vitamin D supplementation in different genetic modes.

		GG	AG	AA	P value in different genetic models		
					Additive	Recessive	Dominant
BMI Percentile	Baseline	62±56	58.4±28	55.7±26	Ns	Ns	Ns
	Follow-up	56±30	54.6±29	49±27			
	Change	-17.6±19	-12.4±26.3	-15.9±22.9			
Blood pressure							
SBP (mm Hg)	Baseline	101±11	100.7±13	99.6±12	Ns	Ns	Ns
	Follow-up	99±13	98.5±13.6	100±12			
	Change	-1±15.5	-1.2±15	2.1±16			
DBP (mm Hg)	Baseline	67±10	67.3±10	67.1±10.5	Ns	Ns	Ns
	Follow-up	65±11	63±10	64.1±11			
	Change	-0.5±14	-4.1±12	-2±9.1			
Serum fasted lipids							
Total Cholesterol (mg/dL)	Baseline	164.3±26	165±31	158±28	Ns	Ns	Ns
	Follow-up	150.5±24.5	156±26.5	153±27			
	Change	-7.4±10.7	-4.6±14.1	-0.1±28			
TG(mg/dL)	Baseline	87.7±41	79.5±29	83±33	0.03	Ns	**0.03**
	Follow-up	80.5±40	81±32	70.7±26			
	Change	-0.8±35.1	4.9±29.6	-7.3±27.1			
HDL(mg/dL)	Baseline	46.4±8	48.9±9	46.1±8	Ns	Ns	Ns
	Follow-up	44.2±8	46.3±10	44.8±7.5			
	Change	-3.2±14.1	-2.5±14.5	-1.7±14.3			
LDL(mg/dL)	Baseline	100.7±20	101.1±27	99.4±21	Ns	Ns	Ns
	Follow-up	89.1±20	92.4±22	93±22			
	Change(%)	-11.9±17	-9.1±19	-7.1±17.1			
Other blood parameters							
FBG	Baseline	90±13	88.6±10	86.6±9	Ns	Ns	**0.05**
	Follow-up	87±12	85±12	85.7±10.6			
	Change	-3±10.8	-4.1±11	-0.9±13.4			
WBC(10^9/L)	Baseline	6.3±1.8	6.06±1.6	6.1±1.5	Ns	Ns	Ns
	Follow-up	6.1±1.6	6.1±1.4	5.5±1.3			
	Change	-1.8±24.6	0.1±27	-4.4±28			
Serum Hs-CRP(mg/L)	Baseline	1.3±1.6	1.1±1.1	1.8±1.7	Ns	0.003	**0.05**
	Follow-up	1.6±2	1.4±1.3	1.1±1.4			
	Change	17.7±13	61.6±17.6	-26.8±8.4			

25-OH vitamin D(nmol/L)	Baseline	27.5±25	24±18	29±34	(AA vs. GG) **0.03** (AA vs. AG) **0.04**	**0.049**	Ns
	Follow-up	99.3±42	86±40	84.4±46			
	Change	447.3±414.6	423.6±380.4	433.0±426.9			
Ca(mg/dL)	Baseline	9.6±0.5	9.4±0.5	9.4±0.7	Ns	Ns	Ns
	Follow-up	9.7±0.5	9.7±0.5	9.7±0.5			
	Change	0.1±0.6	0.3±0.57	0.3±0.8			
Phosphate(mg/dL)	Baseline	4±0.5	3.9±0.4	3.9±0.5	Ns	Ns	Ns
	Follow-up	4±0.4	4±0.4	4±0.4			
	Change	0±0.4	0.1±0.4	0.1±0.4			
Creatinine(mg/dL)	Baseline	10.6±3.8	6.6±11.5	5.6±11.8	Ns	Ns	Ns
	Follow-up	0.7±0.09	0.7±0.1	0.7±0.1			
	Change	8.7±12.9	10.3±15.2	8.0±13			
BUN(mg/dL)	Baseline	12.3±3	12.5±3	12.6±3	Ns	Ns	Ns
	Follow-up	13.8±4	13.6±3.2	14±3			
	Change	16.3±34.1	15.5±35.3	14.4±30.6			

Note: Change = ((Follow up – Baseline)/Baseline)/100; p values presented for the changes in different variables after vitamin D supplementation according to genotypes; Additive genetic model (GG genotype vs. AG genotype vs. AA genotype); Recessive genetic model (GG genotype vs. AG+AA genotypes); Dominant genetic model (GG+AG genotypes vs. AA genotype). BMI: body mass index; TC: total cholesterol; TG: triglycerides; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; Hs-CRP: high-sensitivity Creative protein; FBG: fasting blood glucose; SBP: systolic blood pressure; DBP: diastolic blood pressure; BUN: Blood Urea nitrogen; Ca: Calcium; FBG: Fasting blood glucose.

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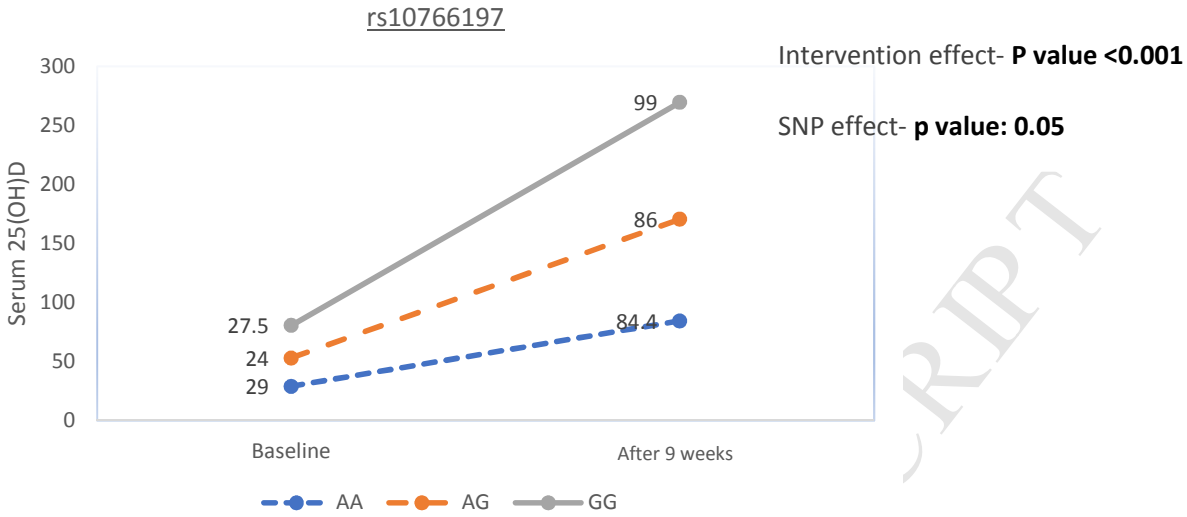

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449 Figure.2.Serum 25(OH)D stratified by a polymorphism in CYP2R1 gene. Values are means ±SD. Two-
450 way ANCOVA repeated measures adjusted for multiple comparisons by Bonferroni test for serum
451 25(OH)D levels. Covariates used: age, BMI percentile and serum 25(OH)D at baseline.

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Table 3. Association of CPY2R1 variant- rs10766197 with the changes in circulation levels of 25(OH)D after 9 weeks of supplementation (under different genetic models)

Additive model	AA	AG	GG
	Reference (Risk group)	OR (CI95%), *p* value	OR (CI95%)
	1	1.6 (0.8-3.3), 0.1	2.1 (1-4.2), **0.03**
Recessive model	AG/AG		GG
	Reference (Risk group)		OR (CI95%), *p* value
	1		1.5 (0.9-2.6), 0.1
Dominant model	AA		AG/GG
	Reference (Risk group)		OR (CI95%), *p* value
	1		1.8 (1-3-.1), **0.05**

Data was adjusted for age, BMI percentile.

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