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Original article

Serum vitamin D and vitamin D receptor (VDR) polymorphisms (FokI and TaqI) as predictors of COVID19 severity: Prospective study in a cohort of Egyptian COVID-19 patients

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ABSTRACT

Background: Severe forms of respiratory failure affect 20% of COVID-19 patients with a death rate of about 65%. Vitamin D deficiency and vitamin D receptor polymorphisms, mainly TaqI (rs731236) and FokI (rs10735810), were associated with respiratory tract infections. **Methods:** A prospective study on 90 confirmed PCR-positive COVID-19 patients was done. **Results:** Statistically significant lower levels of 25OH vitamin D were found in severe to critical COVID-19 and non-survivors than in mild to moderate patients and survivors ($p < 0.001$) and with negative correlations with C-reactive protein, ferritin, procalcitonin, interleukin 6, D-dimer, INR, white blood cells, neutrophils count, and neutrophils/lymphocytes ratio. Serum 25(OH) D was positively correlated with oxygen saturation level, hemoglobin, lymphocyte, and monocyte counts. Mild to moderate and severe to critical patients showed significant differences in both FokI SNP ($p = 0.002$) and TaqI genotypes ($p = 0.010$). FokI recessive mode might be associated with increased severity, while TaqI dominant mode of inheritance and the mutant allele (t) might protect against severe forms of COVID-19. **Conclusion:** 25 OH D and FokI recessive mode of inheritance may be associated with severe forms of COVID-19; however, TaqI dominant mode of inheritance and the mutant allele (t) might protect against COVID-19 severity. It is recommended to apply similar research on different populations together with studying other VDR polymorphisms in addition to clinical trials of vitamin D supplementation in order to generalize these findings.

Introduction

Acute respiratory distress syndrome (ARDS) is one of the prognostic factors of COVID-19 patients that threatens their lives. It usually precedes the development of multiple organ dysfunctions that occur in severe forms of COVID-19 with a high mortality rate. The percent of affection of COVID-19 patients with ARDS is high, about 20% of the hospitalized patients, whether intensive care unit (ICU) or non-ICU patients, although of the great enhancement in ICU strategies, about 65% of these patients die [1].

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Vitamin D has potent antiproliferative, prodifferentiating, and immunomodulatory activities besides its major role in calcium and phosphate regulation. 25-hydroxy vitamin D (25(OH)D) is the useful indicator of vitamin D nutritional status, as it is the most abundant form in circulation, and the plasma level of 1,25 dihydroxy vitamin ($1,25(OH)_2D$) depends on many factors other than nutritional status, mainly renal function, calcium and phosphate levels, and concentrations of intact parathyroid hormone (PTH) [2].

The active form of vitamin D ($1,25(OH)_2D$) exerts its effect through different pathways, either genomic or non-genomic. Vitamin D receptor VDR mediates the genomic actions of ($1,25(OH)_2D$) through binding to vitamin D-responsive element (VDRE), thus modulating the expression of vitamin D-responsive genes [3].

Many organs were found to express the vitamin D receptor (VDR) on their cells, including cells of the immune system. The enzyme that may have an essential role in local production of $1,25(OH)_2D$ (1α-hydroxylase (CYP27B1)) is also found to be expressed in addition to VDR on lung epithelial cells, antigen-presenting cells, and monocytes [4]. Thus, the production of cytokines from these immune cells is thought to be controlled by the level of $1,25(OH)_2D$ [5].

The induction of ARDS through lipopolysaccharide (LPS) may get benefits from vitamin D through its genomic mediation by decreasing the severe inflammatory response, such as the decrease of severe cytokine storm, changing the way of neutrophil action and keeping the alveolar epithelial barrier intact [6,7]. Thus, the decreased production of inflammatory cytokines and chemokines by vitamin D has been reported [8].

Highly expressed levels of 1α-hydroxylase with lowly expressed levels of 24-hydroxylase. $1,25(OH)_2D$ was found in type II alveolar epithelial cells (ACII), thus leading to increased local production of the active form of vitamin D ($1,25(OH)_2D$) and decreased vitamin D inactivation [9-11]. This local production of active vitamin D augments these cells (ACII) in their defense role against viral attack and replication [9]. In an experimental model, it was found that administration of active vitamin D vitiated specific forms of liposaccharides, inducing lung injury through inhibition of epithelial lining metaplasia and apoptosis, adding to the proposed therapeutic role of vitamin D, especially in severe lung injury [7]. In addition, active vitamin D also inhibits epithelial mesenchymal transition (EMT) through reduction of transforming growth factor β (TGF-β) actions [7,12].

The genomic pathway of vitamin was found to play a role in the reduction of SARS-CoV and SARS-CoV-2 entry into the host cells by negative regulation of renin-angiotensin system (RAS), one of the SARS-CoV and SARS-CoV-2 cell surface receptors [13,14]. It was also found to induce angiotensin-converting enzyme 2 and decrease the expression of ACE and Ang II expression in the LPS-induced lung injury [15].

Vitamin D deficiency is a global health problem and adds to the health burden of diseases, such as cancer, immune diseases and infections particularly the upper respiratory tract infections, thus vitamin D supplementation has a great role in decreasing the severity of these diseases [16]. VDR genetic variants were reported to be associated with viral infection susceptibility [17], diabetes mellitus [18], risk of developing cancer [19] and autoimmune diseases [20]. Therefore, the degree of lung injury and patient outcome in COVID-19 may be affected by vitamin D status and VDR polymorphisms.

In addition to vitamin D deficiency's role in the development and progression of many diseases, such as type 2 diabetes, cancer, autoimmune diseases, and other diseases, VDR polymorphisms have also been found to play a role in these diseases, particularly ApaI (rs7975232), BsmI (rs1544410), TaqI (rs731236), and FokI (rs10735810) [21]. Also, these variants were thought to be associated with increases susceptibility to acute lower respiratory infections [22,23].

The VDR FokI polymorphism (C>T rs2228570) represents one of the VDR coding SNPs located within exon 2, which changes the primary sequence of VDR. The absence of this *Fok*I site displays the starting codon as (ACG), resulting in the initiation of the translation at a frame (ATG) three codons downstream. The mutant allele is associated with diminishing the efficiency of vitamin D signaling [24]. The TaqI SNP (T>C rs731236) resides in the coding region, exon 9, near the 3′ UTR of the gene, which generates no amino acid alteration. The risk allele of TaqI is associated with lower levels of mRNA expression as well as more rapid decay or lower stability of VDR mRNA,

which results in a reduction of VDR protein with reduced response to vitamin D [25].

The vitamin D receptor polymorphisms, mainly TaqI (rs731236) and FokI (rs10735810), were mostly associated with respiratory tract infections [26-28].

Data concerning the effect of serum Vitamin D and VDR polymorphisms (TaqI (rs731236) and FokI (rs10735810)) as predictors of COVID-19 severity in Egyptian populations is insufficient up to date. Thus, the present study aimed to find the role of serum Vit D and VDR polymorphisms (FokI and TaqI) as predictors of COVID-19 severity.

Patients and methods

Design: The study was designed as a prospective study.

Population and interventions

Ninety confirmed SARS-CoV-2 PCR positive COVID-19 patients were included in the present study; they were recruited from Alexandria University main hospital. Patients with chronic lung diseases, diabetes mellitus, renal failure, ACE inhibitors medications, malignancy, and previous history of chemotherapy and radiotherapy were excluded from the study.

To all participants, history and clinical examination were done, including anthropometric measures and body mass index calculation. In addition to clinical and CT examinations for assessing COVID-19 severity. Routine laboratory analysis was done, including measurement of serum urea and creatinine levels, serum activities of aminotransferase enzymes (AST, ALT), and inflammatory markers (serum levels of CRP and ferritin), in addition to complete blood picture and D-dimer assessment. Serum levels of 25OH D were measured using ELISA kits [29].

Genetic characterization

Genotyping of VDR polymorphisms was done as follows: DNA extraction was done using EDTA blood samples from peripheral WBCs using the QIAamp® DNA Mini kit (QIAGEN, Cat No. 51304**)** [30]. The concentration and purity of the extracted genomic DNA were determined using the Thermo Scientific NanoDrop™ 1000 Spectrophotometer. Genotyping of VDR gene polymorphisms FokI and TaqI (rs2228570 and rs731236), respectively, was performed using the pre-designed forward and reverse PCR primers followed by the allelic discrimination real-time PCR-SNP genotyping technology with dual labelled fluorogenic TaqMan (MGB) probes. The thermal cycling conditions and the FokI (rs2228570) and TaqI (rs731236) SBPs genotyping according to the fluorescence signals are shown in **table (1)** and **(figure 1)**, respectively.

Ethical considerations

Approval of the Ethical Committee of the Medical Research Institute and Alexandria Main Hospital was taken (approval number IORG0008812), and written consent from all participants was obtained.

Statistical analysis

SPSS program version 20 was used for statistical analysis. The normality of the quantitative data was tested using the Kolmogorov-Smirnov test. The qualitative variables were summarized by frequency and percentage. Student's t-test. A $\chi 2$ test was used to test the statistically significant differences of qualitative variables between the studied groups. For quantitative data, Mann–Whitney for comparing two groups and comparison between more than two groups was done using the Kruskal–Walli's test, and for correlation studies between quantitative variables, the Spearman's correlation test was used. *P*-value of less than or equal to 0.05 was considered significant for all comparisons. Genotype-specific odds ratios (ORs) were computed using logistic regression analysis under codominant, dominant, and recessive genetic models. Other appropriate statistical tests were used whenever indicated.

Results

Upon applying the NIH guidelines of COVID-19 clinical picture, the participants were classified into 40 participants with mild to moderate clinical picture and 50 patients with severe to critical clinical picture. The two studied groups were sex matched; however, severe to critically ill patients were significantly older in age (53.36 ± 13.24 years) than mild to moderately ill patients (40.28 ± 15.51 years) ($p < 0.001$).

The severe to critically ill patients had significantly lower levels of serum 25OHD (9.08 ± 2.74 ng/ml) than mild to moderately ill patients (14.79 ± 3.43 ng/ml) ($p < 0.001$). Also, a higher frequency of 25OHD deficiency (90%) with significant differences than the mild to moderately ill patient group ($p < 0.001$). Upon follow-up of the cases, the non-survivors were found to have significantly lower levels of 25-hydroxyvitamin D

(8.50 ± 2.53 ng/ml) than COVID-19 survivors (12.82 ± 4.07 ng/ml) (p<0.001) **(Table 2) (Figure 2)**.

Negative significant correlations were found between serum 25-hydroxyvitamin D level and other studied parameters including age (r= -o.580, p<0.001) , serum levels of urea (r= -0.304, p= 0.004), AST (r= - 0.363, p<0.001), ALT (r= 0.343, p= 0.001), lactate dehydrogenase (r= -0.246, p=0.019), as well as negative significant correlations with serum levels of C-reactive protein (r= -0.561, p<0.001), ferritin (r= -0.507, p<0.001), procalcitonin (r= -0.421, p<0.001), interleukin 6 (r= -0.626, p<0.001), and negative significant correlations with plasma levels of D-dimer (r= -0.671, p<0.001), in addition to negative significant correlations with INR (r= -0.333, p=0.001), white blood cells (r= -0.381, p<0.001), neutrophils counts (r= -0.505, p<0.001) and neutrophils/lymphocytes ratio (r= -0.624, p<0.001) **(Table 3).**

The 25-hydroxyvitamin D had positive, significant correlations with oxygen saturation level (r = 0.638, p < 0.001), hemoglobin (r = 0.244, p = 0.02), lymphocytic count (r = 0.518, p < 0.001), and monocytic count (r= 0.381, p < 0.001) **(Table 3).**

The observed genotype frequency of FokI and TaqI single nucleotide polymorphisms was in consistency with Hardy-Weinberg equilibrium of the population (p = 0.874) and (p = 0.072), respectively.

Significant differences were found between the mild to moderate patients and severe to critical patients regarding the frequency of the FokI SNP (p = 0.002). The higher frequency was found of the mutant homogenous (ff) genotype in the severe to critical group (18%) than the mild to moderate group (2.5%) with no statistical significance; however, the odds ratio was an OR = 4.667, 95% CI (0.536–40.647), indicating a great association between the mutant (ff) genotype and the severity of COVID-19. The allele frequency of the FokI gene, both F and f alleles, did not vary significantly between both studied groups **(Table 4)**.

On assuming a dominant mode of inheritance of FokI genotypes, where subjects with mutant homozygous and heterozygous (ff) and (Ff) genotypes were compared to subjects with the wild homozygous genotype (FF), there were no significant differences between the studied COVID-19 groups. While, on assuming a recessive mode of inheritance, where subjects with the homo-mutant (ff) genotype were compared to wild homozygous and heterozygous (FF+ Ff) genotypes, there was a significant difference between the mild to moderate and severe to critical COVID-19 patients (p = 0.046) with an (OR = 8.561), 95% CI (1.036–70.753), indicating increased association between the recessive (ff) genotype and increased risk of severity in COVID-19 patients. And on assuming the additive mode of inheritance, where patients with the wild homozygous genotype (FF) were given the lowest value of (0), those with the heterozygous genotype (Ff) were given the value of (1), and finally patients with the mutant homozygous genotype (ff) were given the highest value of (2). No significant difference was observed between patients with mild to moderate and patients with severe to critical clinical pictures **(Table 4).**

Significant differences were found between the mild to moderate patients and severe to critical patients regarding the frequency of TaqI genotypes (p = 0.010). The higher frequency was found of the mutant homogenous (tt) genotype in the mild to moderate group (17.5%) than the severe to critical group (14.0%) with no statistical significance. The odds ratio was an OR = 0.211, 95% CI (0.047–0.947), with no association found. The heterozygous genotype (Tt) also did not show any significant differences between both patient groups, although it was higher in the mild to moderate group (72.5%) than in the other group (48%). Regarding the TaqI allele frequencies, the mutant (t) showed significant differences between both groups (p = 0.035). Also, the (t) allele was lower (38%) than the wild (T) allele (62%) in the severely ill group **(Table 4).**

On assuming a dominant mode of inheritance, when the subjects with heterozygous genotype (Tt) and mutant homozygous genotype (tt) were compared to the wild homozygous genotype (TT), there was a significant difference between mild to moderate and the severe to critical patients (p=0.005). Regarding the recessive mode of inheritance, where the exposed group is subjected only to the mutant homozygous (tt) genotype, there was no observed significant difference between both studied patient groups, while the additive mode showed a significant difference of (p=0.023) **(Table 4)**. No associations were found between both FokI and TaqI genotypes and the outcome of COVID-19 patients.

Serum levels of 25OH vitamin showed significantly lower levels in patients with the mutant homozygous (ff) genotype (9.19 ± 1.94 ng/ml) than other FokI genotypes (p = 0.001).

When comparing the parameters studied with respect to VDR (FokI and TaqI) genotypes, there was a statistically significant difference in the mean serum. 25-hydroxyvitamin D level (ng/ml) between the studied FokI SNP genotypes (p = 0.001). The mutant homozygous (ff) genotype was associated with the lowest mean value of serum 25-hydroxyvitamin D (9.19 ± 1.94 ng/ml). Post hoc tests for exact group differences revealed differences in the serum levels of 25OHd between the wild genotype (FF) and the heterozygous genotype (Ff) (p = 0.004) as well as between the mutant genotype (ff) and the heterozygous genotype (Ff) (p = 0.009). The serum levels of 25OHD did not show significant differences between TaqI genotypes. The post hoc statistical test showed significant differences between the TT and Tt genotypes (p = 0.035) **(Table 5) (Figure 2).**

Significant differences were found between FokI genotypes and other studied parameters, including oxygen saturation (p = 0.014) with post hoc significant differences between (FF) and (Ff) genotypes (p = 0.014), serum CRP (p = 0.043) with post hoc significant differences between (Ff) and (ff) genotypes (p = 0.016), interleukin 6 (p = 0.021) with post hoc significant differences between (Ff) and (ff) genotypes (p = 0.009), and plasma D-dimer (p = 0.005) with post hoc significant differences between (FF) and (Ff) as well as between (ff) and (Ff) genotypes (p = 0.007) and (p = 0.009), respectively **(Table 5).**

Significant differences were found between TaqI genotypes in the serum levels of IL-6 (p = 0.043), with post hoc showing significant differences between (TT) and (Tt) genotypes (p = 0.012) **(Table 5).**

The univariate logistic regression model for prediction of COVID-19 severity revealed a significant protective effect of increased serum 25-hydroxyvitamin D level. (OR= 0.497, p <0.001) and Taql dominant mode of inheritance (OR=0.181, p=0.005), while increased serum interleukin-6 level (OR= 1.301, p=0.001) and plasma D-dimer level (OR = 1.009, p < 0.001) in addition to FokI SNP recessive mode of inheritance (OR = 8.561, p = 0.046), as well as low lymphocytic count (<1.5 ($x10^3/\mu l$), (OR = 5.911, p < 0.001), showed an increased risk for COVID-19 severity. The multivariate logistic regression model for prediction of COVID-19 severity was statistically significant ($\chi 2 = 109.059$, $p < 0.001$); the model explains (94%) of COVID-19 severity (Nagelkerke R Square = 0.947). The only predictors were serum 25-hydroxyvitamin D level (ng/ml) (OR=0.294, p=0.032) and plasma D-dimer level (OR=1.013, p=0.038), while the remaining predictors showed no statistically significant effect **(Table 6).**

Table 1. The thermal cycling conditions and the FokI (rs2228570) and TaqI (rs731236) single nucleotide polymorphisms genotyping according to the fluorescence signals.

The thermal cycling conditions.			
Step	**AmpliTaq Gold Enzyme Activation**	**PCR (40 Cycles)**	
		Denature	**Anneal/Extend**
Temperature	95 °C	95 °C	60 °C
Time	Hold for 10 minutes	15 sec	1 min
The FokI (rs2228570) single nucleotide polymorphism genotyping according to the fluorescence signals			
A substantial increase in	**Indicates**	**Genotyping**	
FAM - dye fluorescence only (FAM / FAM)	Homozygosity for C allele (wild homozygous genotype)	CC/ FF	
VIC - dye fluorescence only (VIC / VIC)	Homozygosity for T allele (mutant homozygous genotype)	TT/ ff	
Both FAM- and VIC- dyes fluorescence (FAM / VIC)	allele C - allele T (heterozygosity)	CT/ Ff	
The TaqI (rs731236) single nucleotide polymorphism genotyping according to the fluorescence signals			
A substantial increase in	**Indicates**	**Genotyping**	
VIC - dye fluorescence only (VIC / VIC)	Homozygosity for A allele (wild homozygous genotype)	TT/ TT	
FAM - dye fluorescence only (FAM / FAM)	Homozygosity for G allele (mutant homozygous genotype)	CC/ tt	
Both FAM - and VIC -dyes fluorescence (FAM / VIC)	allele C - allele T (heterozygosity)	CT/ Tt	

Table 2. Serum 25-hydroxyvitamin D level in the studied groups

25-hydroxyvitamin D level (ng/ml)	**Mild/Moderate COVID-19 patients (n = 40)**		**Severe/ Critical COVID-19 patients (n = 50)**		**Test of Sig.**	***p***
	No.	**%**	**No.**	**%**		
Severe deficiency (<12 ng/ml)	7	17.5	45	90.0	χ^2= 50.970	^{MC}p <0.001*
Deficiency (12– <20 ng/ml)	29	72.5	5	10.0		
Sufficiency (≥20 ng/ml)	4	10.0	0	0.0		
Min. – Max. Mean ± SD.	6.40 – 21.60 14.79 ± 3.43		1.50 – 13.90 9.08 ± 2.74		t= 8.782*	<0.001*
25-hydroxyvitamin D level (ng/ml)	**Patients' outcome**				**t**	***p***
	Non-survivors patients (n = 25)		**Survivors patients (n = 65)**			
Mean ± SD. **Median (Min. – Max.)**	8.50 ± 2.53 **9.30 (1.50 – 12.0)**		12.82 ± 4.07 **12.30 (3.80 – 21.60)**		6.040*	<0.001*

Table 3. Correlation between serum 25-hydroxyvitamin D level and different parameters in all studied patients

25-hydroxyvitamin D level (ng/ml) vs.	**Total (n = 90)**	
	r	***p***
Age (years)	-0.580	<0.001*
Oxygen saturation (%)	0.638	<0.001*
Urea (mg/dl)	-0.304	0.004*
Creatinine (mg/dl)	0.002	0.984
Aspartate aminotransferase (U/L)	-0.363	<0.001*
Alanine aminotransferase (U/L)	-0.343	0.001*
Lactate dehydrogenase (U/L)	-0.246	0.019*
C-reactive protein (mg/L)	-0.561	<0.001*
Ferritin (ng/ml)	-0.507	<0.001*
Procalcitonin (ng/ml)	-0.421	<0.001*
Interleukin 6 (pg/ml)	-0.626	<0.001*
International normalized ratio	-0.333	0.001*
D-dimer (ng/ml)	-0.671	<0.001*
Hemoglobin (g/dl)	0.244	0.020*
Platelets (×10³/µl)	0.183	0.084
White blood cells (×10³/µl)	-0.381	<0.001*
Lymphocytes (×10³/µl)	0.518	<0.001*
Neutrophils (×10³/µl)	-0.505	<0.001*
Neutrophils/ lymphocytes ratio	-0.624	<0.001*
Monocytes (×10³/µl)	0.381	<0.001*

Table 4. Vitamin D receptor polymorphisms FokI and TaqI and their mode of inheritance in the COVID-19 patients studied.

FokI polymorphism significant differences in COVID – 19 Patient groups							
FokI SNP	**Mild/moderate COVID-19 patients (n = 40)**		**Severe/critical COVID-19 patients (n = 50)**		χ^2	p_0	**OR (95% C.I)**
	No.	**%**	**No.**	**%**			
Genotype							
FF	14	35.0	27	54.0	12.670* (0.002)		1.000
Ff	25	62.5	14	28.0		0.008*	0.290 (0.116 – 0.728)
Ff	1	2.5	9	18.0		0.163	4.667(0.536 – 40.647)
Allele							
F	53	66.2	68	68.0	0.062 (0.804)		1.000
F	27	33.8	32	32.0		0.804	0.924 (0.494 – 1.727)
FokI polymorphism mode of inheritance in COVID – 19 Patient groups							
FokI SNP	**Mild/Moderate COVID-19 patients (n = 40)**		**Severe/ Critical COVID-19 patients (n = 50)**		**p**	**OR (95% C.I)**	
	No.	**%**	**No.**	**%**			
Dominant							
FF	14	35.0	27	54.0		1.000	
Ff + ff	26	65.0	23	46.0	0.074	0.459 (0.195 – 1.079)	
Recessive							
FF+ Ff	39	97.5	41	82.0			
Ff	1	2.5	9	18.0	0.046*	8.561 (1.036 – 70.753)	
Additive						0.925 (0.497 – 1.721)	
FF	14	35.0	27	54.0	0.805		
Ff	25	62.5	14	28.0			
Ff	1	2.5	9	18.0			
TaqI polymorphism significant differences in COVID – 19 Patient groups							
	Mild/moderate COVID-19 patients (n = 40))		**Severe/critical COVID-19 patients (n = 50)**		χ^2	**p0**	**OR (95% C.I)**
TaqI SNP	**No.**	**%**	**No.**	**%**			
Genotype							1.000
TT	4	10.0	19	38.0	9.257 (0.010*)		
Tt	29	72.5	24	48.0		0.005*	0.174 (0.052 – 0.582)
Tt	7	17.5	7	14.0		0.042*	0.211 (0.047 – 0.947)
Allele					4.455 (0.035*)		
T	37	46.3	62	62.0			1.000
T	43	53.8	38	38.0		0.036*	0.527 (0.290 – 0.958)
TaqI polymorphism mode of inheritance in COVID – 19 Patient groups							
TaqI SNP	**Mild/Moderate COVID-19 patients (n = 40)**		**Severe/ Critical COVID-19 patients (n = 50)**		***p***	**OR (95% C.I)**	
	No.	**%**	**No.**	**%**			
Dominant					0.005*	1.00 0.181 (0.056 – 0.590)	
TT	4	10.0	19	38.0			
Tt+ tt	36	90.0	31	62.0			
Recessive						1.000 0.767 (0.245 – 2.404)	
TT+ Tt	33	82.5	43	86.0	0.650		
Tt	7	17.5	7	14.0			
Additive						0.437 (0.215 – 0.891)	
TT	4	10.0	19	38.0	0.023*		
Tt	29	72.5	24	48.0			
Tt	7	17.5	7	14.0			

□2: Chi square test OR: Odds ratio *: Statistically significant at $p \leq 0.05$
CI: Confidence interval p0: p value for OR n: number of patients

Table 5. Significance difference of 25-hydroxyvitamin D and some parameters studied in COVID-19 patients regarding vitamin D receptor polymorphisms (FokI and TaqI).

Significance difference of 25-hydroxyvitamin D in COVID-19 patients regarding Vitamin D receptor polymorphisms (FokI and TaqI)						
	N	**25-hydroxyvitamin D (ng/ml)**			**F (p)**	**Pairwise**
		Min. – Max.	**Mean ± SD.**	**Median**		
FokI						
FF	**41**	1.50 – 21.60	10.53 ± 4.27	10.90	7.496* (0.001*)	p FF vs. Ff = 0.004*
Ff	**39**	6.30 – 20.60	13.38 ± 3.84	13.90		p FF vs. ff = 0.594
ff	**10**	6.30 – 11.80	9.19 ± 1.94	9.20		p Ff vs. ff = 0.009*
TaqI						
TT	**23**	3.50 – 20.10	9.80 ± 4.18	9.30	3.210* (0.045*)	p TT vs. Tt = 0.035*
Tt	**53**	1.50 – 21.60	12.38 ± 4.05	12.0		p TT vs. tt = 0.350
tt	**14**	4.50 – 17.50	11.72 ± 4.01	11.45		p Tt vs. tt = 0.855

Significance difference of some of the parameters studied in COVID-19 patients regarding Vitamin D receptor polymorphisms (FokI and TaqI)					
	Genotype of FokI SNP				
	FF (n = 41)	**Ff (n = 39)**	**ff (n = 10)**	**Test of Sig.**	***p***
Oxygen saturation (%)					
Min. – Max.	40.0 – 99.0	40.0 – 98.0	40.0 – 97.0	F= 4.497*	0.014*
Mean ± SD.	72.29 ± 22.40	85.49 ± 18.98	73.10 ± 17.06		
Sig. bet. categories	p_1=0.014*,p_2= 0.993,p_3=0.208				
C-reactive protein (mg/L)					
Min. – Max.	0.70 – 118.0	1.30 – 102.0	1.40 – 151.0	H= 6.272*	0.043*
Median	48.0	20.10	72.0		
Sig. bet. categories	p_1=0.152,p_2=0.131,p_3=0.016*				
Interleukin-6 (pg/ml)					
Min. – Max.	1.10 – 310.0	1.20 – 215.0	2.90 – 180.0	H= 7.688*	0.021*
Median	26.0	4.90	51.0		
Sig. bet. categories	p_1=0.080,p_2=0.132,p_3=0.009*				
D-dimer (ng/ml)					
Min. – Max.	90.0 – 2300.0	88.0 – 2000.0	204.0 – 2000.0	H= 10.542*	0.005*
Median	800.0	224.0	1250.0		
Sig. bet. categories	p_1=0.007*,p_2=0.358,p_3=0.009*				

p: p value for comparing genotype of FOKI and different parameters
p_1: p value for comparing between FF and Ff p_2: p value for comparing between FF and ff
p_3: p value for comparing between Ff and ff

Interleukin-6 (pg/ml)	**Genotypes of TaqI SNP**				
	TT (n = 23)	**Tt (n = 53)**	**tt (n = 14)**	**H**	***p***
Min. – Max.	1.40 – 310.0	1.10 – 215.0	1.50 – 180.0	6.286*	0.043*
Median	43.0	6.70	27.55		
Sig. bet. categories	p_1=0.012*,p_2=0.179,p_3=0.572				

p: p value for comparing genotype of TaqI and different parameters
p_1: p value for comparing between TT and Tt
p_2: p value for comparing between TT and tt
p_3: p value for comparing between Tt and tt

χ^2: Chi square test FE: Fisher Exact *: Statistically significant at $p \leq 0.05$ n: number of patients
H: H for Kruskal Wallis test, Pair wise comparison between each 2 groups was done using Post Hoc Test (Dunn's for multiple comparisons test)
F: F for One way ANOVA test, Pairwise comparison between each 2 groups was done using Post Hoc Test (Tukey's)

Table 6. Univariate and multivariate logistic regression analysis for some studied parameters affecting COVID-19 severity in all studied patients (n= 50 severe/critical patients vs. 40 mild/moderate patients)

	Univariate		#Multivariate	
	p	**OR (95%C.I)**	***p***	**OR (95%C.I)**
25-hydroxyvitamin D level (ng/ml)	<0.001*	0.497 (0.369 – 0.670)	0.032*	0.294 (0.096 – 0.897)
FokI SNP recessive mode of inheritance (ff)	0.046*	8.561 (1.036 – 70.753)	0.935	0.161 (0.0 – 2E+018)
TaqI SNP dominant mode of inheritance (TT + Tt)	0.005*	0.181 (0.056 – 0.590)	0.159	0.005 (0.000 – 7.945)
Age (years)	<0.001*	1.065 (1.030 – 1.102)	0.074	1.167 (0.985 – 1.382)
Interleukin 6 level (pg/ml)	0.001*	1.301 (1.112 – 1.523)	0.069	1.209 (0.986 – 1.484)
D-dimer level (ng/ml)	<0.001*	1.009 (1.004 – 1.014)	0.038*	1.013 (1.001 – 1.025)
Lymphocytes count				
≥1.5 (x10³/µl)		1.000		1.000
<1.5 (x10³/µl)	<0.001*	5.911 (2.368 – 14.758)	0.939	1.185 (0.015 – 93.343)

OR: Odd`s ratio C.I: Confidence interval *: Statistically significant at $p \leq 0.05$ n: number of patients
Nagelkerke R Square= (0.947), Chi-square Model (χ^2= 109.059, p<0.001*)

Figure 1. the FokI (rs2228570) and TaqI (rs731236) single nucleotide polymorphisms genotyping according to the fluorescence signals

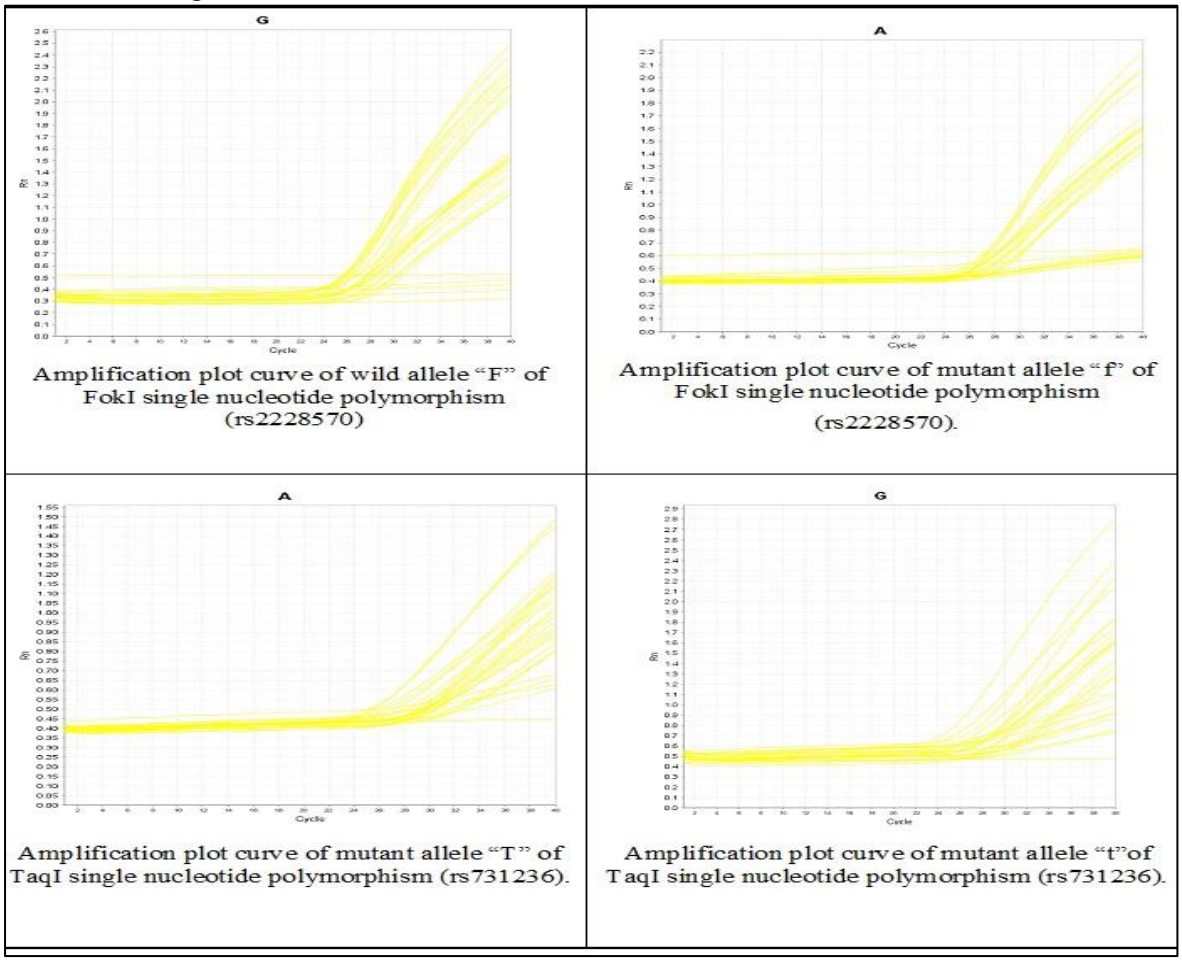
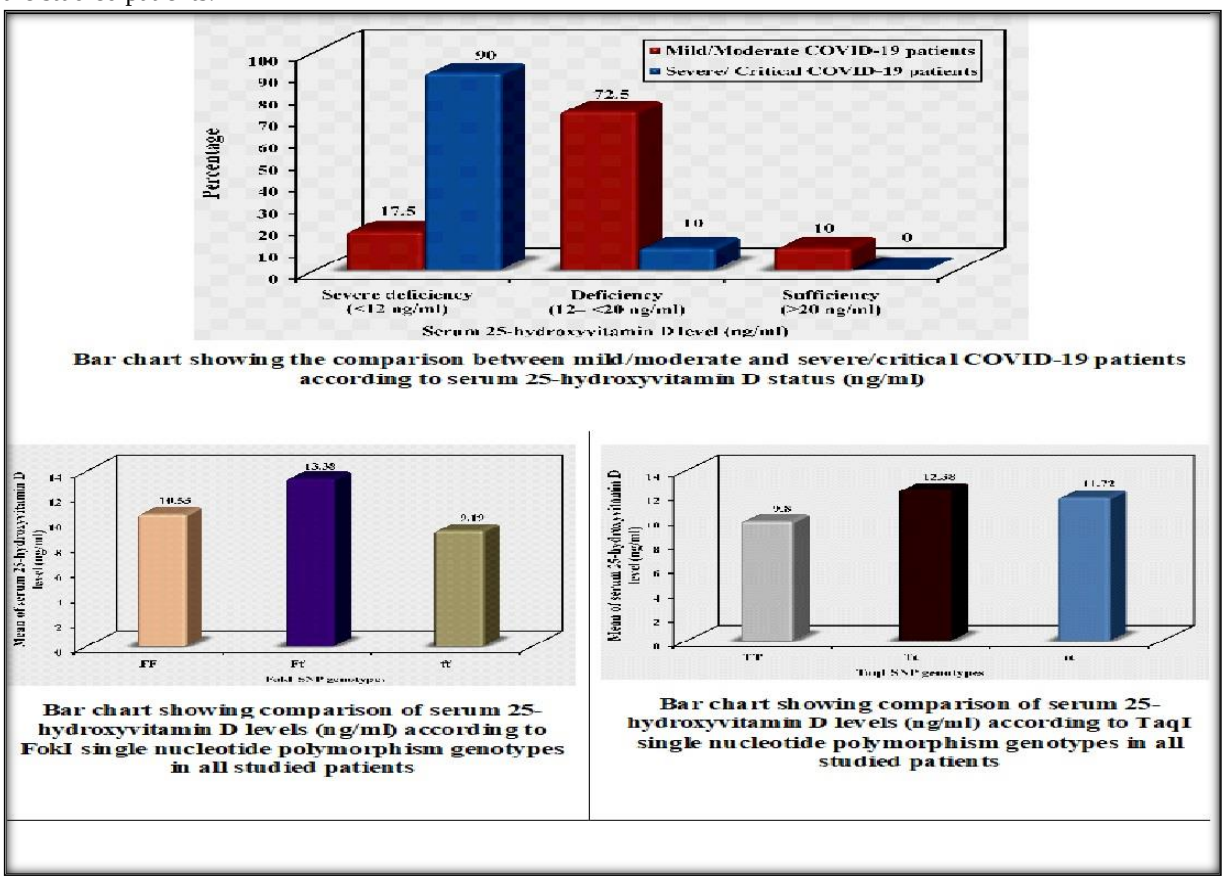


Figure 2. Serum 25 (OH) vitamin D level in relation to COVID-19 severity and FokI& TaqI polymorphisms in the studied patients.



Discussion

The role of serum vitamin D and VDR polymorphisms (FokI and TaqI) as predictors of COVID-19 severity was studied in the present research.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a new member of the beta coronavirus genus [31]. People infected with SARS-CoV-2 have reached over 500 million people, with more than 6 million deaths worldwide [32].

Coronavirus disease-2019 showed a wide range of clinical presentations with generalized organ affection [33]. Severe COVID-19 usually presents with ARDS, a life-threatening condition that occurs as a result of severe rises in plasma levels of pro-inflammatory cytokines and chemokines, resulting in severe lung injury in addition to microthrombi formation [34].

Many mechanisms may be involved in the pathogenesis of SARS-CoV-2 multi-organ injury, through direct viral toxicity, disturbance of the immune regulatory system response, endothelial cell damage, and thromboinflammation, as well as dysregulation of the renin–angiotensin system (RAS) [35].

Vitamin D is a hormone that mainly plays a core role in the maintenance of bone as well as calcium and phosphorus metabolism; in addition, it has a wide spectrum of immunomodulatory, anti-inflammatory, and antioxidant actions [36]. Increased risk of infection has been linked to vitamin D deficiency, and clinical studies of vitamin D supplementation were found to decrease this risk [16]. Respiratory tract infection, mainly viral infection, has been associated with several VDR genetic variants [17]. VDR polymorphisms TaqI (rs731236) and FokI (rs10735810) were mostly associated with respiratory tract infections. [26-28]

Qin et al., 2020 [37], reported no gender difference between severe and non-severe COVID-19 patients, similar to the current results. Severe COVID-19 patients were significantly older than non-severe patients, which may be attributed to the gradual diminution of cilia and ciliated cells in the respiratory tract, in addition to the disruption of innate and adaptive responses as well as the continual production of pro-inflammatory cytokines, which have severe responses, particularly

in the elder populations, that could potentially trigger inflammatory pathogenesis [38].

The higher mortality frequency in severely ill COVID-19 patients than in mild to moderate patients ($p < 0.001$) was also reported by **Yang X.** 2020, **Weiss P.** 2020 [39,40].

The 25-hydroxy vitamin D represents the major circulating form of vitamin D, which makes it the useful indicator of vitamin D nutritional status. [41,42] The $1,25(OH)_2D$ level has a shorter half-life (15 hours) than 25(OH)D (15 days) [43,44]. and depends on many factors other than nutritional status, mainly calcium and phosphate levels, renal function, and parathyroid hormone.[2]

In accordance with the current study, **Campi et al.** 2021 [45] found that patients with severe COVID-19 and non-survivors had a significantly lower 25-hydroxyvitamin D level than patients with mild COVID-19 and survivors. A significant association between vitamin D deficiency and severity of illness in patients with COVID-19 was reported by **Al Kiyumi et al.** 2021 and **Kaya et al.** 2021 systematic reviews [46,47]. **Chiodini et al.** 2021 [48] demonstrated that severe vitamin D deficiency was associated with increased intensive care unit (ICU) admissions in severely ill COVID-19 patients.

Vitamin D deficiency was significantly associated with COVID-19 infection severity and mortality in different populations.

In many other populations, the association between vitamin D deficiency and COVID-19 severity and mortality was also reported. In Nordic countries, the low mortality rate was an exception regarding poorer outcomes in more northerly latitudes, which may be attributed to widespread vitamin D fortification of foods. In addition, more people with dark skin are less likely to have vitamin D deficiency [49].

The $1,25(OH)_2D$ has an immunomodulatory role through targeting various immune cells of innate and adaptive immune responses. It can induce monocyte differentiation into macrophages with enhanced phagocytic and chemotactic capacity [50] , inhibit dendritic cells (DCs) differentiation and maturation [51] , and induce antimicrobial gene expression [52] as well as redox homeostasis [53,54].

Vitamin D supplementation was found to increase T regulatory cells (Tregs) [55] and decrease Th17 cells. [56] Additionally, it stimulates the synthesis of surfactant by alveolar type-II cells, thus reducing the alveolar capillary damage [57,58] and may aid in the prevention of respiratory failure risk in COVID-19 patients [59,60].

Other studies [27,61,62] reported no association between decreased vitamin D and COVID-19 severity or mortality. **Cereda et al.** 2021 [61] reported that 25-hydroxyvitamin D deficiency was not associated with COVID-19 clinical features and outcomes in addition to a significant positive association between increasing 25-hydroxyvitamin D level and in-hospital mortality, which was justified by sample size as well as predominant patients with severe clinical picture and vitamin D deficiency in their study.

A negative correlation was found between age and vitamin D level [63]. This could be explained by decreased cutaneous 7-dehydrocholesterol and response to UV radiation with aging leading to decrease in pre-vitamin D production [2].

In accordance with the current study, a meta-analysis study reported higher levels of interleukin-6, C-reactive protein, ferritin, and D-dimer levels, as well as lactate dehydrogenase activity, in COVID-19 patients with vitamin D deficiency. [64] Another study found an inverse correlation between 25-hydroxyvitamin D and high CRP levels in COVID-19 patients; in addition, severe vitamin D deficiency correction was associated with decreased C-reactive protein level [65].

Hernández et al. 2021 and **Demir et al.** 2021 [66,67] reported a significant negative correlation between 25-hydroxyvitamin D with ferritin and D-dimer levels in COVID-19 patients.

Two cross-sectional studies reported negative significant correlations between serum 25-hydroxyvitamin D level with C-reactive protein level and neutrophils/lymphocytes ratio in hemodialysis patients [68,69]. Another study by **Akbas et al.** 2016 [70] reported a negative significant correlation of neutrophils/lymphocytes ratio with 25-hydroxyvitamin D level. **Skaaby et al.** 2014 [71] reported a higher risk of increasing alanine and aspartate aminotransferase activities in patients with lower vitamin D levels, but it didn't show a statistical significance.

The negative correlation between 25-hydroxyvitamin D and inflammatory markers may

be consistent with the anti-inflammatory effects of vitamin D [72].

The active vitamin D ($1,25(OH)_2D$) was found to inhibit the production of pro-inflammatory cytokines such as IL-6, IFNγ, IL-17, and IL-21 and increase the production of anti-inflammatory cytokines such as IL-10, thus vitamin D is thought to have thromboinflammatory-improving power [73]. Thus, vitamin D may decrease the risk of intravascular coagulopathy and improve lung oxygenation of COVID-19 patients [74, 67].

In contrast to the current study, **Apaydin et al.** 2022 [27] found no correlation between inflammatory markers and 25-hydroxyvitamin D in patients with COVID-19.

Another study found no correlation between 25-hydroxyvitamin D with age, interleukin 6, and high-sensitivity C-reactive protein levels, as well as white blood cell count in patients with chronic kidney disease, which was explained by the association of inflammatory markers to other conditions such as malnutrition and cachexia in vitamin D-deficient patients [75].

Vitamin D receptor gene polymorphisms can result in a dysfunctional VDR, which may affect the vitamin D genomic pathway, thus affecting both vitamin D-mediated innate and adaptive immune responses [27].

The current work showed that the observed genotype frequency of FokI (rs2228570) and TaqI (rs731236) SNPs among studied patients was in agreement with Hardy-Weinberg equilibrium.

In the present study, there was a statistically significant difference between mild to moderate and severe to critical COVID-19 patients regarding FokI SNP (p = 0.002). The mutant genotype (ff) showed a higher frequency (18%) in severely critical than mild to moderate (2.5%) COVID-19 patient groups, with an increased risk of COVID-19 severity as a great association was found by the ODDs ratio. (OR = 4.667), 95% CI (0.536–40.647); however, it did not show a statistical significance (p = 0.163). Also on assessing the effect of mode of inheritance on the degree of COVID-19 severity, the recessive mode of inheritance, where the mutant homozygous (ff) genotype was compared to wild homozygous and heterozygous (FF + Ff) genotypes, showed a significant difference between severely ill and mild COVID-19 patients (p = 0.046), with increased risk of COVID-19 severity (OR = 8.561), 95% CI (1.036–70.753).

A meta-analysis study reported an association between the FokI SNP and susceptibility to enveloped virus infection on assuming a recessive mode of inheritance. An association between the FokI SNP recessive mode of inheritance and respiratory syncytial virus was also found [17]. In addition, **Chen et al**. 2013 [76] reported that the recessive mode of inheritance of FokI SNP was associated with an increased risk of tuberculosis.

It was found that the wild F-allele showed a higher VDR transcription activity by 1.7-fold than the f-allele, assuming that the mutant f-allele is associated with diminished efficient signaling of vitamin D/VDR binding, thus impairing the expression of vitamin D-responsive genes [24]. Moreover, FokI polymorphism may increase the expression of IL-12 mRNA and protein by monocytes and DCs, particularly in response to the short F-allele [77].

In the present study, the non-significant distribution of FokI alleles (F&f) between mild to moderate and severe to critical COVID-19 patients (p = 0.804) may be attributed to the population's genetic predilection, as a lower frequency of the FokI f-allele was found in the African population when compared to Asians [17].

In contrast**, Zacharioudaki et al.** 2021 [78] found no significant association of FokI single nucleotide polymorphism and viral infection, which may be attributed to the small sample size.

The TaqI genotypes were found to be significantly different between the two studied groups (p = 0.010), with a higher frequency of the homogenous (tt) and the heterogenous (Tt) genotypes in the mild to moderate than the severely ill COVID-19 patients (17.5% and 72.5%, respectively), with a higher significant difference regarding the mutant (t) allele in the mild COVID-19 patients (p = 0.035), suggesting a protective effect against COVID-19 severity. In addition to the significant difference in assuming the dominant and the additive mode of inheritance (p = 0.005) (p = 0.023), respectively.

In accordance with the present work, **Apaydin et al.** 2022 [27] found that the TaqI wild homozygous (TT) genotype showed a poor prognosis for admission to ICU, and the heterozygous (Tt) genotype was protective in COVID-19 patients. The mutant (t) all was assumed to have a protective role against tuberculosis in Africans and other populations [79]. Also, the more stable and longer half-life of the (t) allele than the

wild allele results in higher expression of VDR protein and better vitamin D/VDR binding and response [80].

Contrarily, no associations were found between TaqI SNP alleles or genotypes and severe respiratory syncytial virus (RSV) bronchiolitis in a meta-analysis conducted by **McNally et al.** in 2014, most probably due to the small number of research studies included in the study [81].

The significant difference in 25OH vitamin D levels between the FokI genotypes (p=0.001), with the lowest level being found in the (ff) genotype, together with the added results of the post hoc test. In addition to the significant difference in 25OH D between TaqI genotypes (p=0.045), where the lowest level was found in the (TT) genotype together with added value of the post hoc test. May suggest the associations between vitamin D deficiency and VDR (FokI and TaqI) polymorphisms.

A study by **Hamed et al.** 2013 [82] reported that Fokl polymorphism was associated with vitamin D deficiency in type 1 diabetics. Another study demonstrated that more frequent vitamin D deficiency was observed with FokI mutant homozygous (ff) and heterozygous genotypes (Ff) than wild homozygous genotype (FF) in coronary artery disease patients. [83] **Bhanushali et al.** (2009) also reported strong associations between low vitamin D levels with TaqI SNP but not FoqI SNP [84].

In contrast, **Hassan et al. 2019** [85] reported no significant difference in serum vitamin D levels between various genotypes of FokI and TaqI SNPs among patients with vitiligo. This suggests that the mechanism of variation of vitamin D levels in relation to VDR polymorphisms is still unknown [85].

Conclusion

The current study findings suggested that vitamin D deficiency may predispose to more severe presentation of COVID-19, FokI recessive mode of inheritance (ff versus FF+ Ff) may be associated with increased risk for COVID-19 severity, TaqI dominant mode of inheritance (Tt + tt versus TT) and the mutant allele (t) might protect against COVID-19 severity, Thus Vitamin D deficiency and VDR polymorphisms (FokI & TaqI) may be used as predictors of COVID-19 severity. Further studies are needed to confirm these findings.

Recommendations

It is recommended to apply similar research on different populations together with studying other VDR polymorphisms in addition to clinical trials of vitamin D supplementation in order to generalize these findings.

Competing interests' statement

All authors declare no conflict of interest in this study with any business relationships

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