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RESEARCH ARTICLE

Genetic Variants of CYP2R1 Are Key Regulators of Serum Vitamin D Levels and Incidence of Myocardial Infarction in Middle-Aged Egyptians

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Abstract: ***Background***: Myocardial Infarction (MI) is one of the leading causes of morbidity and mortality in Egypt and worldwide. Vitamin D deficiency has long been linked to incidence of cardiovascular diseases. Several factors were reported to contribute to serum vitamin D level including exposure to sunlight. However, genetic variations in the vitamin D metabolic pathways have also been considered as strong determinants of vitamin D levels. CYP2R1 is the major 25-hydroxylase enzyme that is responsible for the 1st activation step of vitamin D.

Objective: to investigate the contribution of polymorphisms in CYP2R1 gene to vitamin D deficiency and incidence of MI in Egyptians.

Methods: The study included 323 subjects; 185 MI patients and 138 healthy controls. Serum 25OHD3, 25OHD2 and total 25OHD levels were measured using LC-MS/MS. SNPs rs2060793 and rs1993116 were determined by polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) which is considered one of the most commonly used techniques in genotyping. SNP rs10766197 was detected using TaqMan allele discrimination assay.

Results: Serum 25OHD3, 25OHD2 and total 25OHD levels were found to be significantly lower in MI patients than controls. The three studied SNPs were associated with significantly different total 25OHD levels and their genotype distributions differed significantly between MI patients and controls where the high risk genotypes were AG/AA for rs2060793, AG/GG for rs1993116 and AG/AA for rs10766197. Additionally, the concurrent presence of high risk genotypes of the three studied SNPs rendered those individuals at extremely higher risk for MI than each individual SNP (OR 14.1, 95% CI (3.1-64.7), p-value = < 0.0001).

Conclusions: Genetic variants of CYP2R1 are key determinants of serum 25OHD levels and are highly associated with MI risk.

Keywords: Polymorphisms, CYP2R1, 25-hydroxyvitamin D, Vitamin D, Myocardial infarction, Egyptians.

1. INTRODUCTION

Over the past two decades, vitamin D deficiency has been reported to be associated with several disorders including cardiovascular diseases, fractures and cancer [1]. Numerous studies have demonstrated the predominance of vitamin D deficiency in patients with cardiovascular disease and its strong relation with mortality [2]. Additionally, low levels of vitamin D were significantly linked to cardiovascular risk factors including hypertension, hyperlipidemia, chronic kidney disease, and diabetes [3].

Myocardial infarction is one of the main causes of death and disability worldwide. Severe vitamin D deficiency in patients with acute coronary syndromes was shown to be remarkably and independently associated with in-hospital death [4]. Preliminary clinical trials reported that vitamin D supplementation was highly beneficial in patients with CVD [5].

Vitamin D is obtained naturally by skin exposure to sun light (UVB radiation coming from sunlight is considered the

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major source of the vitamin) or from diet or supplements. It is biologically inactive and undergoes two hydroxylation steps to proceed to its active form. The 1st hydroxylation step is performed by the enzyme 25-hydroxylase to produce 25(OH)D, the major circulating form of inactive vitamin D [6]. CYP2R1 had been reported as the major 25-hydroxylase enzyme in humans with a 26-fold higher hydroxylation activity toward vitamin D3 than CYP27A1 [7].

Vitamin D deficiency can occur as a result of many genetic and non-genetic factors. Recent genome wide association studies called attention to the potential role of the genetic variants of proteins involved in the vitamin D pathway, whether synthesis, metabolism, transport or elimination, in the control of its circulating levels [8]. Studies on Dutch older population estimated the responsibility of genetic variability for 35% of the differences in 25(OH)D status among their population [9]. Despite of the growing body of evidence that suggests the association of CYP2R1 genetic polymorphisms and increased risk of vitamin D deficiency [10], more studies on different populations are still needed due to limited ethnic groups that were involved in the previous studies.

The major aim of the current study was to explore the effect of rs2060793, rs1993116 and rs10766197 SNPs in CYP2R1 gene on vitamin D levels and the incidence of myocardial infarction in the Egyptians. The 3 SNPs included in the current study were rs2060793 in the promoter region, rs10766197 in the 5' flanking region and rs1993116 in intron 1 of CYP2R1 gene.

2. METHODS

2.1. Study Participants

Three hundred twenty-three subjects participated in the study and were subdivided into two groups. One hundred eighty-five MI patients with a mean age of 54.75 ± 0.7 years (mean ± SEM) were recruited from the inpatients and outpatients of the National Heart Institute (NHI) in Imbaba, Cairo, Egypt. They had either ST elevated myocardial infarction (STEMI); non-ST elevated myocardial infarction (NSTEMI), or Unstable angina that progressed into MI. Random unrelated one hundred thirty-eight healthy volunteers aged 49.49 ± 0.86 years (mean ± SEM) were enrolled in the control group. Exclusion criteria for both groups included musculoskeletal diseases, kidney disease, major organ failure or intake of medications known to affect vitamin D levels or its metabolism. All appropriate steps have been taken in obtaining informed consent of all human subjects participating in the research. Furthermore, the procedures employed were reviewed and approved by our institutional ethics committee.

2.2. Assessments

2.2.1. Clinical Assessment

A wall mounted stadiometer was used to measure participants' height in centimeters. A regular balance was used to measure their weight in kilograms. BMI (kg/m2) was calculated. ECG and cardiac enzymes were used to diagnose ST elevated MI, non-ST elevated MI and unstable angina.

2.2.2. Laboratory Studies

Blood samples were collected from all subjects involved in the study in EDTA vacutainers and centrifuged at 2,500 rpm for 15 minutes at 4°C. The resulting plasma was stored at -80°C until 25(OH)D analysis. Levels of total 25-hydroxyvitamin D (D2 and D3) were determined using liquid chromatography tandem mass spectrometry (LCMS/MS). The instrument Waters ACQUITY Xevo TQD system, which consists of an ACQUITY UPLC H-Class system and XevoTM TQD triple-quadrupole tandem mass spectrometer with an electrospray ionization (ESI) interface (Waters Corp., Milford, MA, USA) was used for detection. Acquity BHE C18 100 mm x 2.1 mm column (particle size, 1.7 µm) was used to separate analytes (Waters, Wexford, Ireland). Subjects identified as having normal/sufficient vitamin D levels if their plasma 25(OH)D concentrations were greater than or equal to 30 ng/mL, whereas insufficient and deficient if their plasma 25(OH)D levels between 20 and 29 ng/mL and less than 20 ng/mL, respectively. Intra-assay coefficient of variations (CVs) are 1.4, 3.09 and 1.98 % at 15, 33 and 100 ng/ml of 25OHD3 and 3.55, 2.56, 3.82 % at 10, 50 and 100 ng/ml of 25OHD2, respectively. Inter-assay CVs are 7.96, 6.29 and 6.58 % at 15, 33, and 100 ng/ml of 25OHD3 and 9.2, 6.76, and 6.67 % at 10, 50, and 100 ng/ml, respectively.

2.2.3. Genetic Studies

2.2.3.1. Extraction and Purification of DNA from Human Blood by Spin Protocol

DNA extraction and purification from whole blood was done using ABIO pure extraction kit (Cat #: M501DP100, Bothell, Washington, USA). The purified DNA was free of protein, nucleases, and other contaminants or inhibitors. DNA was stored at -20°C for PCR analysis.

2.2.3.2. Determination of rs2060793 Polymorphism of CYP2R1

SNP rs2060793 of CYP2R1 gene was detected using PCR-RFLP. The PCR reaction was performed in a final volume of 50 µl containing 10 µl genomic DNA, 3 µl (10 pmole/µl) of each primer, 25 µl of RedTaq Master Mix and 9 µl nuclease free water. PCR cycles were as follows: initial denaturation at 95°C for 5 min. followed by 29 cycles each of denaturation at 95°C for 30 sec, annealing at 61°C for 45 sec and extension at 72°C for 45 sec. A final extension step was carried out at 72°C for 10 min. The primers used were forward (5' → 3'): CCTTCCAACATCGCTGTCTCT and reverse (5' → 3'): CTCTCAGAGGACAAGGTTTGCT. The resulting PCR product size was 336 bp. Enzyme digestion (RFLP) of PCR product was carried out using Thermo Scientific Fastdigest *Hinf*I restriction enzyme (#FD0804, Lot: 00439235, Carlsbad, California, US). The digested products were subjected to electrophoresis on 1.4% agarose gel stained with ethidium bromide. Digested fragments of 237 bp and 99 bp indicate the presence of the G allele, while the appearance of only 1 undigested fragment of 336 bp indicates the presence of A allele (Fig. **1**).

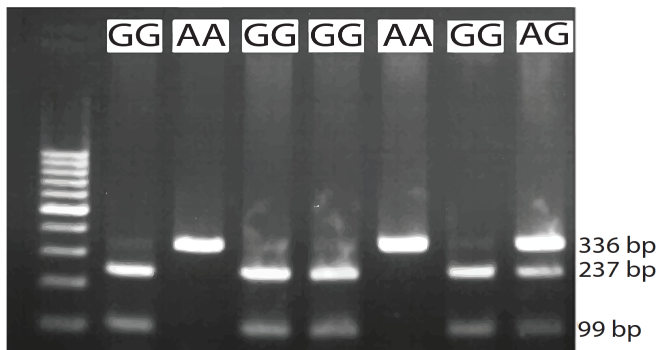

Fig. (1). A representative picture of the outcome of gel electrophoresis to detect SNP rs2060793.

2.2.3.3. Determination of rs1993116 Polymorphism of CYP2R1

SNP rs1993116 of CYP2R1 gene was also determined using PCR-RFLP. The primers used in PCR were forward (5' → 3'): CCTTCCAACATCGCTGTCTCT and reverse (5' → 3'): CTCTCAGAGGACAAGGTTTGCT. The PCR reaction final volume was 50 µl containing 10 µl genomic DNA, 3 µl (10 pmole/µl) of each primer, 25 µl of RedTaq Master Mix and 9 µl nuclease free water. PCR cycles were as follows: initial denaturation at 95°C for 5 min. followed by 29 cycles each of denaturation at 95°C for 30 sec, annealing at 56 °C for 45 sec and extension at 72°C for 45 sec. A final extension step was carried out at 72°C for 10 min. The resulting PCR product size was 552 bp. Enzyme digestion (RFLP) of PCR product was carried out using *Sph*I-*HF* restriction enzyme (New England BioLabs, Lot: 0031608). The digested products were subjected to electrophoresis on 1.4% agarose gel stained with ethidium bromide. In the presence of the A allele the restriction enzyme digests the PCR product to 400 bp and 152 bp fragments while the G allele appears as one undigested fragment of 552 bp (Fig. **2**).

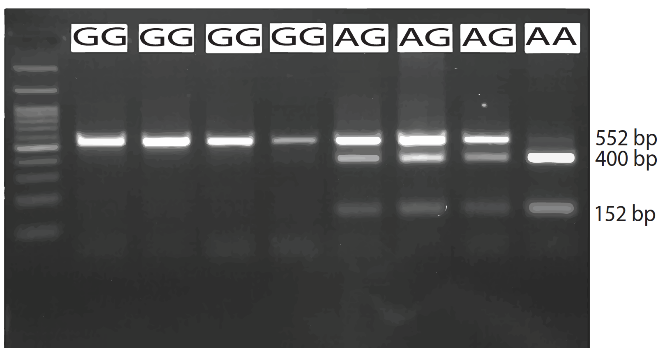

Fig. (2). A representative picture of the outcome of gel electrophoresis to detect SNP rs1993116.

2.2.3.4. Determination of rs10766197 Polymorphism of CYP2R1

SNP rs10766197 was assessed using TaqMan® allele discrimination assay on Applied Biosystems Fast Real Time PCR (CA, USA). Genotyping was performed in duplicate, and the results showed 100% reproducibility.

2.3. Statistical Analyses

Analyses were carried out using GraphPad Prism software, version 6.01and IBM SPSS software, version 22.0. Data are represented as mean ± SEM. D'Agostino and Pearson omnibus test, as well as, Shapiro-Wilk normality test was used to test for normality. Serum 25(OH)D levels were compared for each CYP2R1 SNP using the Kruskal-Wallis test, and Mann-Whitney test was performed to assess the paired difference between two genotypes within each SNP. Multvariate logistic regression analysis was performed to eradicate the influence of the other perplexing factors of vitamin D status (s-25OHD levels) such as age, sex, BMI and smoking. The genotype distribution of each SNP was assessed for Hardy-Weinberg equilibrium (HWE). To compare differences between genotypic groups, odds ratio (Fisher's exact test) was used. *P-values* <0.05 were considered statistically significant.

3. RESULTS

3.1. Baseline Analysis

The baseline characteristics for all study subjects are shown in Table **1**. The mean BMI for patients was found to be remarkably higher than that of the control subjects, and within both groups; women tend to have higher BMI than men. The percentage of smokers was significantly higher in MI patients than the controls. The majority of MI patients enrolled in the study were diagnosed with STEMI (72%).

3.2. Levels of 25OHD3, 25OHD2 and Total 25OHD among Study Participants

The majority of MI patients enrolled in this study (82%) were found to be deficient in vitamin D, contrarily, only very few healthy volunteers (3%) were deficient in vitamin D (Table **1**). In individuals with MI, the percentage of those with insufficient 25OHD levels was 12 % which is also higher than the percentage of those with insufficient 25OHD levels in the control group (9 %). Levels of 25OHD3, 25OHD2 and total 25OHD were significantly lower in MI than in the control group (*p* <0.0001) (Table **1**). No significant difference in 25OHD levels between the different subtypes of MI patients (STEMI, NSTEMI and unstable angina) was observed. However, significantly lower 25OHD3, 25OHD2 and total 25OHD levels were only observed between each subtype of MI patients and the control group (*p* <0.0001 for each subtype vs. controls) (Table **2**).

3.3. Relationship between CYP2R1 Polymorphisms (rs2060793, rs1993116 and rs10766197) and 25(OH)D in all Study Subjects

A significant difference in 25OHD levels was observed between CYP2R1 genotypes in the three studied SNPs. In rs2060793 SNP, total 25OHD level was 31.4 ± 1.4 ng/ml (mean ± SEM) in the major genotype GG, 28.6 ± 1.4 ng/ml in heterozygous and 20.6 ± 1.4 in the minor genotype AA (*p* = 0.0003). A similar trend was observed in SNP rs10766197,

Table 1. Baseline demographics, genotype distribution and clinical characteristics of the study population.

	MI Patients			Healthy Volunteers (Control Group)		
	Overall, N=185	**Men, N= 133**	**Women, N=52**	**Overall, N=138**	**Men, N=123**	**Women, N=15**
Age (years)	54.8 ± 0.7	53.6 ± 0.8	57.7 ± 1.5	49.5 ± 0.86	49.2 ± 0.9	51.6 ± 3.1
BMI (kg/m2)	25.4± 0.3***	24.7 ± 0.4	27.3 ± 0.6	22.1 ± 0.15	22 ± 0.2	22.7 ± 0.4
Smokers / Non-Smokers, N (%)						
Smokers	99*** (54%)	97 (73%)	2 (4%)	41 (30%)	37 (30%)	4 (27%)
Non-smokers	86*** (46%)	36 (27%)	50 (96%)	97 (70%)	86 (70%)	11 (73%)
Other Chronic Conditions						
Diabetes, N(%)	19 (10%)	14 (11%)	5 (10%)	None	None	None
Hypertension, N(%)	13 (7%)	7 (5%)	6 (12%)	None	None	None
Vitamin D						
Serum 25(OH)D (ng/ml)	17.4 ± 0.4***	17.8 ± 0.5	16.4 ± 0.7	43.3 ± 1.1	43.4 ± 1.2	42.4 ± 2.7
25(OH)D ≥ 30 ng/ml, N (%)	10 (6%)	9 (6%)	1 (2%)	121 (88%)	107 (87%)	14 (93%)
25(OH)D 20-30 ng/ml, N (%)	23 (12%)	18 (14%)	5 (10%)	12 (9%)	11 (9%)	1 (7%)
25(OH)D < 20 ng/ml, N (%)	152 (82%)	106 (80%)	46 (88%)	5 (3%)	5 (4%)	None
CYP2R1 Genotype						
rs2060793 SNP						
GG, N (%)	79 (43%)	54 (41%)	25 (48%)	70 (51%)	60 (49%)	10 (67%)
AG, N (%)	67 (36%)	48 (36%)	19 (37%)	53 (38%)	48 (39%)	5 (33%)
AA, N (%)	39 (21%)	31 (23%)	8 (15%)	15 (11%)	15 (12%)	None
rs1993116 SNP						
GG, N (%)	110 (60%)	73 (55%)	37 (71%)	50 (36%)	45 (37%)	5 (33%)
AG, N (%)	67 (36%)	52 (39%)	15 (29%)	57 (41%)	50 (41%)	7 (47%)
AA, N (%)	8 (4%)	8 (6%)	None	31 (23%)	28 (23%)	3 (20%)
rs10766197 SNP						
GG, N (%)	34 (18%)	26 (20%)	8 (15%)	38 (28%)	35 (29%)	3 (20%)
AG, N (%)	96 (52%)	68 (51%)	28 (54%)	69 (50%)	59 (48%)	10 (67%)
AA, N (%)	55 (30%)	39 (29%)	16 (31%)	31 (22%)	29 (23%)	2 (13%)
Clinical Diagnosis						
ST elevated MI, N (%)	134 (72%)	101 (76%)	33 (64%)	None	None	None
Non-ST elevated MI, N (%)	31 (17%)	22 (17%)	9 (17%)	None	None	None
Unstable angina, N (%)	20 (11%)	10 (8%)	10 (19%)	None	None	None

*** Significant difference from the control group at $p < 0.0001$. Age, BMI and serum 25OHD levels are referred to in the table as mean ± SEM.

Table 2. 25OHD levels among different groups of MI patients vs. control subjects. No significant difference is observed among different groups of patients. However, a significant difference is obtained between each of the studied MI patients groups vs. the control group.

	Controls	STEMI	NSTEMI	Unstable Angina
25OHD3 (ng/ml)	31.5 ± 0.9	13.2 ± 0.4 ***	15.4 ± 1.3 ***	13.6 ± 1.1 ***
25OHD2 (ng/ml)	11.8 ± 0.6	3.6 ± 0.2 ***	4.1 ± 0.3 ***	4.3 ± 0.5 ***
Total 25OHD (ng/ml)	43.3 ± 1.1	16.8 ± 0.5 ***	19.5 ± 1.4 ***	17.9 ± 1.2 ***

*** Significant difference from the control group at $p < 0.0001$.

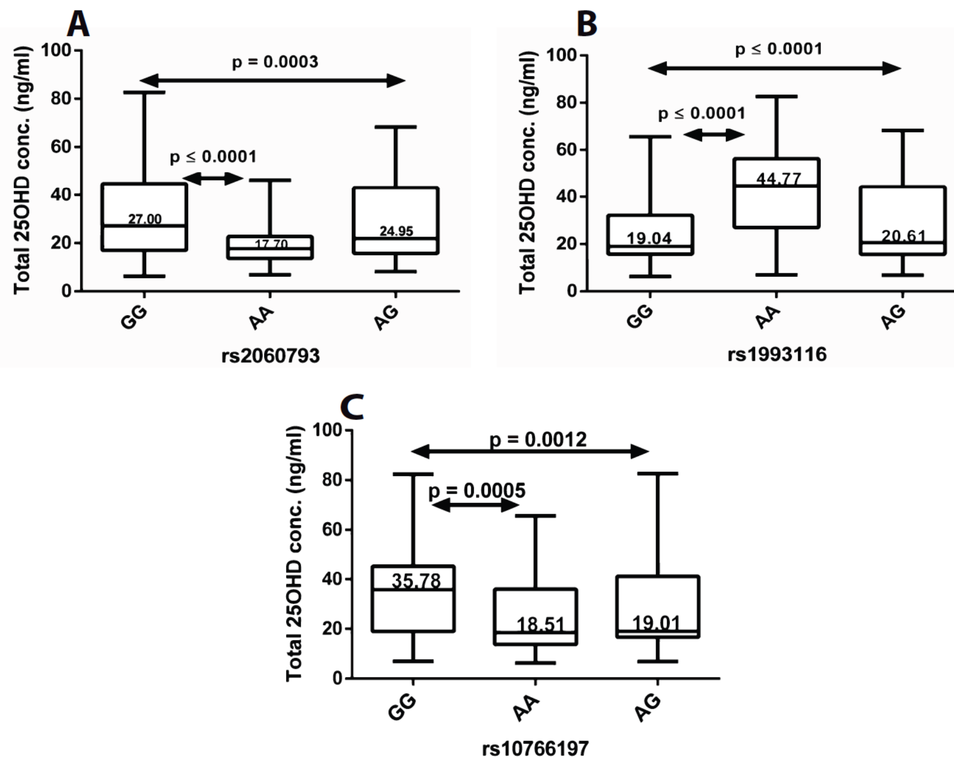

Fig. (3). Boxplots showing the median and interquartile levels (25th and 75th) for 25(OH)D levels in all study subjects (N = 323) according to CYP2R1 genotypes, using **A** rs2060793, **B** rs1993116 and **C** rs10766197 SNPs. Solid black lines represent the median 25(OH)D levels. There was a significant difference in 25(OH)D levels between genotypes in rs2060793 ($p = 0.0003$), rs1993116 ($p \leq 0.0001$), and rs10766197 ($p = 0.001$) by Kruskal-Wallis test. Mann-Whitney test p-values are shown in the body of the figure. The highest levels of 25OHD were observed in subjects who were homozygous for the major genotype (GG) for rs2060793 and rs10766197 SNPs and in subjects who were homozygous for the minor genotype (AA) for rs1993116 SNP.

where the highest total 25OHD was associated with the major genotype GG (33.2 ± 1.9 ng/ml). The total 25OHD was 28.2 ± 1.3 ng/ml in the heterozygous genotype and 25 ± 1.5 ng/ml in the minor genotype AA ($p = 0.001$). A different pattern was observed in rs1993116 where the total 25OHD level was 43.18 ± 3.179 ng/ml in the minor genotype AA, 28.98 ± 1.440 ng/ml in the heterozygous and only 24.6 ± 1 ng/ml in the major genotype GG of ($p < 0.0001$). The median, as well as, the interquartile levels (25th and 75th) for 25(OH)D levels in all study subjects are clearly demonstrated by (Fig. **3**). Multivariate logistic regression analysis showed that each of the three polymorphisms (rs2060793, rs1993116 and rs10766197) is an independent factor associated with s-25OHD levels (P < 0.05).

3.4. Relationship between CYP2R1 Polymorphisms (rs2060793, rs1993116 and rs10766197) and the Incidence of MI among the Egyptian Population

There was no deviation from HWE for the three studied polymorphisms, where $p = 0.31$ for rs2060793, $p = 0.06$ for rs1993116 and $p = 0.98$ for rs10766197, (Table **3**). The genotypic distribution of the three studied polymorphisms differed significantly among patients and controls ($p = 0.04$, 0.04 and < 0.0001 for rs2060793, rs10766197 and rs1993116, respectively). We constructed an analysis model in which the individuals with putatively most advantageous genotypes, low-risk genotypes were considered the reference group. Those individuals were demonstrated to possess homozygous

Table 3. Baseline characteristics of individual SNPs of CYP2R1. All the calculations below are based on the control subjects to detect the original characteristics of the SNP.

SNP	HWE, P	MAF, %	M/m	X2
rs2060793	0.31	30 %	G/A	1.04
rs1993116	0.06	43 %	G/A	3.44
rs10766197	0.98	47 %	G/A	0.0009

Table 4. Genetic variations in CYP2R1 and risk of MI.

Genotype of CYP2R1	Cases (n=185)	Controls (n=138)	OR (95% CI)	*p*-value
rs2060793				
GG	79 (42.7)	70 (50.7)	1	
AG	67 (36.2)	53 (38.4)	1.1 (0.7 - 1.8)	0.71
AA	39 (21.1)	15 (10.9)	2.3 (1.2 - 4.5)	0.02
AA/AG	106 (57.3)	68 (49.3)	1.4 (0.9 - 2.2)	0.18
rs1993116				
AA	8 (4.32)	31 (22.5)	1	
AG	67 (36.2)	57 (41.3)	4.6 (1.9 - 10.7)	0.0002
GG	110 (59.5)	50 (36.2)	8.5 (3.7 - 19.9)	< 0.0001
GG/AG	177 (95.7)	107 (77.5)	6.4 (2.8 - 14.5)	< 0.0001
rs10766197				
GG	34 (18.4)	38 (27.5)	1	
AG	96 (51.9)	69 (50)	1.6 (0.9 - 2.7)	0.16
AA	55 (29.7)	31 (22.5)	2 (1.1 - 3.8)	0.04
AA/AG	151 (81.6)	100 (72.5)	1.7 (0.1 - 2.9)	0.06

Values are presented as number (%) or OR (95% CI).
OR, odds radio; CI, confidence interval.

GG genotype for rs2060793, AA genotype for rs1993116 and GG genotype for rs10766197.

HWE, P refers to the *p*-value for Hardy-Weinberg equilibrium, MAF% represents the minor allele frequency, M/m: major/minor alleles, X2: the goodness of fit "Chi-square" with 1 degree of freedom.

In both rs2060793 and rs10766197 polymorphisms, only the AA genotype was found to be significantly associated with the risk of MI when compared to reference group (OR 2.3; $p = 0.02$ and OR 2; $p = 0.04$, respectively). For rs1993116 polymorphism, the carriers of the G allele showed a higher risk for the incidence of MI (OR 8.53 for homozygous genotype GG ($p < 0.0001$), 4.56 for heterozygous genotype AG ($p = 0.0002$) and 6.41 for GG/AG ($p < 0.0001$)) (Table **4**).

Additionally, we examined possible combined effects of genotypes of the three SNPs on the risk of MI by calculating ORs for all of the combinations of two and three of the risk genotypes. Similar to the previous model, the reference group consisted of individuals with the combinations of the low risk genotypes.

When combinations of two putative high risk genotypes were examined, the concurrent presence of rs2060793 AG/AA and rs10766197 AG/AA high-risk genotypes posed a more than 2-fold risk of MI (OR, 2.5; $p = 0.03$) while the coexistence of rs1993116 AG/GG and rs10766197 AG/AA high-risk genotypes showed more than 4-fold risk of MI (OR, 4.7; $p = 0.009$). The coexistence of rs2060793 AG/AA and rs1993116 AG/GG high-risk genotypes posed a more than 16-fold risk of MI and which is the highest risk of MI amongst all (OR, 17; $p < 0.0001$) (Table **5**).

When three of the putative high risk genotypes were combined, individuals with high-risk genotypes rs2060793 AG/AA, rs1993116 AG/GG and rs10766197 AG/AA were at 14.1-fold risk of MI ($p < 0.0001$) (Table **6**). These results shed the light on the strong synergistic effect of the three studied polymorphisms.

Table 5. Combined effects of two CYP2R1 genotypes (rs2060793, rs1993116 or rs10766197) and risk of MI.

Genotype of CYP2R1 Polymorphisms		Cases/Controls	OR (95% CI)	*p*-value
rs2060793	rs1993116			
GG	AA	2/19	1	
GG	AG/GG	77/51	14.3 (3.2 - 64.3)	< 0.0001
AG/AA	AA	6/12	4.8 (0.8 - 27.5)	0.11
AG/AA	AG/GG	100/56	17 (3.8 - 75.6)	< 0.0001
rs2060793	rs10766197			
GG	GG	13/19	1	
GG	AG/AA	66/51	1.9 (0.9 - 4.2)	0.16
AG/AA	GG	21/19	1.6 (0.6 - 4.1)	0.35
AG/AA	AG/AA	85/49	2.5 (1.2 - 5.6)	0.03
rs1993116	rs10766197			
AA	GG	4/10	1	
AA	AG/AA	4/21	0.5 (0.1- 2.3)	0.42
AG/GG	GG	30/28	2.7 (0.8 - 9.5)	0.15
AG/GG	AG/AA	147/79	4.7 (1.4 to 15.3)	0.01

OR, odds radio; CI, confidence interval.

Table 6. Combined effects of three CYP2R1 genotypes (rs2060793, rs1993116 or rs10766197) and risk of MI.

Genotype			Cases/Controls	OR (95% CI)	*p*-value
rs2060793	rs1993116	rs10766197			
GG	AA	GG	2/4	1	
GG	AA	AG/AA	2/15	3.8 (0.4 - 35.6)	0.27
GG	AG/GG	GG	11/15	5.5 (1. - 29.2)	0.04
AG/AA	AA	GG	2/6	2.5 (0.3 - 22.1)	0.57
GG	AG/GG	AG/AA	66/36	13.8 (3 - 63.5)	< 0.0001
AG/AA	AA	AG/AA	4/6	5 (0.7 - 34.9)	0.15
AG/AA	AG/GG	GG	19/13	11 (2.1 - 56.3)	0.002
AG/AA	AG/GG	AG/AA	81/43	14.1 (3.1 - 64.7)	< 0.0001

OR, odds radio; CI, confidence interval.

4. DISCUSSION

MI is one of the leading causes of morbidity and mortality worldwide. Many research studies reported the effect of vitamin D deficiency on vascular smooth muscle cell proliferation, inflammation, vascular calcification, renin-angiotensin system (RAS), and blood pressure [11]. Other studies examined the relationship between vitamin D levels and the risk of MI in particular. An early Danish study showed that patients with angina or MI had significantly lower 25OHD levels than the control group [12]. In a New Zealand case-control study of patients with MI, the relative risk (RR) of MI decreased in correspondence with the increase in 25OHD levels [13]. In harmony with these results, our study revealed a strong association between 25OHD levels and the risk of MI. The mean serum 25OHD3, 25OHD2 and total 25OHD were less than two-fold in MI patients group than the control group. On the other hand, a recent Polish study suggested that the parathyroid hormone, not the vitamin D, is significantly raised among the patients with heart failure. This discrepancy might be due to the relatively smaller sample size of this study compared to our present study. Parathyroid hormone levels were not assayed in the present study [14].

The mechanism by which vitamin D protects against MI can be explained by its ability to downregulate the expression of renin [15] and angiotensinogen genes by hindering the activity of their promotors [16], thus, causing the activity of RAAS to drop down and protecting against hypertension which is a major risk factor for MI. Moreover, 1,25(OH)2D

acts on the vascular smooth muscle cells and activates the generation of vascular endothelial growth factor (VEGF) which is highly essential for endothelial repair [17]. Vitamin D possesses a potent anti-inflammatory activity as well; it downregulates the expression of pro-inflammatory cytokines, such as tumor necrosis factor (TNF) and IL-6 [18] and upregulates the anti-inflammatory cytokines, IL-10 and induce IL-10 receptor expression [19].

SNPs can be considered the primary contributor to gene variations among individuals. They are commonly known for their ability to induce phenotypic differences between individuals of the same species. Their role in the development of many diseases, as well as, in determination of the response of different individuals to drug treatment has obviously come into view. SNPs can also serve as valuable biological markers [20]. Although several studies were performed to link polymorphisms in vitamin D metabolizing proteins with serum vitamin D levels, limited ethnic groups were involved in these studies. Studies have focused primarily on populations of European descent [21]. The association between the polymorphisms of CYP2R1, the gene responsible for 25-hydroxylation of vitamin D, and 25OHD has been previously studied in European [22], Chinese [23] and very little in Arab populations [24]. The genotypes of the CYP2R1 rs2060793 polymorphism were significantly associated with vitamin D status in northeastern Han Chinese children [23a]. In the current study, we aimed to investigate the effect of three genetic polymorphisms of CYP2R1 on the circulating 25OHD levels and the incidence of MI in the Egyptian population.

Many preceding studies reported an association between 25OHD levels and some polymorphisms of CYP2R1 such as rs2060793[2[illegible]1993116 [26] and rs10766197 [25b]. Interestingly, these findings were highly similar to our results. Our findings also depict a strong association between the three examined polymorphisms in CYP2R1 gene and 25OHD levels. However, in a study done on participants from 11 centers in the continental United States and Puerto Rico, rs10766197 polymorphism of CYP2R1 was the only contributor to serum response to vitamin D supplementation while other genetic factors did not contribute to these changes [27].

The current study results showed that the major genotype (GG) of both rs2060793 and rs10766197 and the minor genotype (AA) of rs1993116 were associated with highest levels of plasma 25OHD. Similar to our results, Zhang et al. reported that carriers of the rs10766197 homozygous genotype (GG) had the highest mean 25OHD concentrations of the study participants (19.48 ng/ml), followed by the heterozygous genotype (18.17 ng/ml) and the least mean 25OHD levels were recorded for carriers of the homozygous variant genotype (17.87 ng/ml) 23b. The A allele of rs2060793 was defined as the strongest SNP-risk allele for vitamin D levels in a genome wide association study[2[illegible]ur results were also similar to a large study that involved more than 60 thousands Singapore Chinese men and women which reported that major allele of rs1993116 was associated with lower 25(OH)D concentrations [28]. On the contrary, SNP rs1993116 did not show any association with vitamin D level in orthopedic outpatients living in Eastern province of the Kingdom of Saudi Arabia [29].

Studies that investigated the role of CYP2R1 in MI susceptibility are scarce. Our research group had previously provided some evidence that SNP rs10741657 of CYP2R1 gene predicted both 25(OH)D levels and CAD incidence in the Egyptian subjects [30]. Both rs1993116 and rs10766197 SNPs of CYP2R1 were not investigated before for their association with the incidence of MI. Our findings revealed a strong association between each of the three examined SNPs in CYP2R1 (rs2060793, rs1993116 and rs10766197) and risk of MI in a very similar pattern to that observed in 25OHD levels. We found out that the same genotypes that are highly linked to low 25OHD levels pose a significantly higher risk to MI incidence.

In addition to examining the individual relationship of each SNP with the incidence of MI, we aimed to assess the combined effects of two and three high risk genotypes on MI incidence. Our findings revealed an extremely higher risk in case of the concurrent presence of two or three high risk genotypes than in the presence of a single high risk genotype, which suggests an additive or a synergistic effect of these genotypes.

CONCLUSION

Vitamin D deficiency is associated with the incidence of MI. The three studied CYP2R1 genetic polymorphisms (rs2060793, rs1993116 and rs10766179) are linked to low serum vitamin D levels and pose a considerable risk for MI in the Egyptian population. Furthermore, the concurrent presence of high risk genotypes rs2060793 AG/AA, rs1993116 AG/GG and rs10766197 AG/AA were associated with an extremely higher risk "about 14 fold higher risk" for MI as a result of their synergistic effect.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All appropriate steps have been taken in obtaining informed consent of all human subjects participating in the research. Furthermore, the procedures employed were reviewed and approved by The German University in Cairo Ethics Committee on 08-May-2016.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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REFERENCES

[1] Muscogiuri, G.; Altieri, B.; Annweiler, C.; Balercia, G.; Pal, H.; Boucher, B.J.; Cannell, J.J.; Foresta, C.; Grübler, M.R.; Kotsa, K. Vitamin D and chronic diseases: The current state of the art. *Archiv. Toxicol.,* **2017,** *91*(1), 97-107.

[2] Wang, T.J. Vitamin D and cardiovascular disease. *Ann. Rev. Med.,* **2016,** *67*, 261-272.

[3] Liu, W.-C.; Wu, C.-C.; Hung, Y.-M.; Liao, M.-T.; Shyu, J.-F.; Lin, Y.-F.; Lu, K.-C.; Yeh, K.-C. Pleiotropic effects of vitamin D in chronic kidney disease. *Clin. Chim. Acta,* **2016,** *453*, 1-12.

[4] Correia, L.C.; Sodré, F.; Garcia, G.; Sabino, M.; Brito, M.; Kalil, F.; Barreto, B.; Lima, J.C.; Noya-Rabelo, M.M. Relation of severe deficiency of vitamin D to cardiovascular mortality during acute coronary syndromes. *Amer. J. Cardiol.,* **2013,** *111*(3), 324-327.

[5] Gotsman, I.; Shauer, A.; Zwas, D.R.; Hellman, Y.; Keren, A.; Lotan, C.; Admon, D. Vitamin D deficiency is a predictor of reduced survival in patients with heart failure; vitamin D supplementation improves outcome. *Europ. J. Heart Fail.,* **2012,** *14*(4), 357-366.

[6] Holick, M.F. Vitamin D deficiency. *N. Engl. J. Med.,* **2007,** *2007*(357), 266-281.

[7] Shinkyo, R.; Sakaki, T.; Kamakura, M.; Ohta, M.; Inouye, K. Metabolism of vitamin D by human microsomal CYP2R1. *Biochemic. Biophysic. Res. Commun.,* **2004,** *324*(1), 451-457.

[8] Jolliffe, D.A.; Walton, R.T.; Griffiths, C.J.; Martineau, A.R. Single nucleotide polymorphisms in the vitamin D pathway associating with circulating concentrations of vitamin D metabolites and non-skeletal health outcomes: Review of genetic association studies. *J. Steroid Biochem. Mol. Biol.,* **2016,** *164*, 18-29.

[9] Brouwer-Brolsma, E.M.; Vaes, A.M.; van der Zwaluw, N.L.; van Wijngaarden, J.P.; Swart, K.M.; Ham, A.C.; van Dijk, S.C.; Enneman, A.W.; Sohl, E.; van Schoor, N.M. Relative importance of summer sun exposure, vitamin D intake, and genes to vitamin D status in Dutch older adults: The B-PROOF study. *J. Steroid Biochem. Mol. Biol.,* **2016,** *164*, 168-176.

[10] (a) Simon, K.C.; Munger, K.; Kraft, P.; Hunter, D.; De Jager, P.; Ascherio, A. Genetic predictors of 25-hydroxyvitamin D levels and risk of multiple sclerosis. *J. Neurol.,* **2011,** *258*(9), 1676-1682; (b) Ye, Z.; Sharp, S.J.; Burgess, S.; Scott, R.A.; Imamura, F.; Langenberg, C.; Wareham, N.J.; Forouhi, N.G.; Consortium, I., Association between circulating 25-hydroxyvitamin D and incident type 2 diabetes: A mendelian randomisation study. *Lancet Diabet. Endocrinol.,* **2015,** *3*(1), 35-42.

[11] Zittermann, A.; Schleithoff, S.S.; Koerfer, R. Putting cardiovascular disease and vitamin D insufficiency into perspective. *Brit. J. Nutrit.,* **2005,** *94*(4), 483-492.

[12] Lund, B.; Badskjaer, J.; Lund, B.; Soerensen, O., Vitamin D and ischaemic heart disease. *Hormone Metabolic Res.,* **1978,** *10*(06), 553-556.

[13] Scragg, R.; Jackson, R.; Holdaway, I.M.; Lim, T.; Beaglehole, R. Myocardial infarction is inversely associated with plasma 25-hydroxyvitamin D3 levels: a community-based study. *Int. J. Epidemiol.,* **1990,** *19*(3), 559-563.

[14] Kolaszko, A.; Nowalany-Kozielska, E.; Ceranowicz, P.; Morawiec, B.; Kubiak, G. The role of parathyroid hormone and vitamin d serum concentrations in patients with cardiovascular diseases. *Dis. Markers,* **2018,** *2018*.

[15] Kienreich, K.; Tomaschitz, A.; Verheyen, N.; Pieber, T.; Gaksch, M.; Grübler, M.R.; Pilz, S. Vitamin D and cardiovascular disease. *Nutrients,* **2013,** *5*(8), 3005-3021.

[16] Danik, J.S.; Manson, J.E. Vitamin D and cardiovascular disease. *Curr. Treat. Options Cardiovasc. Med.,* **2012,** *14*(4), 414-424.

[17] Norman, P.; Powell, J. Vitamin D and cardiovascular disease. *Circ. Res.,* **2014,** *114*(2), 379-393.

[18] Zhang, Y.; Leung, D.Y.; Richers, B.N.; Liu, Y.; Remigio, L.K.; Riches, D.W.; Goleva, E. Vitamin D inhibits monocyte/macrophage proinflammatory cytokine production by targeting MAPK phosphatase-1. *J. Immunol.,* **2012,** *188*(5), 2127-2135.

[19] Canning, M.O.; Grotenhuis, K.; de Wit, H.; Ruwhof, C.; Drexhage, H.A. 1-alpha, 25-Dihydroxyvitamin D3 (1, 25 (OH)(2) D (3)) hampers the maturation of fully active immature dendritic cells from monocytes. *Europ. J. Endocrinol.,* **2001,** *145*(3), 351-357.

[20] Komar, A.A. Single nucleotide polymorphisms. *Methods Mol. Biol.,* **2009,** *578*.

[21] Gozdzik, A.; Zhu, J.; Wong, B.Y.; Fu, L.; Cole, D.E.; Parra, E.J. Association of vitamin D binding protein (VDBP) polymorphisms and serum 25(OH)D concentrations in a sample of young Canadian adults of different ancestry. *J. Steroid Biochem. Mol. Biol.,* **2011,** *127*(3-5), 405-412.

[22] Ramos‐Lopez, E.; Brück, P.; Jansen, T.; Herwig, J.; Badenhoop, K. CYP2R1 (vitamin D 25‐hydroxylase) gene is associated with susceptibility to type 1 diabetes and vitamin D levels in Germans. *Diabet./Metabol. Res. Rev.,* **2007,** *23*(8), 631-636.

[23] (a) Zhang, Y.; Wang, X.; Liu, Y.; Qu, H.; Qu, S.; Wang, W.; Ren, L. The GC, CYP2R1 and DHCR7 genes are associated with vitamin D levels in northeastern Han Chinese children. *Swiss Med. Wkly.,* **2012,** *142*, w13636; (b) Zhang, Z.; He, J.W.; Fu, W.Z.; Zhang, C.Q.; Zhang, Z.L. An analysis of the association between the vitamin D pathway and serum 25‐hydroxyvitamin D levels in a healthy Chinese population. *J. Bone Mineral Res.,* **2013,** *28*(8), 1784-1792.

[24] Elkum, N.; Alkayal, F.; Noronha, F.; Ali, M.M.; Melhem, M.; Al-Arouj, M.; Bennakhi, A.; Behbehani, K.; Alsmadi, O.; Abubaker, J. Vitamin D insufficiency in Arabs and South Asians positively associates with polymorphisms in GC and CYP2R1 genes. *PLOS One,* **2014,** *9*(11), e113102.

[25] (a) Lu, L.; Sheng, H.; Li, H.; Gan, W.; Liu, C.; Zhu, J.; Loos, R. J.; Lin, X. Associations between common variants in GC and DHCR7/NADSYN1 and vitamin D concentration in Chinese Hans. *Human Genetics,* **2012,** *131*(3), 505-512; (b) Bu, F.-X.; Armas, L.; Lappe, J.; Zhou, Y.; Gao, G.; Wang, H.-W.; Recker, R.; Zhao, L.-J. Comprehensive association analysis of nine candidate genes with serum 25-hydroxy vitamin D levels among healthy Caucasian subjects. *Human Genetics,* **2010,** *128*(5), 549-556.

[26] Ahn, J.; Yu, K.; Stolzenberg-Solomon, R.; Simon, K.C.; McCullough, M.L.; Gallicchio, L.; Jacobs, E. J.; Ascherio, A.; Helzlsouer, K.; Jacobs, K.B. Genome-wide association study of circulating vitamin D levels. *Human Mol. Genet.,* **2010,** *19*(13), 2739-2745.

[27] Rees, J.R.; Mott, L.A.; Barry, E.L.; Baron, J.A.; Bostick, R.M.; Figueiredo, J.C.; Bresalier, R.S.; Robertson, D.J.; Peacock, J.L. Lifestyle and other factors explain one-half of the variability in the serum 25-hydroxyvitamin D response to cholecalciferol supplementation in healthy adults. *J. Nutrit.,* **2016,** *146*(11), 2312-2324.

[28] Robien, K.; Butler, L.M.; Wang, R.; Beckman, K.B.; Walek, D.; Koh, W.-P.; Yuan, J.-M. Genetic and environmental predictors of serum 25-hydroxyvitamin D concentrations among middle-aged and elderly Chinese in Singapore. *Brit. J. Nutrit.,* **2013,** *109*(3), 493-502.

[29] Sadat-Ali, M.; Al-Turki, H.A.; Azam, M.Q.; Al-Elq, A.H. Genetic influence on circulating vitamin D among Saudi Arabians. *Saudi Med. J.,* **2016,** *37*(9), 996.

[30] Hassanein, S.I.; Abu el Maaty, M.A.; Sleem, H.M.; Gad, M.Z. Triangular relationship between single nucleotide polymorphisms in the CYP2R1 gene (rs10741657 and rs12794714), 25-hydroxyvitamin d levels, and coronary artery disease incidence. *Biomarkers,* **2014,** *19*(6), 488-492.