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The association of megalin and cubilin genetic variants with serum levels of 25-hydroxvitamin D and the incidence of acute coronary syndrome in Egyptians: A case control study

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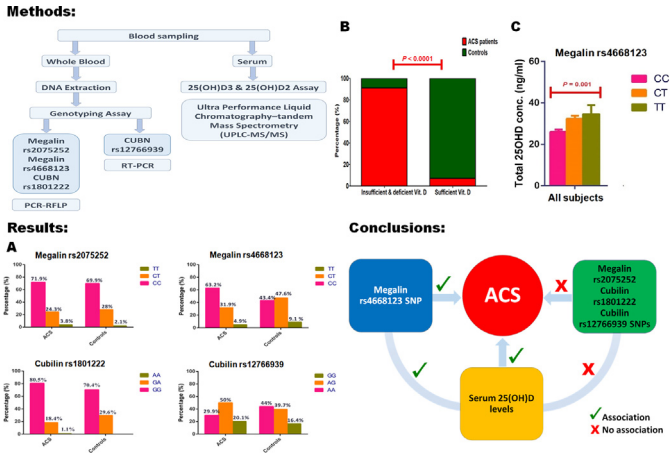
HIGHLIGHTS

- The manuscript describes the link of vitamin D deficiency to acute coronary syndrome.
- It tackles megalin and cubilin genetic variants in ACS Egyptians.
- It explores the effects of megalin and cubilin polymorphisms on serum 25 (OH)D levels.
- Megalin rs4668123 (CC) can be considered an independent risk factor forACS incidence.
- Megalin rs4668123 (CC) was linked to lower 25(OH)D levels.

GRAPHICAL ABSTRACT



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ABSTRACT

Megalin and cubilin are two receptors that mediate endocytosis of 25-hydroxyvitamin D (25(OH)D) for its final activation by hydroxylation. The aim of the present study was to evaluate the association of polymorphisms in megalin (rs2075252 and rs4668123) and cubilin (rs1801222 and rs12766939) with the circulating serum levels of 25(OH)D and with the early incidence of acute coronary syndrome (ACS) in Egyptians. The study included 328 subjects; 185 ACS patients aged between 27 and 60 years, and 143 healthy age-matched controls. Genotyping of cubilin rs12766939 Single Nucleotide Polymorphism (SNP) was performed using Real-Time Polymerase Chain Reaction (qPCR) and for megalin rs4668123 and rs2075252 and cubilin rs1801222 by Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (PCR-RFLP). 25(OH)D levels were measured by Ultra Performance Liquid Chromatography- Tandem Mass Spectroscopy (UPLC-MS/MS). Results showed that vitamin D deficiency

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Polymorphisms
Vitamin D receptor

was highly linked to ACS incidence ($P < 0.0001$). The megalin rs4668123 CC, cubilin rs1801222 GG and cubilin rs12766939 GG + GA genotypes are associated with a higher ACS incidence and can be considered risk factors, according to Chi-squared test ($P = 0.0003$, 0.0442, 0.013 respectively). Conversely, the megalin rs2075252 SNP was not associated with increased ACS incidence. However, after performing multiple logistic regression analysis, only the megalin rs4668123 SNP was considered an independent ACS risk factor. Furthermore, the megalin rs4668123 CC genotype was associated with lower 25(OH)D levels ($P = 0.0018$). In conclusion, megalin rs4668123 (CC) was linked to lower 25(OH)D levels and can be considered an independent risk factor for incidence of ACS.

Introduction

Due to a series of revolutionary discoveries in the last decade, vitamin D has stepped out from the shadows of bone diseases to emerge as a prominent player in non-calcemic actions. Vitamin D exists in two forms, D_2 and D_3, where D_3 is the principal form in humans, photosynthesized from 7-dehydrocholesterol precursor in the epidermal and dermal cells [1]. Vitamin D is biologically inert and must undergo two activation phases: 25-hydroxylation in the liver and 1α-hydroxylation in the kidney [2,3]. These steps yield the biologically active form, 1,25-dihydroxyvitamin D (1,25 (OH)D2) [4].

Unfortunately, almost 1 billion people worldwide, across all ethnicities and age groups, are vitamin D deficient and nearly 50% are vitamin D insufficient [5]. Paradoxically, although the Middle East receives abundant sunshine throughout the year, one third to one half of its inhabitants have deficient serum levels of 25-hydroxyvitamin D (25(OH)D) [6]. Numerous studies have linked vitamin D deficiency with insidious long-term consequences that can imprint on children and adults for the rest of their lives, such as increased risk for type1 diabetes, multiple sclerosis, cancers and cardiovascular diseases (CVDs) [3]. Cardiovascular disease is recognized as the leading cause of death and disability worldwide accounting for 30% of all global deaths per year [7]. Among all CVDs, acute coronary syndrome (ACS) is the number one killer in both genders, accounting for 46% of cardiovascular deaths among men and 38% among women [8]. ACS is the umbrella term for the clinical signs and symptoms of: unstable angina together with myocardial infarction, including non-ST-segment elevation myocardial infarction (NSTEMI) and ST-segment elevation myocardial infarction (STEMI) [9].

It would be of value to mention the positive influence of vitamin D with relevance to the cardiovascular system. Vitamin D induces vascular endothelial growth factor production, thus promotes endothelial repair. Vitamin D also protects against atherosclerosis by increasing cholesterol efflux from macrophages to extracellular high density lipoprotein [10], inhibiting vascular smooth muscle cells proliferation and migration [11], and suppressing pro-inflammatory cytokines [12,13]. Furthermore, it reduces NADPH oxidase expression, therefore diminishes oxidative stress [14] and increases endothelial nitric oxide production [10]. Additionally, $1,25(OH)_2D$ suppresses markers of cardiac hypertrophy and regulates myocardial extracellular matrix turnover, thus protecting against cardiac fibrosis [15]. $1,25(OH)_2D$ also suppresses the renin-angiotensin-aldosterone system [16]. Antidiabetic properties of vitamin D include increasing insulin secretion and sensitivity [17].

Vitamin D pathway incorporates several proteins. Two of these, megalin and cubilin, are receptors localized in the kidney proximal tubule that mediate the uptake of the filtered Vitamin D Binding protein (DBP) −25(OH)D complex through apical clathrin-coated pits into coated vesicles and subsequently to endosomes. There, the DBP-25(OH)D dissociates from the receptors due to acidification of the endocytic compartment. The receptors are recycled and returned to the apical plasma membrane. The DBP proteins are degraded in lysosomes, and 25(OH)D diffuses to the cytosol. The 25(OH)D is either secreted into the circulation or hydroxylated by 1α-hydroxylase in the mitochondria to $1,25(OH)_2D$ before release into the interstitial fluid at the basolateral membrane to complex with DBP. Cubilin greatly facilitates the endocytic process by sequestering the steroid-carrier complex on the cell surface before its association with megalin and internalization of the cubilin-bound 25(OH)D-DBP. Some 25(OH)D-DBP binds directly to megalin. $1,25(OH)_2D$ increases megalin expression, protects against renal anaemia and has renoprotective effects on kidney podocytes, thus lowering chronic kidney disease risk [18]. There are only a few investigations that have examined the genetic factors of the relationship between vitamin D deficiency and CVD [19]. Accordingly, patients with either cubilin or megalin gene mutations show low serum levels of $1,25(OH)_2D$, disturbed calcium homeostasis, and severe bone-formation defects, including growth retardation and decreased bone mineralization [20,21].

The aim of the present study was to evaluate the association of polymorphisms in megalin (rs2075252 and rs4668123) and cubilin (rs1801222 and rs12766939) with the circulating serum levels of 25(OH)D and with the early incidence of ACS in Egyptians.

Patients and methods

Subjects

In total, 185 patients, aged between 27 and 60 years and with confirmed ACS, were recruited from in-patient and out-patient settings of the National Heart Institute (NHI) in Imbaba, Cairo. ACS was verified as either a history of myocardial infarction or of unstable or stable angina. A second group of 143 age-matched (22–59 years) persons with no diagnostic signs of ACS were selected by convenience sampling to serve as controls. The baseline characteristics for all study subjects are represented in Table 1.

Both groups of subjects had controlled blood pressure of below 140/90 mmHg; only a few patients were hypertensive and were taking antihypertensive medication not known to interfere with circulating levels of 25(OH)D. All subjects were Egyptians and were therefore population matched. The presence of other chronic diseases such as, kidney, heart and liver diseases as well as diabetes milletus, was the exclusion criterion in this study. All subjects were informed of the nature of the study and provided written consent that conformed to the Helsinki declaration. Study approval was obtained from both the NHI and the German University in Cairo.

Sample collection

The obtained blood samples were collected in EDTA coated vacutainers. Whole blood was used for DNA isolation. Plasma

Table 1
The baseline characteristics of the study groups.

	ACS patients			Control subjects		
	Overall, N = 185	Men, N = 133	Women, N = 52	Overall, N = 143	Men, N = 128	Women, N = 15
Age (years)	54.75 ± 0.7	53.62 ± 0.78	57.65 ± 1.45	49.49 ± 0.86	49.24 ± 0.89	51.6 ± 3.14
BMI (kg/m^2)	25.4 ± 0.33***	24.6 ± 0.37	27.29 ± 0.63	22.05 ± 0.15	21.97 ± 0.15	22.71 ± 0.43
Clinical Diagnosis						
ST elevated MI, N (%)	134 (72.43%)	101 (75.94%)	33 (63.46%)	None	None	None
Non-ST elevated MI, N (%)	31 (16.76%)	22 (16.54%)	9 (17.31%)	None	None	None
Unstable angina, N (%)	20 (10.81%)	10 (7.52%)	10 (19.23%)	None	None	None
Season of sample collection, N (%)						
Fall	4 (2.16%)	2 (1.5%)	2 (3.85%)	52 (36.36%)	46 (35.94%)	6 (40%)
Winter	29 (15.68%)	21 (15.79%)	8 (15.38%)	50 (35%)	48 (37.5%)	2 (13.33%)
Spring	79 (42.7%)	60 (45.11%)	19 (36.54%)	7 (4.9%)	5 (3.91%)	2 (13.33%)
Summer	73 (39.46%)	50 (37.59%)	23 (44.23%)	34 (23.78%)	29 (22.66%)	5 (33.33%)
Smokers/Non-Smokers, N (%)						
Smokers	99 (53.51%)***	97 (72.93%)	2 (3.85%)	46 (32.17%)	42 (32.81%)	4 (26.67%)
Non-smokers	86 (46.49%)***	36 (27.07%)	50 (96.15%)	97 (67.83%)	86 (67.19%)	11 (73.33%)
Other chronic conditions						
Diabetes, N(%)	19 (10.27%)	14 (10.53%)	5 (9.62%)	None	None	None
Hypertension, N(%)	13 (7.03%)	7 (5.26%)	6 (11.54%)	None	None	None

MI: myocardial Infarction, N: Number, BMI: Body Mass Index.
Age and BMI are expressed as mean ± SEM.
***BMI in patients with ACS is significantly different from the control group at $P < 0.001$ calculated by Mann-Whitney test.
***Number of smokers and non-smokers is also significantly different between patients and controls at $P < 0.001$ calculated by Chi-squared test.

was obtained by centrifugation of whole blood at 2500 rpm (Eppendorf Fixed-angle rotor F-34-6-38, 3500 g)for 10 min at 4 °C and was stored at −80 °C until 25(OH)D analysis.

Selection of the candidate genes

The candidate genes and polymorphisms were selected based on a prior knowledge of their involvement in the metabolic pathway of 25(OH)D in humans. SNPS were only included if they were validated and non-synonymous, indicating potential functionality, if they had a known minor allele frequency (MAF) > 0.1, or if they had a previously shown association with 25(OH)D levels. The selected SNPs were megalin (rs2075252 and rs4668123) and cubilin (rs1801222 and rs12766939).

Genotyping

Genomic DNA was extracted from whole blood samples using an ABIOpureTM Genomic DNA Blood/Cell Culture Extraction Kit following the manufacturer's instructions (Alliance Bio Inc., Washington, USA). Absorbance at 260 nm was measured with the FLUOstar® Omega NanoDrop instrument (BMG Labtech, Ortenberg, Germany) and DNA concentration was calculated using the NanoDrop nucleic acid application module. DNA purity was assessed by 260/280 absorbance ratios. A ratio of ~1.8 is generally accepted as "pure" for DNA and 100 ug/mL was the minimum DNA concentration accepted. DNA extraction was repeated for samples which failed to meet the minimum DNA concentration and purity recommended for genotyping.

The polymorphisms rs2075252 and rs4668123 in the megalin gene and rs1801222 in the cubilin gene were genotyped using a polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) method. The National Center for Biotechnology Information primer designing tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast) was used to design specific primers for the polymorphisms rs2075252, rs4668123, and rs1801222, which are listed in Table 2. The thermal profile of the polymerase chain reaction (PCR) of the three SNPs was initiated with a 5 min denaturation period at 95 °C, followed by 35 cycles of denaturation at 95 °C, annealing (temperatures in Table 1) and extension at 72 °C, with each step lasting 30 sec. The thermal profiles end with a final extension period of 10 min at 72 °C. Successful amplification was checked by electrophoresis. The PCR products were purified using a PureLink™ PCR Purification Kit (Invitrogen, California, USA) following the manufacturer's instructions. Restriction enzymes (New England BioLabs, Hitchin, UK) are summarized in Table 1. The reaction was conducted by combining 10 μL of PCR product for each SNP with 17 μL nuclease-free water, 2 μL CutSmart Buffer and 1 μL of the restriction enzyme. Post restriction, the products were electrophoresed on a 2% agarose gel and viewed under ultraviolet gel documentation system. To confirm the accuracy of genotyping, a randomly selected sample (15%) of the study cohort was reanalyzed.

The genotyping for the rs12766939 SNP was performed in 96-well plates using the validated fluorogenic 5′-nuclease genotyping TaqMan assay C_3135025_10 (which includes designed probes and primers as supplied by Applied Biosystems, Massachusetts, USA) using a Stratagene Mx3005P qPCR – Agilent Genomics real-time qPCR system. The assay was carried out according to the manufacturer's instructions. Each reaction mix consisted of the following: 20 ng of genomic DNA diluted to 11.25 μL with DNase-free water, 13.75 μL of SNP reaction mixture consisting of 12.5 μL of TaqMan Universal PCR Mastermix and 1.25 μL of 20× working stock of SNP genotyping assay (probe and primer solution). The PCR program started with an initial denaturation at 95 °C for 10 min for activating the AmpliTaq Gold enzyme followed by 40 cycles of denaturation at 92 °C for 15 sec and an annealing/extension temperature at 62 °C for 1 min. Allelic discrimination assays were performed using two TaqMan MGB probes (VIC/FAM dye) that target the SNP sites. The obtained genotyping data were presented according to the NCBI SNP cluster reports. Positive control samples (homozygote for wild alleles, heterozygote, or homozygote for variant alleles) and the negative control sample were included in each batch of samples. Discordant samples were repeated.

25(OH)D determination

25(OH)D levels were measured using UPLC-MS/MS, which permitted an individualized assessment of both vitamin D metabolite forms: 25(OH)D_2 and 25(OH)D_3 [22,23]. Waters Acquity Xevo TQD

Table 2
A summary of the PCR thermal profile and RFLP conditions for the megalin and cubilin polymorphisms.

Rs	Alleles	Primers for PCR amplification (5′-3′)	Annealing temperature (C)	PCR product length (bp)	RFLP analysis	
					Restriction enzyme	Restriction fragment length (bp)
Megalin polymorphisms						
rs2075252	C/T	F: TGTTTGTTTACAGGTAGCTCTCC R: AGAAAGAAATCAGGAAAGCTTGG	61.0	352	*Ava*I	T = 352 C = 213 + 139
rs4668123	C/T	F: ACAAATTGGGGAATTGGGGC R: ATCAGCAGCTTCCGTATCCT	61.0	550	*Fsp*I	T = 550 C = 305 + 245
Cubilin polymorphisms						
rs1801222	G/A	F: TGACTTACAGTTCTTGATTGTTGTT R: TGTGGCCCTGAGAATGTACC	57.0	451	*Bbs*I	A = 451 G = 298 + 153

System instrument (Waters Corporation Milford, MA, USA) was used for detection which consists of an ACUITY UPLC H-Class system and Xevo™ TQD triple-Quadropole tandem mass spectrometer with an electrospray ionization (ESI) interface. An Acuity UPLC BEH C18 Phenyl Column (2.1 × 10 cm, particle size: 1.7 μm) was used to separate analytes (Waters Corporation Milford, MA, USA). According to agreed-upon standards, subjects were categorized as normal when their total 25(OH)D concentration were equal to or exceeded 30 ng/μL, whereas insufficient and deficient subjects had 25(OH)D levels of 21–29 ng/μL and less than 20 ng/μL, respectively [2,5,13,24–27]. Intra-assay coefficients of variations (CVs) were 1.4, 3.09, and 1.98% at 15, 30 and 100 ng/mL respectively of 25 $(OH)D_3$ and 3.55, 2.56, 3.82% at 10, 50 and 100 ng/mL respectively of $25(OH)D_2$. Inter-assay CVs were 7.96, 6.29 and 6.58% at 15, 30 and 100 ng/mL respectively of $25(OH)D_3$ and 9.2, 6.76, and 6.67% at 10, 50 and 100 ng/mL respectively of $25(OH)D_2$. The lower limit of detection (LOD) of either metabolite was 1.5 ng/mL. The lower limit of quantification (LOQ) for these compounds was 5 ng/mL. The recovery of $25(OH)D_2$ and $25(OH)D_3$ ranged from 86% to 98% over the analytical range of the assay.

Statistical analyses

Analyses were performed using Graphpad Prism statistics software, version 6.01 and SPSS 13.0. The D'Agostino and Pearson omnibus test was used to test for normality. Odds ratio (Chi-squared test) was used to measure the risk of susceptibility to ACS. The association of different genotypes of a SNP with 25(OH)D concentrations was tested using the Kruskal-Wallis test. To test the independent association of the studied SNPs with the incidence of ACS and vitamin D status, multiple logistic regression analysis was performed to eradicate the influence of age, gender, BMI and smoking on both the incidence of ACS and vitamin D status in addition to the influence of season of sample collection on vitamin D status only. The Hardy-Weinberg equilibrium was calculated for both patients and controls for the four SNPs. Statistical significance was defined as a *P*-value of less than or equal to 0.05.

Results

The baseline characteristics of the studied subjects are presented in Table 1.

Vitamin D status

The total vitamin D status of patients and controls was categorized as deficient, insufficient, or normal, as listed in Table 3. The prevalence of vitamin D insufficiency and deficiency significantly increased the ACS risk by more than 100 folds ($P < 0.0001$).

Genotyping

Representative gels of PCR-restriction fragment length polymorphism products of the megalin rs2075252 and rs4668123 SNPs and the cubilin rs1801222 SNPs are presented in Fig. 1.

The genotypes distribution pattern of the megalin rs2075252 and rs4668123 SNPs and cubilin rs1801222 and rs12766939 SNPs were consistent with the Hardy Weinberg equilibrium ($P > 0.05$). Allelic and genotypic distributions of the four polymorphisms among patients and controls are presented in Table 4.

Genotype and allelic distributions of megalin rs2075252 were not different between patients and controls ($P > 0.05$) as shown in Table 4. Individuals with the megalin rs4668123 CC genotype have a 2.2 times higher risk of incidence of ACS when compared with individuals with either TT or CT genotypes ($P = 0.0003$). The C variant also represented a higher risk for ACS than did the T variant ($P = 0.0005$; OR = 1.863). After performing multiple logistic regression, the adjusted *P* value was 0.001, indicating that the megalin rs4668123 polymorphism may be a risk factor for ACS.

As for cubilin rs1801222 polymorphism, Chi-squared test revealed that individuals with the GG genotype have 1.7 times the risk of incidence of ACS than did individuals with the AA or GA genotypes. ($P = 0.0442$) Overall, 80.5% of the patients with ACS were high risk GG genotype carriers, while 18.4% were GA genotype carriers. Regarding controls 70.4% were GG, while 29.6% were GA genotype carriers. However, multiple logistic regression revealed that cubilin rs1801222 might not be an independent risk factor for ACS (adjusted $P > 0.05$).

Statistical analyses revealed the association of the cubilin rs12766939 with ACS incidence with the predominance of the G alleles ($P = 0.0337$; OR = 1.4) and the predominance of G carrier genotypes (AG and GG) ($P = 0.0182$; OR = 1.8) in ACS patients. Similar to the cubilin rs1801222 SNP, multivariate logistic regression revealed that cubilin rs12766939 SNP might not be an independent risk factor for ACS ($P > 0.05$).

The association of each SNP with 25(OH)D levels was assessed by investigating the connection separately in patients, then in controls and finally by pooling all subjects together. The megalin rs4668123 influenced $25(OH)D_3$, $25(OH)D_2$ and total 25(OH)D concentrations, when pooling all subjects together, and the TT genotype exhibited the highest concentrations of serum vitamin D, followed by the CT genotype. The carriers of the CC genotype showed lower values of serum vitamin D and higher frequencies of vitamin D insufficiency and of vitamin D deficiency when compared to the other genotypes. After performing multivariate logistic regression, the adjusted *P* value was 0.007 for total 25(OH)D. By contrast, the megalin rs2075252 SNP was not associated with 25 $(OH)D_3$, $25(OH)D_2$ or total 25(OH)D levels in either the patient or the control groups in the genotypic model. Similarly, cubilin

Table 3
25(OH)D levels in the study groups: patients with ACS and healthy controls.

	ACS patients			Control subjects		
	Overall, N = 185	Men, N = 133	Women, N = 52	Overall, N = 143	Men, N = 128	Women, N = 15
Serum Vitamin D levels						
Serum 25(OH)D (ng/mL)	17.37 ± 0.42	17.76 ± 0.52	16.42 ± 0.67	43.48 ± 1.06	43.43 ± 1.15	42.41 ± 2.68
25(OH)D >30 ng/mL, N (%)	10 (5.4%)	9 (6.8%)	1 (1.9%)	126 (88.1%)	112 (87.5%)	14 (93.3%)
25(OH)D 20–30 ng/mL, N (%)	23 (12.4%)	18 (13.5%)	5 (9.6%)	12 (8.39%)	11 (8.59%)	1 (6.7%)
25(OH)D <20 ng/mL, N (%)	152 (82.2%)	106 (79.7%)	46 (88.5%)	5 (3.5%)	5 (3.91%)	None
	Comparison of Vitamin D status among patients and controls					
	Overall Patients with ACS, N = 185	Overall Controls, N = 143	Odds Ratio (95% CI)	*P* value		
Insufficient and Deficient 25(OH)D <30 ng/mL, N (%)	175 (94.6%)	17 (11.89%)	Insufficient and Deficient Vs. Sufficient 129.7 (57.5–292.8)	<0.0001****		
Sufficient 25(OH)D > 30 ng/mL, N (%)	10 (5.4%)	126 (88.1%)				

The serum 25(OH)D results are expressed as mean ± SEM. *P* value was calculated by Chi-squared test.

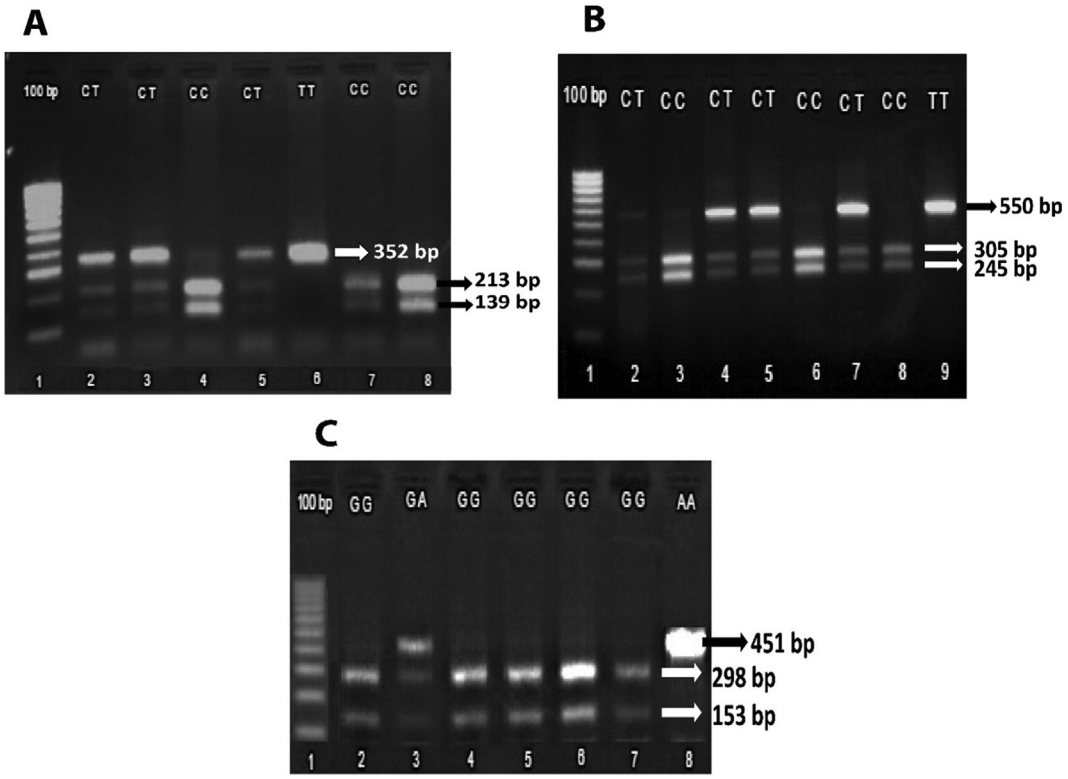

Fig. 1. Representative 2% agarose gel electrophoresis of restriction digestion products. (A) Megalin rs2075252 polymorphism. Lane 1, a 100-bp ladder; lanes 2, 3 and 5, are a CT heterozygote with 139 bp, 213 bp and 352 bp fragments; lanes 4, 7 and 8 are a CC homozygote with 139 bp and 213 bp fragments; and lane 6 is a TT homozygote with a single 352 bp fragment. (B) Megalin rs4668123 polymorphism. Lane 1, a 100-bp ladder; lanes 2, 4, 5 and 7 are a CT heterozygote with 245 bp, 305 bp and 550 bp fragments; lanes 3, 6 and 8 are a CC homozygote with 245 bp and 305 bp fragments; and lane 9 is a TT homozygote with a single 550 bp fragment. (C) Cubilin rs1801222 polymorphism. Lane 1, a 100-bp ladder; lane 3 is a GA heterozygote with 153 bp, 298 bp and 451 bp fragments; lanes 2, 4, 5, 6 and 7 are a GG homozygote with 153 bp and 298 bp fragments; and lane 8 is a AA homozygote with a single 451 bp fragment.

rs1801222 and rs12766939 SNPs lacked 25(OH)D predictive abilities (Table 5).

The results are expressed as mean ± SEM. Vitamin D levels were compared between genotypes in each SNP using the non-parametric Kruskal Wallis test. Adjusted *P* values for association of SNP genotypes and total 25(OH)D (ng/mL) were calculated using multiple logistic regression analysis.

Discussion

Coronary Artery Diseases, which include ACS, are complex multifactorial polygenic disorders that are thought to result from interactions between a person's genetic makeup and various environmental factors [28]. This case control study investigated associations of serum 25(OH)D concentrations and functionally relevant genetic variants in megalin and cubilin genes, that encode for the two kidney receptors, in the vitamin D metabolic pathway, with ACS risk in Egyptians.

Vitamin D receptors have been found in all the major cardiovascular cell types including cardiomyocytes, immune cells and arterial wall cells [29]. Emerging evidence indicates that vitamin D beneficially modulates the heart and blood vessels as well as systems associated with CVD risk factors including the renin-angiotensin-aldosterone system, the parathyroid glands, and the

Table 4
Genotypic and allelic distribution of megalin and cubilin SNPs in patients with ACS and healthy controls.

	ACS patients (n = 185)	Control subjects (n = 143)	Odds ratio (95% CI)	*P*-value	Adjusted *P*-value
Megalin rs2075252 (E4049K)					
CC (%)	133 (71.9%)	100 (69.9%)	CC vs. CT + TT	0.6977	0.107
CT (%)	45 (24.3%)	40 (28%)	1.1 (0.7–1.8)		
TT (%)	7 (3.8%)	3 (2.1%)			
C (%)	311 (84%)	240 (83.9%)	1.0 (0.7–1.5)	0.9619	
T (%)	59 (16%)	46 (16.1%)			
Megalin rs4668123 (T2872A)					
CC (%)	117 (63.2%)	62 (43.4%)	CC vs. CT + TT	****0.0003	***0.001
CT (%)	59 (31.9%)	68 (47.6%)	2.2 (1.4–3.5)		
TT (%)	9 (4.9%)	13 (9.1%)			
C (%)	293 (79.2%)	192 (67.1%)	1.9 (1.3–2.7)	****0.0005	
T (%)	77 (20.8%)	94 (32.9%)			
Cubilin rs1801222 (S253F)					
GG (%)	149 (80.54%)	81 (70.43%)	GG vs. GA + AA	**0.0442	0.867
GA (%)	34 (18.38%)	34 (29.57%)	1.7 (1.0–3.0)		
AA (%)	2 (1.08%)	0 (0%)			
G (%)	332 (89.7%)	196 (85.2%)	1.5 (0.9–2.5)	**0.0982	
A (%)	38 (10.3%)	34 (14.8%)			
Cubilin rs12766939					
AA (%)	55 (29.9%)	51 (44%)	AA vs. AG + GG	**0.013	0.996
AG (%)	92 (50%)	46 (39.7%)	1.8 (1.1–3.0)		
GG (%)	37 (20.1%)	19 (16.4%)			
A (%)	202 (54.89%)	148 (63.79%)	1.4 (1.0–2.0)	**0.0313	
G (%)	166 (45.1%)	84 (36.21%)			

P-value of odds ratio was calculated by Chi-squared Fisher's exact test to compare the allele frequencies and genotypic differences between ACS and control groups. Multiple logistic regression was done to test the association of each individual studied SNP and the incidence of ACS Megalin rs2075252 Megalin rs4668123, Cubilin rs1801222 and Cubilin rs12766939 to eliminate the confounders: age, sex smoking and BMI (r = 0.766, 0.756, 0.754 and 0.754 for Megalin rs2075252, Megalin rs4668123, Cubilin rs1801222 and Cubilin rs12766939 respectively). The adjusted *P*-value was calculated using multivariate multiple logistic regression.

Table 5
Comparison between the distribution pattern of megalin and cubilin polymorphism genotypes and the serum $25(OH)D_3$, $25(OH)D_2$ and total 25(OH)D concentrations in all study subjects.

SNP ID	Genotype	$25(OH)D_3$ (ng/mL)	*P*-value	$25(OH)D_2$ (ng/mL)	*P*-value	Total 25(OH)D (ng/mL)	*P*-value	Adjusted *P*-value
Megalin E4049K (rs2075252)	CC	21.24 ± 0.79	0.6595	7.63 ± 0.49	0.205	28.86 ± 1.06	0.6746	0.487
	CT	22.26 ± 1.35		7.46 ± 0.63		29.71 ± 1.79		
	TT	22.19 ± 5.43		4.76 ± 1.28		26.94 ± 5.94		
Megalin T2872A (rs4668123)	CC	19.25 ± 0.81	***0.0021	6.7 ± 0.49	**0.0128	26 ± 1.12	***0.0018	***0.007
	CT	24.14 ± 1.15		8.24 ± 0.6		32.38 ± 1.48		
	TT	25.34 ± 3.28		9.26 ± 2.13		34.59 ± 4.32		
Cubilin S253F (rs1801222)	GG	20.15 ± 0.79	0.4215	6.47 ± 0.38	0.0449	26.62 ± 1	0.2032	0.980
	GA	22.37 ± 1.57		8.76 ± 1.02		30.97 ± 2.1		
	AA	13.66 ± 8.17		3.5 ± 2.12		17.16 ± 10.29		
Cubilin rs12766939	AA	21.56 ± 1.24	0.7261	8.29 ± 0.75	0.162	29.85 ± 1.7	0.6532	0.315
	AG	20.12 ± 1		6.51 ± 0.51		26.63 ± 1.26		
	GG	21.31 ± 1.63		6.44 ± 0.57		27.75 ± 2.04		

nitric oxide system [10]. In the current study, the prevalence of vitamin D insufficiency and deficiency significantly increased the ACS risk by more than 100 folds in agreement with previously published studies that have implicated vitamin D deficiency as a potential risk factor for cardiovascular diseases. A number of large epidemiologic studies have indicated that the occurrence of cardiovascular disorders, such as ischemic heart disease, heart attack, stroke, heart failure, cardiac arrhythmia, and hypertension, and mortality from these disorders are significantly higher in patients with low vitamin D levels than in patients with adequate levels [2,26,30–33]. The prevalence of cardiovascular risk factors, such as hypertriglyceridemia, hypercholesterolemia, diabetes, inflammatory markers, and high body mass index, was also increased [26]. The cardiovascular risk also increased across the categories of 25(OH)D deficiency, so that subjects with levels 10 to <15 ng/mL had a 53% higher risk, and in those with the levels <10 ng/mL had an 80% higher risk [31]. A study that monitored 18,225 men for 10 years showed that low levels of 25(OH)D were associated with a high risk of myocardial infarction [30,34].

Till now, there are no studies on Egyptians that have examined the association of vitamin D deficiency on the risk of ACS. However, there are some that studied the association of vitamin D deficiency on other disorders such as Non Alcoholic Fatty Liver Disease (NAFLD) [35] and rheumatoid arthritis [36]. Both studies portrayed results similar to those displayed in the current study. Vitamin D levels in patients with NAFLD were 18.76 ± 14.37 ng/mL and were 40.36 ± 22.24 ng/mL in the study's controls. Results also showed that low Vitamin D levels were frequent in rheumatoid arthritis patients (22 ± 9.2 ng/mL) compared to the controls (28.7 ± 9.6 ng/mL).

Although several studies investigated several genetic determinants of vitamin D and their non-skeletal outcomes, studies investigating the relationship of polymorphisms in the vitamin D pathway and ACS are limited. The focus of the present study was the triangular relationship between the two megalin genetic vari-

ants (rs2075252 and rs4668123) and the two cubilin genetic variants (rs1801222 and rs12766939), circulating vitamin D levels and ACS susceptibility in Egyptians.

This is one of the first epidemiological studies to examine megalin and cubilin, two structurally different multiligand endocytic receptors that actively transport 25(OH)D into the renal proximal cells [37]. Megalin and cubilin are two receptors localized on the apical membrane of the proximal tubular cells in the kidney and work in concert to mediate the uptake of the complexes of vitamin D binding protein (DBP) and 25(OH)D that are filtered by the kidneys. The receptors are recycled back to the membrane, whereas the 25(OH)D ligands are either secreted into the circulation or hydroxylated by 1 α -hydroxylase in the mitochondria to the active 1,25(OH)2D form [20,21].

Regarding megalin rs2075252 SNP, very similar genotype distribution and allele frequencies were observed in patients with ACS and controls suggesting a lack of association between rs2075252 polymorphism and ACS. Moreover, the serum levels of 25(OH)D_3, 25(OH)D_2 and total 25(OH)D did not differ significantly among different rs2075252 genotypes in both study groups. By contrast, CC genotype of megalin rs4668123 SNP was associated with an increased incidence of ACS in patients and the C allele was a risk factor compared to the T allele. Compared with the homozygous CC genotype, carrying the heterozygous CT or homozygous TT genotypes provided a significant protective effect against developing ACS.

On comparing the genotype distribution of the studied SNPs among the control subjects of the current study to published studies, the distribution pattern of rs2075252 SNP and rs4668123 SNP were different from Asian subjects [38]. Another study reported that rs2075252 SNP genotype distribution in German controls was: CC 55% CT 38% and TT 7% which differed from the genotypic pattern of this study's Egyptian controls. The rs4668123 SNP genotype distribution in German controls was 53% CC, 37% CT and 10% TT. The allele distribution of SNP rs4668123 was similar to that in the Egyptians in the present study, where the T-allele frequency in the controls group was 28% and 32% in Germans and Egyptians respectively [39].

Serum vitamin D levels also differed significantly among the various megalin rs4668123 genotypes in all study subjects. The serum 25(OH)D_3, 25(OH)D_2 and total 25(OH)D were the highest in TT genotype and lowest in the CC genotype, and intermediate in the CT genotype. Multiple logistic regression revealed that megalin rs4668123 genotype represents an independent determinant for total 25(OH)D. Taken together, these results suggest that the non-synonymous megalin rs4668123 SNP could potentially alter the functionality of the encoded megalin protein to affect the reabsorption of 25(OH)D-DBP complex from the proximal tubular lumen. The result would be a phenotype that has altered levels of total 25(OH)D in the plasma. Consequently, the TT genotype in megalin rs4668123 SNP would greatly promote the reabsorption of 25(OH)D into the cells, thereby increasing the total 25(OH)D in plasma and acting as an independent cardioprotective factor, in contrast to the CC genotype. Further studies are required to confirm this.

To the best of found knowledge, this is the first study to examine polymorphisms within the megalin gene for associations with ACS and vitamin D levels. Other studies have examined megalin polymorphisms in association with other non-skeletal diseases. A study reported the association of TT genotypes of the megalin rs2075252 SNP and elevated central adiposity in non-Hispanic white US adults. Elevated central adiposity increases the incidence of ACS [40]. This contrasts with this study's conclusion that the TT genotype in megalin rs4668123 is a protective genotype with respect to the susceptibility to ACS.

Regarding cubilin rs1801222, the GG genotype was associated with a 1.737-fold increase in the incidence of ACS compared to individuals with the AA or GA genotypes. The genotype distribution in the Egyptian controls was 70.4% homozygous GG and 29.6% heterozygous AG. Likewise, a Chinese study showed a genotype distribution of GG 61%, GA 34%, and AA 5% in a Shanghai population and GG 72%, GA 26%, and AA 2% in a in Shandong population [41]. 85% of control subjects in this study were G-allele carriers and 15% were A-allele carriers. These findings differed from those of another study where a population of a European descent had an A-allele frequency of 34% [42].

The serum levels of 25(OH)D_3, 25(OH)D_2 and total 25(OH)D did not differ significantly among different cubilin rs1801222 genotypes in either group. These results were contradicted by a study that reported that GG genotype possessed significantly lower 1,25$(OH)_2D_3$ plasma levels [43].

The cubilin rs12766939 SNP showed a significant difference in both genotype distribution patterns and allele frequencies between the patients with ACS and the controls. The G allele presented a higher risk of ACS incidence than did the A allele; thus, individuals with GG + AG genotypes were more prone to develop ACS. The serum levels of 25(OH)D_3, 25(OH)D_2 and total 25(OH)D were not significantly different among different rs12766939 genotypes in either group. A Cardiovascular Health Study Discovery Cohort, by Levin et al., conducted on 1514 participants from the US, identified interactions between cubilin rs1801222 and rs12766939 and low circulating 25(OH)D levels and increased risk of the studied diseases, including myocardial infarction (MI) [44]. The current study only acknowledged the association of those SNPs had on ACS risk but found no association with circulating vitamin D levels.

Other factors that might have influenced the vitamin D status such as usage of sunscreen, vitamin D supplementation and alcohol consumption are lacking. Smoking also might had an impact on vitamin D levels [45]. These might be considered the study's limitations. No ECG data were available for control subjects. There was also no data on lipid profiles of the subjects available. Moreover, genotypic variants also might have an association with survival rates after ACS, However, the study was cross sectional and survival rates after developing ACS weren't tracked.

Conclusions

The results of the present study indicate that megalin rs4668123 SNP genotype CC, cubilin rs1801222 SNP genotype GG and cubilin rs12766939 SNP genotype GG are associated with a higher ACS incidence and can be considered risk factors, according to Chi-squared test. Conversely, the megalin rs2075252 SNP was not associated with increased ACS incidence. However, after performing multiple logistic regression analysis, only the megalin rs4668123 SNP was considered an independent ACS risk factor. Furthermore, the megalin rs4668123 CC genotype was associated with lower 25(OH)D levels. Replication of these findings in different populations, including populations of other racial and ethnic groups, will be of interest for confirming the biological significance of these polymorphisms in relation to vitamin D levels and the incidence of ACS.

Compliance with Ethics Requirements

The project was evaluated by the Ethics Committee of the German University in Cairo with regard participation of human patients or animals and/or clinical samples obtained from humans or animals, and with the respect of anonymity guaranteed to patients from whom any samples are being obtained.

Declaration of Competing Interest

The authors have declared no conflict of interest.

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