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Original Article

Association of promoter region polymorphism in *CYP19* gene to cardiovascular disease risk in Saudi cohort

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Abstract: Introduction: Cardiovascular diseases (CVD) are multifactorial in nature, where genetic susceptibility and environmental factors are required for the disease to occur. Several genetic loci have been implicated as possible susceptibility loci for CVD development, but differences in different populations are frequently reported. Since aromatase is responsible for the synthesis of estrogens from androgens which provide a protective influence against CVD development, we hypothesized that it may play a role in the development of CVD and that polymorphisms in the *CYP19* gene that encodes aromatase may influence the development of the disease state. The study group comprised of 120 cardiovascular patients and 132 normal healthy controls. Five polymorphic sites [-81371 A>G (rs4774585), -45965 C>G (rs936308), R264C (rs700519), 80 A>G (rs700518), +32226 A>C (rs4646)] in the *CYP19* gene were analysed using TaqMan Genotyping assay. Genotype and allele frequencies were calculated and the results in the patients and control groups were compared. The G allele of rs4774585 and G allele of rs936308 were significantly protective, where the wild type alleles (A and C) for both SNPs increased the risk of CVD. The other studied SNPs did not show any significant difference in frequency between the patients and controls. These results show that the A>G mutation at the rs4774585 site and C>G mutation at the rs936308 site, both in the promoter region, probably increase the level of *CYP19* gene expression and hence increase the amount of aromatase, thus increasing the amount of estrogens formed from androgens. The plasma lipid levels and renal function tests were compared in the different genotypes of each SNP and very little associations were observed. It is suggested that there is a need to conduct investigations on gene expression of *CYP19* in different mutants, in an attempt to determine the mechanism behind these associations.

Keywords: Aromatase, estrogens, androgens, *CYP19*, plasma lipids, cardiovascular disease

Introduction

Cardiovascular diseases (CVD)are considered as multifactorial disorders, where both genetic and environmental factors are required for the disease causation [1]. The genetic basis is shown to be complex since atherosclerosis and thrombosis, the two primary processes leading to clinically manifest CVD, involve several different cells, organs and distinct pathophysiological processes [2]. Extensive studies have been conducted to identify the genetic loci involved in the development of CVD and several population specific differences have been reported [3-5]. Endogenous hormones, such as the sex hormones, seem to play an important role in providing either protection or predisposition to CVD development, where the role of estrogens in providing protection against CVD development is widely documented in the females [6, 7]. Estrogen, a female sex hormone, also formed in the males, is synthesized from cholesterol in a multistep reaction, where androstenedione and testosterone are first formed and are converted to estrone and estradiol, respectively, under the catalytic action of the enzyme aromatase [8, 9]. This step involves hydroxylation of the 19-methyl group of andro-

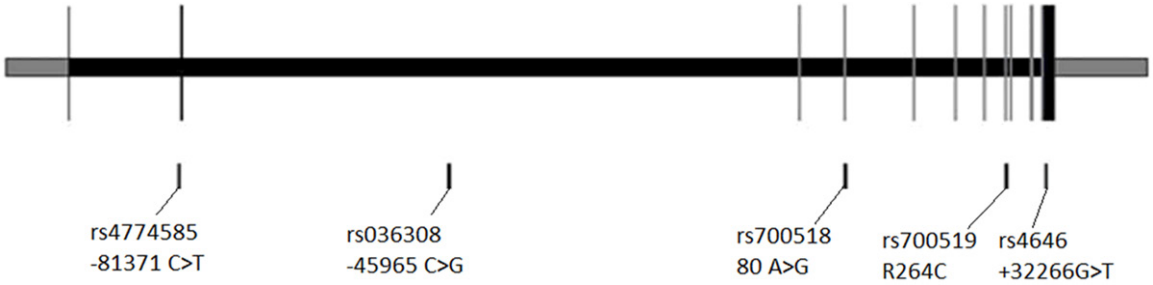

Figure 1. Schematic presentation of the location of the five SNPs in the *CYP19* gene investigated during this study.

gens, elimination of the methyl group as formate and aromatization of the A-ring. Aromatase (EC 1.14.14.1), also known as estrogen synthetase, is a member of the cytochrome P450 superfamily which are monooxygenases that catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids [10-12]. Elevation in aromatase activity is reported to occur with age, stress and alcohol intake [13].

Aromatase is encoded by *CYP19* gene located on chromosome 15q21.1 [14]. Mutations in this gene can result in either increased or decreased aromatase activity or the amount and the associated phenotypes suggest that estrogen functions both as a sex steroid hormone and in growth and differentiation [15]. Several common genetic polymorphisms in *CYP19* have been identified and linked to different diseases, such as insulin resistance, hypertension, prostatitis, CVD, autoimmune disease, and cancer [16-18].

We hypothesized that aromatase, due to its role in estrogen synthesis, may play a role in CVD development. To confirm, we initiated this study on Saudi CVD patients and healthy controls in an attempt to study the role played by five reported polymorphic sites, in the *CYP19* gene, in susceptibility to CVD development in Saudis.

Materials and methods

The study was approved by the ethical committee of the institution. The study group consisted of 120 CVD patients and 132 controls attending clinics at the King Khalid University hospital for routine checkup. All patients had been diagnosed in the outpatients' clinics and had volunteered to be included in the study after informed consent. The controls were healthy Saudi individuals with no apparent disease and no history of CVD. All patients and controls were requested to remain in an overnight fast prior to the day of blood extraction. Blood was extracted by venipuncture in plain tubes and part of the drawn blood was placed in an EDTA tubes. The serum was carefully removed by centrifugation from the clotted blood and used for the estimation of biochemical parameters using an autoanalyser (American Monitor) at the Biochemistry Laboratory, College of Science, King Saud University, Riyadh, Saudi Arabia. The blood collected in EDTA was used for DNA extraction using DNA extraction kit (QIAmp DNA blood Mini Kit, Qiagen, Valencia, CA). The extracted DNA was checked for purity and used for the genotype analysis.

Five single nucleotide polymorphisms (SNPs) in and around the *CYP19* gene were investigated using TaqMan genotyping assay using the ABI 7500 (Applied Biosystems, Foster City, CA, USA). These SNP included: two SNPs located in the promoter region: -81371 C>T (rs4774585) and -45965 G>C (rs936308); one nonsynonymous SNP R264C (rs700519); one synonymous SNP: 80 A>G (rs700518); and one SNP located in the 3' UTR: +32226 G>T (rs4646), as shown schematically in **Figure 1**.

The genotype data was used to calculate genotype and allele frequencies for each SNP in the total patients and control group and in the males and females separately. The results obtained in the patients was compared to the results obtained for the controls and odds ratio (OR) (and *p* value), 95% Confidence Interval (CI%), X^2 and *p* values were obtained, to investigate the significance of the difference between the results of the patients and controls. The biochemical data in the patients was grouped according to the genotype of the SNP and mean, standard error of the means (SEM) were obtained. Significance of the difference between any two groups (p) was obtained

Table 1. Genotype and allele frequencies for the SNPs in the *CYP19* gene in CVS patients and controls

Frequency	Frequency*		Control vs. CVD			*p*-value
	Control No. (%)	CVD No. (%)	OR	CI	χ^2	
Genotype			rs4774585 G>A			
A/A	102	104	Ref			
A/G	24	14	0.572	0.280-1.168	2.39	0.12201
G/G	6	2	0.327	0.064-1.658	2.00	0.15721
Allele frequency						
A	228	222	Ref			
G	36	18	0.514	0.283-0.93	4.95	0.02612
Genotype			rs936308 G>C			
C/C	5	10	Ref			
C/G	45	49	0.544	0.17-1.715	1.10	0.29399
G/G	82	60	0.366	0.12-1.126	3.27	0.07044
Allele frequency						
C	55	69	Ref			
G	209	169	0.645	0.429-0.97	4.48	0.03432
Genotype A>G			rs700518			
A/A	55	44	Ref			
A/G	60	54	1.125	0.655-1.93	0.18	0.66935
G/G	16	21	1.641	0.766-3.51	1.64	0.20082
Allele frequency						
A	170	142	Ref			
G	92	96	1.249	0.869-1.79	1.45	0.22865
Genotype C>T			rs700519			
C/C	123	116	Ref			
C/T	6	4	0.707	0.195-2.57	0.28	0.59665
T/T	0	0	1.060	0.02-53.86	0.01	1
Allele frequency						
C	252	236	Ref			
T	6	4	0.712	0.198-2.55	0.27	0.75318
Genotype			rs4646 A>C			
A/A	13	8	Ref			
A/C	32	38	1.930	0.71-5.237	1.69	0.19308
C/C	83	74	1.449	0.569-3.69	0.61	0.43515
Allele frequency						
A	58	54	Ref			
C	198	186	1.009	0.66-1.537	0.02	0.96682

using students 't' test. P<0.05 was considered statistically significant. Arlequin version 3.5.1 programs [31] were used for haplotyping analysis.

Results

This study was carried out on Saudi CVD patients and the results of genetic analysis were compared to the results obtained in the controls from the same population. In the patients group, there were 78 males (age: 62.2±1.38 yrs) and 42 females (age: 63.2±1.61 yrs). Genotyping data for the five SNPs was used to calculate the genotype and allele frequencies in the males and female patients and controls. The difference between the genders was not significant (results not shown), so the results were grouped and the results in the total patients and total controls are presented in **Table 1**. Comparison of the results in the patients and control groups showed that the allele frequencies differed significantly for two SNPs, i.e.-81371 A>G (rs4774585) and -45965 C>G (rs936308) (P<0.05). For the SNP rs4774585, a A>G transition in the promoter region, where A, the ancestral allele, had 1.91 times higher risk of CVD, while the mutant G allele was protective with an OR of 0.514. For rs936308, a C>G transversion in the promoter region of the *CYP19* gene, the ancestral C allele had the risk of CVD 2.33 times more than the mutant G allele, which was protective (OR =0.64). The other three SNPs investigated did not show a statistically significant difference between the patients and the control groups. **Table 2** presents the results obtained on application of Hardy Weinburg equilibrium (HWE) to the results of the five SNPs in the CVD patients and controls.

Haplotypes were constructed and 10 different haplotypes were observed in the patients and control group (**Table 3**). Two major haplotypes were selected and results were compared between the case and control. Both the haplotypes, showed statistically significant difference in the frequency in the patients and control group as shown in **Table 4**.

Table 2. Distribution of genotypes of five SNPs in Aromatase gene among Saudi CVD patients and controls and application of Hardy-Weinburg equilibrium (HWE)

Genotype	Cases	HWE *P*-value	Controls	HWE *P*-value
rs4774585				
A/A	104	0.081246	102	0.008785
A/G	14		24	
G/G	2		6	
rs936308				
C/C	10	0.999254	5	0.700383
C/G	49		45	
G/G	60		82	
rs700518				
A/A	44	0.532511	55	0.953315
A/G	54		60	
G/G	21		16	
rs700519				
C/C	116	0.852705	123	0.786834
C/T	4		6	
T/T	0		0	
rs4646				
A/A	8	0.313571	13	0.001182
A/C	38		32	
C/C	74		83	

Table 3. Haplotypes identified in the CVD patients and controls

Haplotype	Patients		Controls	
	No.	Frequency	No.	Frequency
GCCGC	31	0.303	28	0.281
GCTGC	27	0.272	29	0.285
GGTGA	12	0.119	7	0.073
GGTGC	9	0.092	12	0.117
GCTGA	7	0.069	8	0.083
GGCGC	6	0.061	0	0
ACCGC	3	0.034	5	0.054
ACTGA	3	0.033	5	0.054
GGTAC	0	0	2	0.018
ACTGC	0	0	2	0.016
ACCGA	0	0	1	0.013

The biochemical parameters were estimated in the CVD patients, and the patients were grouped according to the different genotypes for the SNP, and the data for plasma lipids and renal function tests were compared in the different genotypes of each SNP. The results are presented in **Table 5**. [Results in the males and female patients were analyzed separately but no differences were observed (results not shown)].

Discussion

Cardiovascular diseases are disorders that involve the heart or blood vessels (arteries, capillaries and veins) and in recent years have become a major cause of morbidity and mortality in the developed countries. Even in the developing countries these diseases are assuming an equally significant role [19]. The etiological factors are diverse but atherosclerosis and/or hypertension are the most common predisposing factors [20]. Environmental factors, such as diet, exercise, avoidance of smoking, weight loss, less stressful living, seem to play an important role in preventing arteriosclerosis and hence CVD. Since CVD are multifactorial, where genetic susceptibility is necessary for the disease causation under the effects of the environmental factors [21], it has been repeatedly stated that early and presymptomatic diagnosis of multifactorial disorders including CVD, followed by improvement in life style and avoidance of the precipitating factors, can delay or even prevent the development of the disease [22, 23]. With the view to identify genetic susceptibility, several studies have explored the human genome. Though several susceptibility loci have been identified, but differences reported in different populations and ethnic groups, warrant further studies [24].

With the aim to study the role played by polymorphic loci in the aromatase gene (*CYP19*), we investigated five SNPs distributed in different regions in the *CYP19* gene. All five SNPs in the patients group obeyed HWE. In the control group, three SNPs obeyed HWE, but two did not. Since the patients were the same, it could not be explained why the two SNPs were not obeying HWE.

Our genotyping results showed that polymorphisms in the promoter site, plays an important role in influencing susceptibility to development of CVD. Two SNPs, both located in the promoter region of *CYP19* gene showed a statistically significant contribution to disease development. These were rs4774585 and rs936308. The rs4774585 (rs58750380 has merged into rs4774585) is an A>G transition and lies in the intron 1, -81371 bp prior to the exon II of *CYP19*

Table 4. Major haplotype frequencies of aromatase gene polymorphism in CVD cases and controls

SNP	Variant	BR Cases (Freq)	Controls	OR	CI	χ^2 Value	*P*-Value
Hap1 (29)	AA	46 (0.32)	279 (0.428)	1.045	0.809-1.350	0.11	0.73760
	AB	65 (0.45)	167 (0.256)	2.361	1.546-3.606	16.30	0.00005
	BB	33 (0.23)	206 (0.316)	0.972	0.600-1.573	0.01	0.90678
	AB+BB	118	373	1.594	1.086-2.337	5.74	0.01654
Hap1 (65)	AA	123 (0.38)	279 (0.428)	1.480	1.225-1.788	16.55	0.00005
	AB	51 (0.16)	167 (0.256)	0.693	0.474-1.012	3.63	0.05667
	BB	150 (0.46)	206 (0.316)	1.652	1.225-2.227	10.91	0.00096
	AB+BB	201	373	1.222	0.931-1.606	2.08	0.14893

Haplotype 1: 29 CVD samples (5 SNPs (5*29=145), Haplotype 2: 65 CVD samples (5SNPs (5*65=325) compared with control (all) sample frequencies for risk.

gene, which harbors the promoter region and the rs936308 is a C>G transversion and lies -45965 bp prior to the exon II of *CYP19* gene. The CYP19 gene is comprised of 10 exons. The exons II to X are the 9 coding exons, while there are a number of alternative non-coding first exon which are expressed in a tissue specific manner. At rs477585, our results show that the frequency of the genotypes AA, AG and GG do not differ significantly between the CVD patients and controls, though AA almost reached significance being higher in the patients group (P=0.054). Furthermore, in the results of the genotypes an interesting observation is made. When the wild type allele A is mutated to G, the risk of developing CVD decreases, as the OR decreases to 0.327, in the homozygous state and to 0.59 in the heterozygous individuals, compared to 1.91 in individuals carrying the AA genotype. In addition, the presence of Gallele has a dominant effect, since presence of G even in the heterozygotes is playing a protective role (OR=0.59). Allele frequencies of the A and G alleles are significantly different in the two groups (P=0.026), with A having an OR of 1.95 and G an OR of 0.51. Comparison of the frequencies of G and C in Saudis with those reported by NCBI in different population groups showed several differences with the European, Sub-Saharan and Kenyan populations, though the frequencies were similar to Asians and Chinese [25].

For rs936308, significant difference is also observed in the allele frequency. The ancestral allele C occurs at a low frequency in the Saudi population both in the CVD patients and the control group. Presence of this allele in homozygous state increases the OR to 2.33, compared to 0.62 in the homozygous GG and 0.544 in the heterozygous state. This predicts a dominant manner of inheritance for the G allele which has a protective effect against cancer development. The frequency of this allele has been reported to range from 0.045 in Hispanic population to 0.719 in the Sub-Saharan Africans. In this study on Saudis the frequency of the wild type C allele in the normal controls was 0.2 and was significantly less than in the patients group (0.289). The 1000 Genome project reports the frequency of the G allele as 0.338, which is significantly lower than the one observed during this study in Saudis [26].

Both these polymorphisms are protective against development of CVD, and this can be explained as follows: aromatase, encoded by *CYP19*, catalyzes the aromatization of androstenedione and testosterone to estrone and estradiol, respectively [9]. The polymorphic change in both sites discussed above, possibly results in an increase expression of the aromatase, thus increasing the estrogen levels and hence plays a protective role. The estrogens have a protective effect against development of CVD, since in females, as aging occurs and the level of estrogens decrease the prevalence of CVD reaches the same level as in the males [27]. Since mutations in the promoter region influence gene expression, we suggest that the two SNPs effect gene expression of the aromatase, decreasing the amount produced and hence result in a decrease in estrogen production and an increase in the prevalence of CVD. Once mutated, the gene expression increases, elevating estrogen levels and the mutant alleles provide protection against CVD development. Further gene expression studies are warranted to confirm this suggestion.

The three other SNPs investigated during this study i.e. R264C (rs700519); 80 A>G (rs70-

Table 5. Value of biochemical parameters in CVD patients with different genotypes of the SNPs studied during this investigation

SNP	Biochemical Parameters									
Geno-type	TAG	Chol	LDL-C	HDL-C	FBS	Creatinine	Urea	Na^+	K^+	Cl^-
Rs4774585										
AA 86	1.55 ± 0.07	4.6 ± 1.51	4.4 ± 0.34	0.79 ± 0.04	8.4 ± 0.44	88.5 ± 2.9	7.02 ± 0.69	137.6 ± 0.32*	4.5 ± 0.05	101.9 ± 0.34
AG 11	1.23 ± 0.15	4.1± 0.28	2.35 ± 0.23	1.11 ± 0.01	7.7 ± 1.2	87.3 ± 8.4	6.70 ± 0.56	137.6 ± 0.33*	4.9 ± 0.15	101.8 ± 0.84
GG 2	1.06±0.27	4.2± 0.12	2.6 ± 0.096	0.99 ± 0.03	7.2 ± 2.2	81.5 ± 3.5	5.6 ± 0.1	139.0 ± 0.10*	4.25 ± 0.35	103.5 ± 0.50
Rs936308										
CC 5	1.8 ± 0.49	4.22 ± 0.47	2.63 ± 0.39	0.83 ± 0.02*	9.0 ± 1.14	85.0 ± 12.8	5.4 ± 0.942	139.5 ± 1.6	4.4 ± 0.125	104.0 ± 1.08
CG 45	1.4 ± 0.09	4.20 ± 0.15	2.59 ± 0.14	1.00 ± 0.04*	8.4 ± 0.62	90.6 ± 6.6	7.8 ± 1.29	137.6 ± 0.5	4.55 ± 0.08	101.8 ± 0.56
GG 98	1.5 ± 0.10	4.20 ± 0.17	2.56 ± 0.14	1.02 ± 0.05*	8.0 ± 0.58	86.5 ± 3.6	6.4 ± 0.41	137.5 ± 0.31	4.50 ± 0.06	109.9 ± 0.36
Rs700518										
AA 39	1.6 ± 0.11	4.2 ± 0.19	2.47 ± 0.15	0.99 ± 0.04	8.7 ± 0.72	85.4 ± 3.6	6.3 ± 0.46	137.7 ± 0.48	4.60 ± 0.08	102.3 ± 0.50
AG 44	1.37 ± 0.08	4.3 ± 0.16	2.70 ± 0.13	1.03 ± 0.05	8.2 ± 0.58	73.7 ± 4.6	7.9 ± 1.3	137.2 ± 0.46*	4.50 ± 0.06	101.5 ± 0.50
GG 15	1.57 ± 0.2	4.3 ± 0.27	2.72 ± 0.28	0.90 ± 0.04	7.4 ± 0.89	79.5 ± 6.5	5.7 ± 0.44	138.7 ± 0.52*	4.5 ± 0.11	102.5 ± 0.80
Rs700519										
CC 96	1.51 ± 0.07	4.3 ± 0.11	2.6 ± 0.09	1.0 ± 0.03	8.3 ± 0.4	87.0 ± 2.7*	6.9 ± 0.63	137.6 ± 0.27	4.5 ± 0.045	101.9 ± 0.32
CT 3	1.1 ± 0.36	3.2 ± 0.41	2.3 ± 0.17	0.7 ± 0.15	8.5 ± 2.0	131 ± 14.1*	8.5 ± 2.0	137.0 ± 2.3	5.0 ± 0.45	102.3 ± 1.30
TT --	--	--	--	--	--	--	--	--	--	--
Rs4646										
AA 7	CC 60	3.65 ± 0.52	2.14 ± 0.44	0.97 ± 0.07	6.6 ± 0.60*	71.5 ± 5.5*	5.2 ± 0.56	139.0 ± 1.29*	4.37 ± 0.17	102.7 ± 0.70
AC 32	1.4 ± 0.10	4.30 ± 0.14	2.62 ± 0.18	1.0 ± 0.40	6.9 ± 0.47*	92.9 ± 4.8*	8.58 ± 0.8	138.3 ± 0.47*	4.54 ± 0.08	103.0 ± 0.60
CC 60	1.6 ± 0.09	4.25 ± 0.18	0.76 ± 0.10	1.0 ± 0.40	9.2 ± 0.58*	87.9 ± 3.5*	6.34 ± 0.32	137.1 ± 0.37*	4.55 ± 0.06	101.4 ± 0.40

*Difference: Statistically significant ($P<0.05$).

0518); and +32226 G.T (rs4646) showed polymorphism but there were no significant differences in the frequencies when the CVD patients were compared to the control.

Only a few studies on *CYP19* gene polymorphism have been reported in literature. Beitelshees et al [28] conducted a study on patients with cardiovascular disease and documented a significant interaction between *CYP19A1* genotype and sex on the outcome of the cardiovascular disease. They reported in white men, that the variant allele -81371 C>T was associated with a significant increase in mortality and in women the variant allele was associated with a nonsignificant decreased risk of mortality. Our study in Saudis did not show any difference in the genotype and allele frequencies between the males and females and we did not study the disease outcome. Peter et al [29] reported that in men, there was a significant association between *CYP19* polymorphisms and estradiol and testosterone levels, and the estradiol to testosterone ratio. Specifically, carriers of common haplotype *rs700518* [*G*]-*(TTTA)$_n$* [*L*]-*rs726547*[*C*] had higher estradiol levels, lower testosterone levels, and a higher estradiol to testosterone ratio compared with the *rs700518*[*A*]-*(TTTA)$_n$* [*S*]-*rs726547*[*C*] carriers. Letonja et al [30] reported that the tetranucleotide repeat (TTTA) polymorphism of the CYP19 gene does not qualify as genetic marker for premature coronary artery disease in Caucasians. Since Rosano et al have suggested that androgens have a protective effect in men against CVD development, we can suggest that the mutants of the two SNPs [-81371 A/G (rs477585) and -45965 C >G (rs936308)] in the promoter region of CYP19 gene, by increasing gene expression of the aromatase gene may be elevating the testosterone levels by decreasing the conversion to estrogens and hence providing a protective effect. Further studies are warranted to confirm this hypothesis.

The biochemical parameter levels were compared in the different genotypes of the five investigated alleles in the CVD patients and few significant differences were observed. SNP rs-4774585 genotypes differed significantly in the level of urea, Na^+ and K^+, while rs936308 genotypes differed in the HDL-cholesterol level and the genotype GG had the lowest level. High HDL-cholesterol plays a protective role against development of CVD and this could be a possible mechanism whereby the GG genotype predisposed to CVD development. Significant differences were also seen in the Na^+ level between the GG and GA genotypes of rs700318, while creatinine was higher in the CT genotype of rs700519. Finally, genotypes of rs4646 also had an effect on the creatinine and blood glucose level, where CC had significant elevation (glucose: 9.19 ± 0.58 mmol/l and creatinine: 87.9 ± 3.5 μmol/l). These finding have highlighted an important effect of the different genotypes of the SNPs investigated during this study and indicate that aromatase may be involved in different functions in different tissues and variations in the CYP19 gene may be leading to different abnormalities. As stated above detailed tissue specific gene expression studies need to be carried out to confirm these findings.

In conclusion, this is the first study showing a link between polymorphism at rs477585 and rs936308 of *CYP19* and CVD in Saudi population. Further studies on a larger sample size and in different disease states are required to confirm these associations.

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Disclosure of conflict of interest

None.

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