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Lack of Association between Endothelial Nitric Oxide Synthase Gene Polymorphisms and Coronary Artery Disease among Egyptian Population

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Authors' contributions

This work was carried out in collaboration between all authors. All authors designed the study, wrote the protocol and supervised the work, carried out all laboratories work and performed the statistical analysis. All authors managed the analyses of the study, wrote the first draft of the manuscript, managed the literature searches and edited the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Controversial results regarding the association of e NOS gene polymorphisms with Coronary Artery Disease (CAD) have been reported up to now, there has been conflicting data regarding the association between two clinically relevant polymorphisms (T-786C) in the promoter region and intron 4 variable number of 27-bp tandem repeats (VNTR) of the eNOS gene and coronary artery disease (CAD). The present study was undertaken to investigate association of these two eNOS gene polymorphisms with susceptibility to CAD in Egyptian population. A total of 80 patients with

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CAD and 40 healthy controls were included in this study. CAD patients were divided according to their body mass index (BMI) limits into 2 main groups: normal weigh group (BMI: 18.5–24.9, n=40) and abnormal weight group (BMI≥25, n= 40), the last group was further sub-divided into overweight group (BMI: 25.0–29.9, n= 20) and obese group (BMI≥ 30, n= 20).
The T-786C and intron 4 VNTR polymorphisms were analyzed by polymerase chain reaction (PCR) using specific primers. The T-786C polymorphism frequencies for T/T, T/C and C/C genotypes were 27.5%, 28.75%, 43.75% respectively in the CAD patients, and 35%, 37.5%, 27.5% in the control subjects, while allele frequencies for C, T were 58.12%, 41.87% in the CAD patients and 46.25%, 53.75%, in the control group. No significant differences were observed in genotype frequencies between CAD patient group when compared to control group, or between them according to allele frequency. Also, there was no significant differences in the genotype distribution and allele frequencies of T786-C polymorphism among the overweight and obese cases compared to normal weight subjects. The genotype frequencies for eNOS b/b, a/b, a/a were 45%, 50% and 5% in the CAD patients, and 35%, 65% and 0% in the control subjects, with no significant difference between the two groups. Also, there were no significant difference in the allele frequencies between the CAD patients (a: b = 30%:70%), and controls (a: b = 32.5%:67.5%) as well as in the distribution of genotype and alleles frequencies of intron 4 VNTR of eNOS gene when overweight and obese patient groups were compared with the normal weight patient group. Thus the present study suggested that the two eNOS gene polymorphisms (the eNOS T-786C and intron 4 VNTR polymorphisms) were unlikely to be major genetic susceptibility factors for CAD in the Egyptian population even after classification of the CAD patients according to their BMI. Further studies with larger sample size are required to be done to confirm these findings.

Keywords: CAD; eNOS; VNTR.

1. INTRODUCTION

Myocardium (the muscle tissue that forms the wall of the heart) as any muscle tissue, requires oxygen to operate. This is supplied by the coronary arteries surrounding the heart. Over time a stenosis, i.e. a partial or complete occlusion, may develop in one or more of the coronary arteries due to various causes. A patient with stenosis in one or more arteries is said to have coronary artery disease (CAD) [1]. Like other common chronic diseases, CAD has a complex etiology that is postulated to involve both genetic and environmental factors [2]. Coronary atherosclerosis, a prerequisite for the development of CAD, results from a defective endothelial function, which is attributed mainly to an altered production of NO, a vasodilator and atheroprotective molecule [3,4]. NO is one of the most versatile molecules and plays an important role in almost every biological system [5,6]. It can inhibit the adhesion, aggregation and recruitment of platelets; vascular smooth muscle cells migration and growth, also regulates some vessel-platelet interactions and limits the oxidation of atherogenic low density lipoproteins cholesterol (LDL-C) [7]. Therefore, the reduced bioavailability of NO is common to CAD, and defects in NO production and function correlate well with the incidence of CAD [8,9].

NO is synthesized from l-arginine catalyzed by nitric oxide synthase (NOS) [10]. There are at least three isoenzymes of NOS: constitutive neuronal NOS (nNOS or NOS-1), inducible NOS (iNOS or NOS-2), and constitutive endothelial NOS (eNOS or NOS-3), which constitute a “gene family”, located on different chromosomes and expressed in different cell lines [7,10,11]. Human eNOS is located on chromosome 7q35 to 36 with a total size of 21 kb and encoded by a 26 exon gene [7]. Because eNOS availability is regulated at transcriptional and posttranscriptional levels and owing to its role in the production of NO, eNOS gene is considered to be a potential candidate for CAD [12]. The eNOS gene presents some polymorphisms that have been previously associated with angiographically assessed CAD [13].

On the basis of close interrelationship between oxidative stress, inflammation, and atherosclerosis, several studies have been performed to investigate the benefits of nutrients and food components with known antioxidant effects on cardiovascular health [14]. The antioxidant properties of vitamins C, E, and A seemed to be effective against different conditions able to promote CVD, that is, high blood pressure, impaired glucose and lipid profile, smoke abuse, with a positive influence on every step of atherosclerotic progression

(endothelial dysfunction, LDL-C oxidation, monocyte, and smooth muscle cell activity) [14].

Among several polymorphisms in eNOS gene, a single nucleotide polymorphism (SNP), T–786C, was identified in the 5 flanking region of the eNOS gene involving a substitution of thymine (T) to cytosine (C) at a locus 786 base pairs upstream [15]. It is associated with reduction in the promoter efficiency and the level of expressed enzyme leading to increased CAD risk [16]. Variable number of tandem repeat (VNTR) polymorphism located in intron 4 of eNOS (eNOS4b/a polymorphism) were also known to be associated with excess of risk of CAD [17].

Numerous epidemiological studies evaluated the eNOS polymorphisms in patients with CAD, but the results are often conflicting [8]. Ethnic background is known to influence polymorphism frequencies and their effects on the disease [18].

The present study was there for undertaken to investigate the possible association between CAD and e NOS gene polymorphisms in Egyptian population. To the best of our knowledge, the current investigation is the first to evaluate such association in the Egyptian population.

2. SUBJECTS AND METHODS

2.1 Subjects

This study included 80 patients with coronary artery disease (CAD) (mean age: 47.86±0.34 year, male / female: 43/37) and 40 healthy controls (mean age: 46.15±0.56 year, male / female: 13/27). Subjects diagnosed with stenosis 50% or more by coronary angiography in any of the coronary arteries or their branches were classified as CAD patients. This classification was defined according to the world health organization (WHO) recommendations [19]. BMI was determined by dividing the subject's mass by the square height, typically expressed in metric units. BMI = kilograms / meters^2 [19]. CAD patients were recruited from health insurance and private polyclinics (Mansoura), as well as, Naser institute, and Cardiology National institute, Cairo, Egypt. Control subjects were proven to have no history of cardiac disease, diabetes, hypertension and they were normal weight individuals. Subjects who had smoking habits were excluded from the study. All patients and normal subjects gave their informed consent to participate in this study.

2.2 Methods

2.2.1 Clinical investigation

For all participants, echocardiography (Echo) and electrocardiography (ECG) were performed at rest and/or stress in order to define the presence, distribution, and functional status of CAD. Segmental Wall Motion Abnormalities (SWMA) has been used to define myocardial ischemia, detect coronary artery perfusion impairment, and assess cardiac function. Coronary angiography was performed in CAD patients for the assessment of the extent and severity of CAD [20].

2.2.2 Samples collection

Venous blood samples were collected from each examined subject after overnight fasting in polyethylene tubes containing 0.1% ethylene diaminetetracetic acid (EDTA) solution as an anticoagulant for DNA extraction.

2.2.3 Molecular investigations

2.2.3.1 DNA extraction

Genomic DNA was extracted from samples of whole blood by the the Generation DNA Purification Capture Column Kit (Gentra systems, USA).

2.2.3.2 Genotyping

2.2.3.2.1 Detection of $T^{786\rightarrow}C$ polymorphism in the 5'- flanking region of the e NOS gene

The presence of the T→C conversion at nucleotide position 786 in the 5- flanking region of the eNOS gene was determined by using (Amplification Refractory Mutation system) ARMS “PCR” amplification. For genotyping of T-786C, a newly developed allele-specific PCR was used. The oligonuleotide primers used in the reaction are listed in Fig. 1. Amplification was performed in a total volume of 25 µl containing 5 µl genomic DNA,1 µl 2684T and 1 µl 2684C primers, 0.5 µl T0 and 0.5 µl C0 primers, 12 µl 2X Taq complete master mix (Fermentase) and 5 µl PCR grade H2O. After 1 cycle initial denaturation at 96℃ for 5 minutes, amplification was achieved by 35 cycles at 94℃ for 30 seconds, 60℃ for 30 seconds, and 72℃ for 20 seconds, then final extension was done at 72℃ for 5 minutes .The products of the PCR process were separated by electrophoresis on a 3%

agarose gel and visualized with UV transilluminator, PCR generate T and C alleles at 176-bp and 250-bp respectively; the gel then be photographed with a digital camera (Fig. 2).

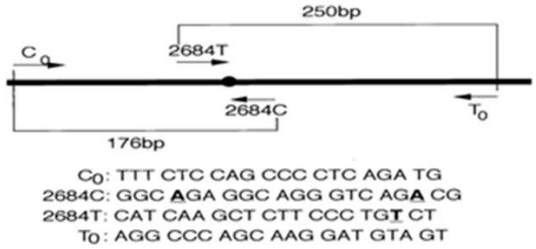

Fig. 1. Shows the primers used in the reaction and the expected PCR products

Fig. 2. 3% agarose gel stained with ethedium bromide showing bands of allele –specific PCR for T-786C polymorphism in e NOS gene, analyzed by electrophoresis

- *Lane (M):Sibenzyme M15, 100 bp DNA ladder.*
- *Lane (1&2): Show CC homozygote polymorphism of the T-786C e NOS gene at 250 bp.*
- *Lane (3 &4): Show TC heterozygote polymorphism where T at 176 bp &C at 250 bp.*
- *Lane (5 &6): Show TT homozygote polymorphism at 176 bp*
- *Lane (7): Negative control*

2.2.3.2.2 Detection of 27-bp repeat polymorphism in intron 4 of the e NOS gene [21]

PCR amplification was used for genotyping of the 27-bp repeat polymorphism in intron 4 VNTR of the e NOS gene with the primers 5'- GCC CTATGG TAG TGC CTT -3'(forward) and 5'- CTC TTAGTG CTG TGG TCA C -3' (reverse). A 25 µl volume was used for each PCR reaction.We added 1 µl of each primer, together with 5 µl genomic DNA,12 µl 2X Taq complete master mix, and 6 µl PCR grade H2O .Each reaction mixture was heated for 1 cycle at 94℃ for 5 min for initialization, followed by 40 cycles of 94℃ for 30 sec, 68℃ for 30 sec and 72℃ for 30 sec, and a final extension of 72℃ for 8 min. The PCR products were separated by electrophoresis on a 3% agarose gel. The alleles 4b and 4a produced bands of 418 bp and 391 bp respectively; the 418 bp band indicated five repeats of the 27-bp (b allele), and a 391 bp band indicated four repeats (a allele) (Fig. 3).

Fig. 3. 3% agarose gel stained with ethedium bromide, showing bands of allele –specific PCR for intron 4 VNTR polymorphism in eNOS gene, analyzed by gel electrophoresis

- *Lane (M): Sibenzyme M15, 100 bp DNA ladder.*
- *Lane (1, 2, 4, 6 & 7): Show bb homozygote polymorphism of the intron 4 VNTR in e NOS gene at 418 bp.*
- *Lane (3 & 5): Show a b heterozygote polymorphism where a at 391 bp & b at 418 bp.*

2.2.4 Statistical analysis

Data were statistically analyzed using SPSS statistical computer package version 10 software [22]. Quantitative variables were expressed as mean ± SE, while the qualitative variables were presented as numbers and percentages. Comparison of qualitative data was done using chi-square test ($\chi 2$). Quantitative data were

compared using Independent- Samples T test or One Way ANOVA test. Statistical significance was set at p≤0.05.

3. RESULTS

The clinical characteristics of the study subjects are shown in (Table 1a). CAD patients presented elevated values of systolic and diastolic blood pressure (SBP &DBP) and higher prevalence of traditional risk factors for CAD, including male gender, diabetes and BMI. Significant increases in these parameters were showed in obese and overweight patient groups when compared with the normal weight group. However, these changes together seemed to be more drastic in the obese patients comparing with patients with normal weight profile (Table 1b). Also the prevalence of female gender in the obese group seemed to be significantly higher.

3.1 Endothelial Nitric Oxide Synthase (eNOS) Gene Polymorphisms

Distribution of genotype and allele frequency of T786-C and intron 4 VNTR polymorphisms among the CAD patients and controls are shown in Tables 2a and 2b. Table 2a showed that the genotype distribution of T786-C polymorphism in CAD patients and controls were 43.75% vs. 27.5% (CC), 28.75% vs. 37.5(CT) and 27.5% vs. 35 (TT), respectively, and was found to be statistically non-significant (p =0.23) Allele frequencies of T786-C polymorphism in CAD patients and controls were 58.12% vs. 46.25%(C allele) and 41.87% vs. 53.75%(T allele), respectively, (C allele) tend to be higher but non-significant in patients than controls (p =0.21). Table 2b showed that there was no significant difference in the genotype distribution and allele frequencies of T786-C polymorphism among the overweight cases compared to normal weight subjects (Table 2b) CC, TC and TT genotypes (45% vs. 40%, 25% vs. 32.5% and 30% vs. 27.5%,p=0.23, respectively). On the other hand, the frequencies of C and T alleles was slightly but non significantly differ between the two groups (57.5% vs. 56.25% and 42.5% vs. 43.75%, p=0.12, respectively). Also, there was no significant differences in the genotype distribution of the T-786C polymorphism in the obese group compared to normal weight group CC, TC and TT genotypes (50% vs. 40%, 25% vs. 32.5% and 25% vs. 27.5%, p= 0.24, respectively). While, the comparison of the frequencies of C and T alleles between these groups revealed no significant difference (62.5% vs. 56.25% and 37.5% vs. 43.75%, p=0.18, respectively).

Table 3a showed that the genotype distribution of eNOS intron 4 VNTR polymorphism in CAD patients and controls was 5% vs. 0% (aa), 50%vs. 65% (ab) and 45% vs. 35% (bb), respectively, and was found to be non-significant (p =0.15). Allele frequencies of intron 4 VNTR polymorphism in CAD patients and controls were 30% vs.32.5% (a allele) and 70% vs. 67.5% (b allele), respectively (p =0.12). In Table 3b, there were no significant differences observed in the distribution of genotype and alleles frequencies of the intron 4 VNTR polymorphism when overweight patient group were compared with normal weight patient group, the genotype distribution was 10% vs. 2.5% (aa), 45%vs. 55% (ab) and 45% vs. 42.5% (bb), respectively (p =0.28). Allele frequencies of intron 4 VNTR polymorphism in overweight and normal weight patients were32.5% vs.28.75% (a allele) and 67.5% vs. 71.25% (b allele), respectively (p =0.18). In addition, no differences were found when obese group were compared with normal weight patient group according to genotype or allele frequency. The genotype distribution was 5% vs. 2.5% (aa), 45% vs. 55% (ab) and 50% vs. 42.5% (bb), respectively (P=0.43). Allele frequencies of this polymorphism in obese and normal weight patients were 27.5% vs.28.75% (a allele) and 72.5% vs. 71.25% (b allele), respectively (p =0.23).

In Table 3b, there were no significant differences observed in the distribution of genotype and alleles frequencies of the intron 4 VNTR polymorphism when overweight patient group were compared with normal weight patient group, the genotype distribution was 10% vs. 2.5% (aa), 45%vs. 55% (ab) and 45% vs. 42.5% (bb), respectively (p =0.28). Allele frequencies ofintron 4 VNTR polymorphism in overweight and normal weight patients were32.5% vs.28.75% (a allele) and 67.5% vs. 71.25% (b allele), respectively (p =0.18). In addition, no differences were found when obese group were compared with normal weight patient group according to genotype or allele frequency. The genotype distribution was 5% vs. 2.5% (aa), 45% vs. 55% (ab) and 50% vs. 42.5% (bb), respectively (P=0.43). Allele frequencies of this polymorphism in obese and normal weight patients were 27.5% vs. 28.75% (a allele) and 72.5% vs. 71.25% (b allele), respectively (p =0.23).

Table 1a. Clinical characteristics of the study subjects

		Control (n=40)	CAD (n=80)	P value
Age , Years		46.15±0.56	47.86±0.34	0.33
Gender	Male, n (%)	13(32.5%)	43(53.75%)	0.02
	Female, n (%)	27(67.5%)	37(46.25%)	
BMI(Kg/m2)		23.57±0.36	27.39±0.54	0.00
SBP(mm/Hg)		120.88±1.06	133.25±1.45	0.00
DBP(mm/Hg)		76.00±1.05	86.19±0.94	0.03
Diabetic, n (%)		0(0%)	27(33.75%)	0.00

Data are presented as mean± SEM. n: number of cases, (%): percentage of cases. CAD: Coronary artery Disease; BMI: Body mass index, SBP= Systolic blood pressure, DBP=Diastolic blood pressure. p≤0.05 (significant)

Table 1b. Clinical characteristics of CAD patients after classification according to BMI

		CAD (N=80)			*P1* value	*P2* value
		Normal weight (N=40)	Over weight (N=20)	Obese (N=20)		
Age , Years		47.75±0.53	47.60±0.65	48.35±0.54	0.99	0.22
Gender	Male, n (%)	23(42.5%)	16(80%)	4(20%)	0.07	0.00
	Female, n (%)	17(42.5%)	4(20%)	16(80%)		
BMI(Kg/m2)		23.92±0.39	27.76±0.50	33.97±0.80	0.03	0.04
SBP(mm/Hg)		131.50±1.70	133.00±3.37	137.00±3.27	0.03	0.03
DBP(mm/Hg)		79.50±0.62	91.25±1.53	94.50±1.08	0.03	0.00
Diabetic, n (%)		11(27.5%)	8(40%)	8(40%)	0.24	0.24

Data are presented as mean± SEM. N: number of cases, (%): percentage of cases. CAD: Coronary artery Disease; BMI: Body mass index, SBP= Systolic blood pressure, DBP=Diastolic blood pressure.p≤0.05 (significant).P1=If comparing overweight patients with normal weight patients. P2=If comparing obese patients with normal weight patients

Table 2a. Genotype distribution and allele frequencies of T-786C polymorphism of the eNOS gene in the study subjects

Genotype		Control (n=40)	CAD (n=80)	*P* value
Genotype	CC, n (%)	11(27.5%)	35(43.75%)	0.23
	TC, n (%)	15(37.5%)	23(28.75%)	
	TT, n (%)	14(35%)	22(27.5%)	
Allele	C allele, n (%)	37(46.25%)	93(58.12%)	0.21
	T allele, n (%)	43(53.75%)	67(41.87%)	

Data are presented as number and percentage (%). CAD: Coronary artery Disease. P ≤0.05 (significant)

Table 2b. Genotype and allele frequencies of the T-786C polymorphism of the eNOS gene in CAD patients after classification according to BMI

		CAD (N=80)			*P 1* value	*P2* value
		Normal weight (n=40)	Overweight (n=20)	eObes (n=20)		
Genotype	CC, n(%)	16(40%)	9(45%)	10(50%)	0.23	0.24
	TC, n(%)	13(32.5%)	5(25%)	5(25%)		
	TT, n(%)	11(27.5%)	6(30%)	5(25%)		
Allele	C allele, n(%)	45(56.25%)	23(57.5%)	25(62.5%)	0.12	0.18
	T allele, n(%)	35(43.75%)	17(42.5%)	15(37.5%)		

Data are presented as number and percentage (%). CAD: Coronary artery Disease. P ≤0.05 (significant). P1=If comparing overweight patients with normal weight patients. P2= If comparing obese patients with normal weight patients

Table 3a. Genotype distribution and allele frequencies of 4b/a 27-bp repeats variant polymorphism of the eNOS gene in the study subjects

Genotype		Control (n=40)	CAD (n=80)	*P* value
Genotype	bb, n(%)	14(35%)	36(45%)	0.15
	ab, n(%)	26(65%)	40(50%)	
	aa, n(%)	0(0%)	4(5%)	
Allele	b allele, n(%)	54(67.5%)	112(70%)	0.12
	a allele, n(%)	26(32.5%)	48(30%)	

Data are presented as mean± SE. n: number of cases, (%) = percentage of cases. CAD: Coronary artery Disease. p≤0.05 (significant)

Table 3b. Genotype distribution and allele frequencies of eNOS 4b/a 27-bp repeats variant in CAD patients after classification according to BMI

		CAD (N=80)			*P 1* value	*P2* value
		Normal weight (n=40)	Overweight (n=20)	Obese (n=20)		
Genotype	bb, n(%)	17(42.5%)	9(45%)	10(50%)	0.28	0.43
	ab, n(%)	22(55%)	9(45%)	9(45%)		
	aa, n(%)	1(2.5%)	2(10%)	1(5%)		
Allele	a allele, n(%)	23(28.75%)	13(32.5%)	11(27.5%)	0.18	0.23
	b allele, n(%)	57(71.25%)	27(67.5%)	29(72.5%)		

Data are presented as number and percentage (%). CAD: Coronary artery Disease .p≤0.05 (significant). P1= If comparing overweight patients with normal weight patients. P2= If comparing obese patients with normal weight patients

4. DISCUSSION

Coronary artery disease (CAD) is multifactorial disorder with genotype and environmental interactions having an important role in its development [2]. Therefore in addition to established risk factors such as obesity, hypertension, diabetes mellitus and sex, and genetic risk factors may have important roles in the pathogenesis of CAD [23]. Identification of these genetic risk factors is expected to enhance our understanding of the molecular basis for CAD. The epidemiologic studies performed during the last 50 years showed that there are many genes responsible in the etiology of coronary atherosclerosis [24]. Over the last decade, a remarkable evidence has accumulated; offering that NO plays a pivotal role in CAD [10]. The e NOS polymorphism may alter gene expression and may reduce the bioavailability of endothelial NO, thereby resulting in endothelial dysfunction [25]. Due to the importance of e NOS in the generation of NO, several epidemiological studies have investigated the relation between eNOS gene polymorphisms and CAD and have produced varied or contradictory results [26,27].

Two polymorphisms in e NOS gene are reportedly associated with CAD: a 27-bp repeat [variable number of tandem repeat (VNTR)] polymorphism located in intron 4 of eNOS gene (eNOS 4b/a), and the T-786C single nucleotide polymorphism (SNP) in the 5′-flanking region [28].

It is well accepted that endothelial dysfunction occurs in response to cardiovascular risk factors and precedes the development of atherosclerosis [29]. Mean while, it was indicated that point mutation of thymine (T) to cytosine(C) at nucleotide 786 (T-786C) in the 5′-flanking region of the eNOS gene could result in a significant reduction in the promoter activity by 50% [30], suggesting that in many carriers of the mutant allele, the l-arginine/NO pathway does not function properly, leading to endothelial dysfunction [31]. Thus, it is possible that the T-786C mutation of the eNOS gene may make carriers susceptible to the development of endothelial dysfunction and in turn to CAD [32]. Data from other studies demonstrated that homozygosity (CC allele) for this polymorphism is associated with a deficit of eNOS expression in endothelial cells, as well as with a reduced NO-

mediated vasomotor function, because the C allele creates a binding site for a replication protein A-1 that acts as a repressor of gene transcription [30].

Consistent with those findings, Nakayama et al [15] suggested that the T-786C polymorphism of the eNOS gene contribute to CAD. The same authors recently reported that this mutation could decrease the concentration of serum nitrite/nitrate (NOx) significantly [33]. Rios et al [16] indicated that a variant T-786C may be the most relevant eNOS polymorphism for the development of cardiovascular disease in the Brazilian population. These results agree with those described by Tanus-Santos et al. [34] in the Caucasian population. A meta-analysis involving 22 studies that evaluated the association between the T-786C polymorphism and CAD showed that this genetic variant was associated with an increased risk of CAD [35]. The T-786C mutation was also found to be significantly associated with CAD in Italian population [31,36], although, each study condition was slightly different. Younan et al. [37] showed that polymorphism of Glu2983Asp and T7863C of the eNOS gene does not increase the susceptibility to coronary and carotid arteries disease in Egyptian patients.

In the current investigation a non-significant difference in the distribution of genotypes (cases: 43.75% CC, 28.75% CT, 27.5% TT) and control (27.5% CC, 37.5% CT, and 35% TT) was observed Although The CAD patients showed remarkable proportion of mutant allele frequency of T-786C mutation, were the C allele frequency of T-786C polymorphism was higher in CAD than control individuals (58.12% vs. 46.25%, p= 0.21) it was not significant.

Comparable results were found by Nassar et al [38] in the Canadian population, where non significant increase in the frequency of the variant T-786C allele in the patients with premature CAD was recorded Likewise, neither Poirier et al. [39] in the French population, nor Granath et al. [40] in the Australian Caucasian population found significant difference among CAD cases and controls with respect to eNOS T-786C polymorphism.

Further research regarding association of intron 4 VNTR polymorphism with CAD was considered. The intron 4 VNTR polymorphism have been demonstrated to bind nuclear proteins as an enhancer or repressor, resulting in promotion or suppression, respectively of the transcription efficiency [41]. The intron 4 VNTR polymorphism is associated with altered plasma NO levels, influencing both NO and enzyme production and data from literature reported that it accounted for 25% of the variance of NO circulating levels [42]. Wang et al. [41] have demonstrated that the intron 4 VNTR polymorphism could also be potentially functional, since it not only binds to certain nuclear proteins, but also affects promoter efficiency. In the current study the genotype frequencies for intron 4 VNTR b/b , a/b and a/a were 45%, 50% and 5%, respectively for the CAD patients, and 35%, 65% and 0%, respectively for controls.

In the present study, the frequency of 4a allele was slightly higher but non significant in CAD patients compared to control individuals (30% vs. 32.5%) respectively. Consistent with these findings, Vasilakou et al. [27] did not find any association between the mutant alleles of eNOS 4b/a polymorphisms and the risk for premature CAD in the Greek population. Studies on German [43] or Japanese [44] patients also failed to observe such an association.

The molecular analysis of intron 4 VNTR polymorphism of demonstrated that this genetic variant was not associated with CAD in the studied population. In agree with current results, Sigusch et al. [45] and Hwang et al. [46] did not detect a link between this polymorphism and CAD in German and Taiwanese populations. In addition, Granath et al. [40] found no evidence for an association between this polymorphism and CAD in the Australian Caucasian population.

In contrast, Wang et al. [41] detected a significant association between intron 4 VNTR polymorphism and CAD. Yoon et al [5] also identified an association of this polymorphism with the risk of CAD. Lee et al. [47] suggested that intron 4 VNTR polyporphism was significantly associated with the development of CAD in Korea. Other reports suggested potential associations of the intron 4 VNTR polymorphism with vascular disease Fatini et al. [48] or endothelial dysfunction. Li and Forstermann [49] in Iranian population with CAD showed that eNOS4b/a polymorphisms were associated.

In contrast, the intron 4 VNTR polymorphism was found to be associated with coronary atherosclerosis in Australian [50], Japanese [51], and Afro-American patients [52]. Positive associations of (a) allele compared with (b) allele were reported in Caucasians with CAD [53]. This

discrepancy between different studies with eNOS gene polymorphism it could be the result of differences in ethnicity [54]. Ethnic background is known to influence polymorphism frequencies and their effects on the disease. Additionally, These different results are likely a consequence of differences in exposure to risk factors, differences in sampling methods, different selection criteria adopted for patients and controls, in particular clinical presentation, extent of disease, age, race, geographical area, and concomitant environmental risk factors with in all can explain such discrepancies.

The present study extended also in order to determine the genotype distribution of the eNOS T-786C and intron 4 VNTR gene regions when CAD patients were classified according to their BMI into normal weight patients, overweight patients and obese patients groups, As far as we are aware it has not been previously reported that CAD is classified into subgroups according to BMI for the evaluation of polymorphisms in T-786C and intron 4 VNTR of the eNOS gene. When the control group and the whole CAD patient group (prior classification) were compared, no statistically significant difference was found between the genotype frequencies of the T-786C promoter and intron 4 VNTR. The genotype distribution of the intron 4 "a a" allele was found to be 2.5%, 10%, and 5%, respectively in the normal weight, overweight, and obese groups. The distribution of this genotype in overweight and obese patients was found to be non-significantly differed when compared with the normal weight group. Distribution of the "bb" genotype in the normal weight, overweight, and obese groups was found to be 42.5%, 45% and 50%, respectively, however distribution of the "a b" genotype in the normal weight, overweight, and obese groups was found to be 55%, 45%, and 45%, respectively. The total proportion of individuals carrying the bb and ab genotype in the overweight and obese groups was statistically non-significant when compared the normal weight group. Accordingly no relationship was detected between eNOS T-786C gene polymorphism and different subgroups of CAD patients.

5. CONCLUSION

In conclusion, our results indicated that the eNOS T-786C and intron 4 VNTR polymer-phisms are not independent predisposition risk factors for CAD in Egyptian population.

6. STUDY LIMITATION AND RECOMMENDATIONS

Our study is restricted by its sample size. Other factors, such as severity of disease, duration and coexistence of other diseases were also likely to influence the eNOS gene polymorphism. Thus, larger studies are required to confirm the real association of these polymorphisms and CAD.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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