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Investigation of the Effect of Two Major *eNOS* Polymorphisms (4a/b and T786C) on Coronary Artery Disease in North Lebanon

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Abstract

Endothelial nitric oxide synthase is a crucial gene associated with coronary artery disease, owing to the important functions of nitric oxide in vessel protection and vasodilation. Three "Single Nucleotide Polymorphisms" were found to be significantly associated with CAD: 'the 4a/b polymorphism in intron 4', 'G894T (GLU298ASP) in exon 7', and 'the T786C replacement in the flanking region'. This study aimed to explore the relationship between the '4a/b polymorphism of the *eNOS* gene', the 'T786C polymorphism of the *eNOS* gene', and 'the combined effect of both 4a/b and T786C' with the risk of CAD in the Northern Lebanese region. A total of 91 CAD cases and 36 Control healthy individuals were gathered to investigate the allelic frequency and genotypic distribution of the 27VNTR gene polymorphism. 70 of 91 cases and 24 of 36 healthy participants were considered for the T786C polymorphism investigation. Peripheral blood samples were collected, and the 4a/b polymorphism genotypes were determined using "polymerase chain reaction". Genotypes for the T786C polymorphism were determined using "polymerase chain reaction-restriction fragment length polymorphism". A comparison was made between the two groups regarding the distributions of genotypes and allele frequencies. In our sample, the "a allele" of the 4a/b polymorphism was observed in 14.17% of individuals, while the "C allele" of the T786C polymorphism was found in 27.7%. The Control and CAD groups showed no significant differences in the distributions of genotype and allele frequencies for the T786C and 4a/b gene polymorphisms. No additive effect of both polymorphisms was noted. Therefore, *eNOS* polymorphisms, in our study, do not show a significant association with CAD.

Keywords: 4a/b Polymorphism, Coronary Artery Disease-CAD, Endothelial Nitric Oxide Gene *eNOS,* Nitric Oxide-NO, T786C Polymorphism.

Introduction

The foremost contributor to worldwide deaths from cardiovascular disease (CVD) is coronary artery disease (CAD), also known as ischemic heart disease. It results from decreased blood flow and oxygen deficiency caused by the partial obstruction of the coronary arteries. CAD has a complex, heterogeneous background involving gene-environment and gene-gene interactions (1). Blood pressure, hypercholesterolemia, obesity, diabetes, smoking, socio-economic status, physical inactivity and alcohol consumption are considered as modifiable risk factors of CAD. Regarding non-modifiable risk factors, family history, ethnicity, gender, age, and, most significantly, genetic susceptibility appear to be the primary contributors to this disease. Studies indicate that most of these risk factors significantly impact cardiovascular health by affecting endothelial function (2). The endothelium regulates vascular homeostasis by controlling relaxation and producing vasoactive enzymes like nitric oxide (NO) (3), which is a cellular signaling molecule in gaseous form (4). According to the tissues from which they were first cloned, three isoforms of NO synthase have been named after being discovered (5). The main isoform that influences various cellular processes, such as the ones related to vascular cells, is the endothelial isoform (NOS3, eNOS), synthesized in endothelial cells (6, 7). It controls blood pressure, maintains blood vessel dilation, and prevents the adhesion of leucocytes and the aggregation of platelets (8). *eNOS* also promotes angiogenesis and regulates nerve function and neurotransmission (9, 10). Moreover, it controls vascular smooth muscle proliferation (11). NO has the potential to hyperpolarize the cell

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membrane and lead to vasodilation by activating the K^+ and Ca^{++} channels (12). All these actions repress the development of CAD. Nitric oxide derived from the endothelium is produced from the amino acid, with the L-citrulline byproduct (13). The primary target of nitric oxide (NO) is the soluble guanylate cyclase in smooth muscle cells (SMCs), which upon activation stimulates the synthesis of cyclic guanosine monophosphate (cGMP) in SMCs, which causes their relaxation and ultimately results in vasodilation (14). Evidence suggests that the reduction in the bioavailability of NO is often present in CAD (15). In 1993, the *eNOS* gene was cloned, sequenced and located on chromosome 7q35-7q36 (6, 16). 25 introns and 26 exons, encoding 4.1 kb mRNA and covering 21-22 Kb genomic DNA, comprise the *eNOS* gene of humans (6, 17). Three major *eNOS* polymorphisms, Glu298ASP (894 G/T) (rs1799983), VNTR polymorphism in intron 4 (4a/b) (rs61722009) and T786C (rs2070744) are considered to be highly attributed with CAD. The mutation where Glutamate is replaced by Aspartate at position 298 is the *eNOS* G894T (18). According to literature, by promoting selective proteolytic cleavage in vascular and endothelial tissues, Aspartate may decrease *eNOS* bioavailability, which results in reduced synthesis of NO (19). The VNTR (variable number of tandem repeats) 27 forms the 4a/b polymorphism of the *eNOS* gene. It is found on the 7th chromosome in the genomic region 150997170-150997268 of intron 4. siRNA, also known as small interfering RNA, is proved to be responsible for transcription regulation (15). Based on the specific alleles present, polymorphisms at this site lead to variety of functions. The occasional b-allele has been linked to higher levels of plasma NO metabolites in comparison to the a-allele, with the 4b allele comprising five 27 bp tandem repeats and the 4a allele consisting of four (20). Some studies were unable to establish this association, leaving the role of the *eNOS* 4a/b polymorphism in various physiological conditions unclear and subject to debate. The *eNOS* 4a/b polymorphism was first associated with CAD in a 1997 study, which found that homozygosity for the 4a-allele increases the risk of developing the condition (21). It is additionally connected to a higher risk of myocardial infarction (MI) in African-Americans (22). Likewise, it was observed that the 4a allele is more prevalent among Japanese male patients compared to the control group, who had no traditional risk factors such as a BMI below 27 kg/m^2, or a history of diabetes mellitus, hypertension, or hypercholesterolemia. Conversely, in women with more than one risk factor for myocardial infarction, the 4a allele showed no association with the condition (23). Insignificant association of the *eNOS* 4aa genotype with myocardial infarction was also confirmed by a Japanese study, which involved myocardial infarction patients and healthy control subjects matched by gender and age, revealing no link between this polymorphism and an increased risk of MI (24). In Taiwan, comparable findings were observed (25). Additional studies have observed no connection between the *eNOS* 4a/b gene and the occurrence of cardiovascular problems (26, 27). In Germany as well, and among controls, myocardial infarction, and coronary artery disease patients, allele frequencies were similarly distributed (28). Upstream of the transcription initiation point, the rs2070744 single nucleotide polymorphism is a functional site positioned at -786 (29). Research suggests that the T786C polymorphism reduces *eNOS* mRNA levels as well as serum nitrite and nitrate concentrations (30). Other studies have indicated that this polymorphism is strongly linked to reduced *eNOS* promoter activity, leading to a decrease in nitric oxide concentration (19). Multiple studies have demonstrated a correlation between CAD and the T786C polymorphism in populations from Iran, Italy, China, and Africa-Brazil (31-34). In contrast, a research conducted on the Caucasian-Australian population found no evidence of this association (35). Our current research seeks to help the public health and medical bodies in Lebanon to better understand the causes behind CAD by examining the 27 VNTR polymorphism in the intron region and the T786C polymorphism in the promoter region of the *eNOS* gene, as done in similar geographic regions, in addition to exploring their combined impact on CAD. As far as we are aware, the present investigation is the first of its kind conducted in North Lebanon.

Materials and Method

This study included 127 participants, by convenience sampling, comprising 91 cases of CAD and 36 controls that are healthy, to examine the

eNOS 4a/b polymorphism. Using the polymerase chain reaction (PCR) technique, the genotype of this polymorphism was determined. To genotype the T786C polymorphism, blood samples from 70 of the 91 CAD cases and 24 of the 36 control subjects were analyzed using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. CAD patients were taken from different hospitals in North Lebanon and diagnosed by cardiologists using the coronary angiography technique according to the Lebanese medical protocols. The control group comprised healthy volunteers, excluding individuals with hypertension, diabetes, high cholesterol, or a first-degree family history of coronary artery disease, to minimize the potential for biased results. The study received approval from the local ethics committee, and informed consent with handwritten signature was secured from both the CAD cases and the healthy control participants. Blood samples were anonymous and encoded by participant ID for confidentiality.

Figure 1: *eNOS* Polymorphism Sequences (A) The sequence of the *eNOS* 4a/b-27 VNTRs Polymorphism. Primer Sequences are Highlighted in Bold, while the 27-bp repeats, which are absent in the VNTR 4a Polymorphism, are shown in Italics. (B) The Sequence of the *eNOS* T786C Polymorphism. Primer sequences are highlighted in bold, Italicized Letters Indicate the Restriction Enzyme Site, while the Underlined Letter Represents the single Mutation

PCR and DNA Extraction

DNA was extracted from whole blood using the GenElute™ DNA Kit. The *eNOS* 4a/b polymorphism was genotyped using the PCR technique with specific oligonucleotide primers (Forward: TGGTTATCAGGCCCTATGGT; Reverse: GAAGCCTTCTCTCTTGGGG) (Figure 1A) which surround the 27 bp VNTR region of intron 4. On the other hand, the T786C polymorphism was genotyped by the PCR-RFLP method, using the oligonucleotide primers (Forward: ACCTGCATTCTGGGAACTGT; Reverse: ATGACTCAAGTGGGGGACAC) (Figure 1B). PCR for both mutations was performed in a 50 µL-reaction mixture (REDTaq® ReadyMix™ (R2523)), which included Taq polymerase (25 µL), DNA (5 µL), reverse primer (RV) (3 µL), forward primer (FW) (3 µL), and water (14 µL). We used, for both mutations, the thermal cycler (Bio-RAD C1000 thermal cycler); the thermocycling conditions included denaturation (1 minute-95°C), annealing (1 minute-60°C), and elongation (10 minutes-72°C). 36 repetitions were done for the cycle. 12 µL of PCR products were examined through 3% "agarose gel electrophoresis" at 100 V for 45 minutes and stained with "ethidium bromide" for visualization.

Genotyping the 4a/b Polymorphism

The PCR technique was enough to genotype the insertion/deletion polymorphism (4a/b), where the larger allele, *eNOS* 4b, consists of 5 tandem 27 bp repeats, while the smaller allele, *eNOS* 4a, contains four repeats. The PCR products measured 279 bp for the *eNOS* 4b allele and 252 bp for the *eNOS* 4a allele (Figure 2A). For the T786C polymorphism, a fragment of 205 bp was visualized as a PCR product (Figure 2A).

Genotyping the T786C Polymorphism (RFLP)

Regarding the issue of genotyping the T786C polymorphism, the used restriction enzyme NaeI (BioLabs, LEBANON) is designed to cut the muted gene only once. The amplified 205 bp of the T786C *eNOS* polymorphism was examined by the NaeI RE

that cleaves whenever T786C polymorphism is present. The products of RFLP were examined using electrophoresis on a 3% agarose gel and visualized under ultraviolet light. 3 forms of genotypes were identified for the T786C polymorphism. For the two copies of the *eNOS* gene position at 786 of the promoter region, the non-mutant T allele preserves the 205 bp fragment-length in presence of the RE, while the mutant type (C allele) rs2070744 (T786C) is cleaved, and the digestion products are 133 bp and 72 bp. In the heterogenic state, a mixed product of cleavage resulted in 3 bands: 205 bp, 133 bp and 72 bp (Figure 2B).

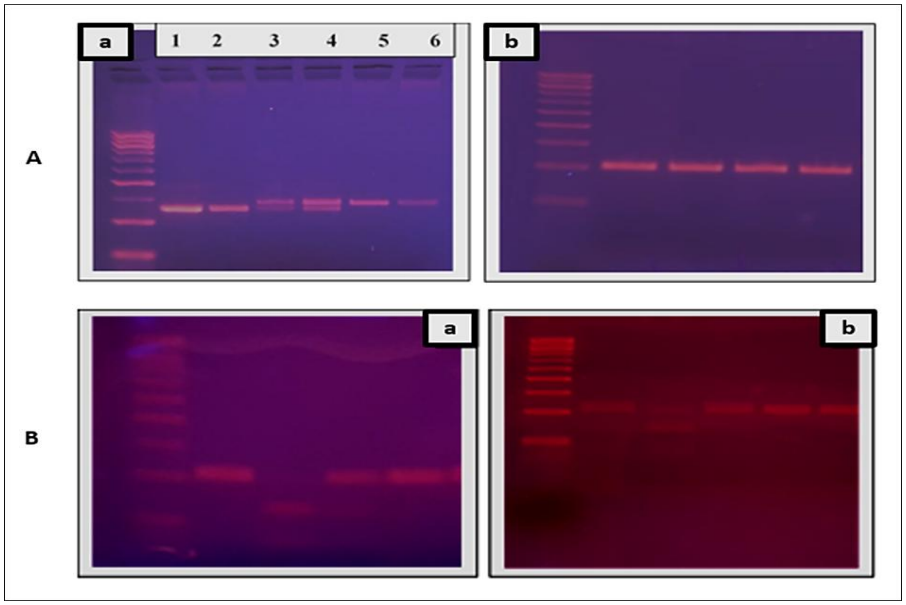

Figure 2: PCR and Genotyping (A) PCR Products of *eNOS* 4a/b and T786C Polymorphisms. a: *eNOS* 4a/b: Lane 1, 2: 252 bp (4a); Lane 3, 4: 279 bp + 252 bp (4a/b); Lane 5, 6: 279 bp (4b). b: *eNOS* T786C: A band of 205 bp (B) Genotyping T786C Polymorphism using RFLP. a: Lane 1,3,4: 205 bp (TT), Lane 2: 133 bp and 72 bp (CC). b: Lane 1,3: 205 bp (TT), Lane 2: 205 bp, 133 bp and 72 bp (TC)

Statistical Analysis

Recordings, for both the patient and control groups of the genotype data, were done. The statistical study and analysis were conducted using SPSS (version 25) platform from IBM. For categorical variables, data was presented as frequencies and percentages. To verify that the genotype distribution is in agreement with the distribution anticipated by the equilibrium of Hardy-Weinberg, comparison of the expected and actual genotype frequencies was performed by the Chi-squared test. Binary logistic regression analysis was used to evaluate the odds ratio (OR) by means of 95% confidence interval (CI) for potential genotype risk factors associated with CAD. A p-value of less than 0.05 was considered statistically significant for all tests.

Results

The three genotypes of each polymorphism, (4a/a, 4a/b, 4b/b) for the 4a/b SNP and (TT, TC, CC) for the T786C SNP, were distributed in accordance to Hardy-Weinberg equilibrium (p-value > 0.05) in the total sample sizes of 127 for the 4a/b study and 94 for the T786C study (Table 1). This indicates no significant deviation between the observed and expected genotype frequencies, suggesting that the samples were representative of the population. Table 2 presents the Control and CAD genotypes for both mutations. Encompassing patients and controls, the genotyping method was used to calculate the frequencies of each genotype in the overall sample of the study. It was observed that the homozygous non-mutant genotype (4b/b) accounted for 74% of the sample, while the heterozygous mutant (4a/b) and homozygous mutant (4a/a) genotypes were present in 23.6% and 2.4% of the sample, respectively. Additionally, the 4a allele was detected in 14.17% of the 254 chromosomes, whereas the 4b allele was found in 85.83%. Regarding the T786C polymorphism, a percentage of 47.8% for the combined genotypes (TC and CC) was obtained. More clearly, 27.7% C alleles, among 188 chromosomes, was observed in comparison to the wild type T allele (72.3%) (Table 2).

Table 1: The Equilibrium of Hardy-Weinberg analysis for the Genotype Distribution

(a) Actual and Expected Frequencies of Genotypes in The Two Groups (Sample Size=127)				
Genotypes	4a/a	4a/b	4b/b	
Actual-Percentages (Actual-Frequencies)	2.36 (03.0)	23.62 (30.0)	74.02 (94.0)	χ^2= 0.107 p= 0.948
Expected-Percentages (Expected-Frequencies)	2.01 (02.6)	24.33 (30.9)	73.66 (93.6)	
(b) Actual and Expected Frequencies of Genotypes in The Two Groups (Sample Size=94)				
Genotypes	TT	TC	CC	
Actual-Percentages (Actual-Frequencies)	52.13 (49.0)	40.42 (38.0)	7.45 (07.0)	χ^2= 0.010 p= 0.995
Expected-Percentages (Expected-Frequencies)	52.33 (49.2)	40.02 (37.6)	7.65 (07.2)	

Table 2: Prevalence of the Genotypes and Alleles of the Polymorphisms of the *eNOS* Gene (4a/b & T786C)

(a) The 4a/b *eNOS* Polymorphism Prevalence	
Genotype	Percentage
4b/b	74.0
4a/b	23.6
4a/a	02.4
Allele	Percentage
4b	85.83
4a	14.17
(b) The TC *eNOS* Polymorphism Prevalence	
Genotype	Percentage
TT	52.2
TC	40.4
CC	07.4
Allele	Percentage
T	72.3
C	27.7

The Relationship between the 4a/b *eNOS* Polymorphism and CAD Risk

In our sample of size 127, the frequency distribution of the 4a/b *eNOS* polymorphism is detailed in Table 3. In the CAD group, 24 out of 91 carry the 4a/b polymorphism distributed as frequencies of 2 and 22, for the homozygous and the heterozygous mutants, respectively. As for the controls, 9 out of 36 carry the mutation (1 homozygote and 8 heterozygotes). Taking the 4b/b genotype as the reference for the OR computation, the risk of CAD in the ab genotype (OR=1.108, CI:0.440-2.793) is greater than the risk in the aa genotype (homozygous) (OR=0.806, CI:0.700-9.262), while the risk of CAD in the ab and aa groups together (OR=1.074, CI:0.443-2.609) is slightly greater than the risk in the reference bb group (OR=1). The risk of CAD associated with the *eNOS* 'a' allele (OR=1.033, CI:0.471-2.269) is slightly greater than the risk associated with the reference *eNOS* 'b' allele (OR=1) (Table 3).

The Relationship between the T786C *eNOS* Polymorphism and CAD Risk

Table 3 presents the distribution of the frequencies, in the sample of 94 participants, of the *eNOS* T786C polymorphism. Regarding the CAD patients, 32 out of 70 carry the 786C polymorphism, distributed as 7 homozygous mutants and 25 heterozygous mutants. As for the healthy controls, thirteen out of 24 individuals exhibit the mutation. In all carrier samples, the mutation is inherited in a heterozygous form, with no cases of homozygous mutation identified. Considering the TT genotype as the reference of our OR study, the risk of CAD is 0.557 (CI:0.216-1.437) in the TC genotype, while the OR in the CC and TC combined together is 0.713 (CI:0.281-1.807). As for the C allele, the risk of CAD is 1.040 (CI: 0.498-2.170), slightly greater than that of the reference *eNOS* 786T allele (OR=1) (Table 3).

Table 3: The Risk of CAD in Relation to Each Polymorphism (4a/b and T786C)

(a) The Relationship between the 4a/b Polymorphism and CAD Risk				
Frequencies of Genotypes and Alleles	**Control Group (group size=36)**	**CAD Group (group size=91)**	**OR (Cl/95%)**	**p-value**
Genotype Percentages (Frequencies)				
4b/b	75.0 (27)	73.6 (67)	1.000 (Reference)	
4a/a	02.8 (01)	02.2 (02)	0.806 (0.700-9.262)	0.863
4a/b	22.2 (08)	24.2 (22)	1.108 (0.440-2.793)	0.828
4a/a+4a/b	25.0 (09)	26.4 (24)	1.075 (0.443-2.609)	0.874
Allele Percentages (Frequencies)	72 chromosomes	182 chromosomes		
4b	86.1 (62)	85.7 (156)	1.000 (Reference)	
4a	13.9 (10)	14.3 (026)	1.033 (0.471-2.269)	0.935
(b) The Relationship between the T786C Polymorphism and CAD Risk				
Frequencies of Genotypes and Alleles	**Control Group (group size =24)**	**CAD Group (group size =70)**	**OR (Cl/95%)**	**p-value**
Genotype Percentages (Frequencies)				
TT	45.8 (11)	54.3 (38)	1.000 (Reference)	
CC	00.0 (00)	10.0 (07)	-------	-------
TC	54.2 (13)	35.7 (25)	0.557 (0.216-1.437)	0.226
CC+TC	54.2 (13)	45.7 (32)	0.713 (0.281-1.807)	0.475
Allele Percentages (Frequencies)	48 chromosomes	140 chromosomes		
T	18.6 (35)	53.7 (101)	1.000 (Reference)	
C	06.9 (13)	20.7 (039)	1.040 (0.498-2.170)	0.918

Table 4: The Relationship between CAD and both the 4a/b and T786C *eNOS* Polymorphisms

Frequencies of Genotypes and Alleles	**Control Group (sample size=24)**	**CAD Group (sample size=70)**	**OR (Cl/95%)**	**p-value**
Genotype Percentages (Frequencies)				
4b/b,TT	45.8 (11)	44.2 (31)	1.000 (Reference)	
4a/a,TT	00.0 (00)	00.0 (00)	-------	-------
4a/a,TC	00.0 (00)	01.4 (01)	-------	-------
4a/a,CC	00.0 (00)	00.0 (00)	-------	-------
4a/b,TT	00.0 (00)	10.1 (07)	-------	-------
4a/b,TC	16.7 (04)	07.1 (05)	0.444 (0.101-1.956)	0.283
4a/b,CC	00.0 (00)	02.8 (02)	-------	-------
4b/b,TC	37.5 (09)	25.7 (18)	0.710 (0.247-2.039)	0.524
4b/b,CC	00.0 (00)	08.5 (06)	-------	-------
4a/a,TC+4a/a,CC +4a/b,TC+4a/b,CC	16.7 (04)	11.4 (08)	0.710 (0.178-2.830)	0.627
Allele Percentages (Frequencies)	96 alleles	280 alleles		
4b,T	68.7 (66)	63.9 (179)	1.000 (Reference)	
4a,T	04.2 (04)	07.5 (021)	1.936 (0.641-5.850)	0.242
4a,C	04.2 (04)	03.9 (011)	1.014 (0.312-3.295)	0.982
4b,C	22.9 (22)	24.7 (069)	1.156 (0.663-2.018)	0.609

Investigation of CAD with the additive effect of both polymorphisms of *eNOS* (4a/b & T786C)

The same sample as that of the T786C polymorphism (94 individuals: 70 CAD patients and 24 healthy controls) is utilized to detect the 4a/b mutation (27 bp tandem repeat with variable copy numbers). The primary aim was to determine if the coexistence of both polymorphisms has an additive effect on CAD. Regarding our CAD group, 39 individuals (55.8%) carried at least one of the two *eNOS* polymorphisms. On the other hand, 13 controls (54.2%) turned out to have at least one of the two polymorphisms of interest in our study. Moreover, it was noted that 11.4% CAD patients and 16.7% healthy controls carried both polymorphisms (Table 4). To study the OR, the presence of both 4b/b and TT genotypes together (4b/b,TT) was taken as the reference. The calculated risk associated with carrying the heterozygous genotype for both mutations simultaneously, 4a/b,TC, appeared to be 0.444 (CI:0.101-1.956), and that of holding the genotypes 4b/b and TC together is 0.710 (CI:0.247-2.039). Moreover, the risk of holding both polymorphisms (4a/a,TC+4a/a,CC+4a/b,TC+4a/b,CC) turned out to be 0.710 (CI:0.178-2.830). However, regarding the allele ORs, taking the presence of the 4b and T alleles together (4b, T) as a reference for calculations, the obtained risks are 1.936 (CI:0.641-5.850), 1.014 (CI:0.312-3.295) and 1.156 (CI:0.663-2.018) for the presence of alleles '4a with T', '4a with C' and '4b with C', respectively (Table 4).

Discussion

The intricacy of CAD surpasses its role as merely the leading cause of mortality globally. The polygenic and multifactorial nature of the disease makes it difficult to diagnose, predict, and prevent. Research has demonstrated that it is a complex condition influenced by environmental, lifestyle, and genetic factors (36). Studies have shown that 70% of smokers develop CAD, in contrast to non-smokers. In fact, Smoking and obesity appear to be the leading risk factors for CAD (36, 37). The imbalance between the environmental factors and the lifestyle makes Lebanon an alarming country in the region regarding CAD (36, 38-40). Remarkably, works showed that risk factors related to genetics present up to 60% vulnerability to CAD (41). In 2007, GWAS (Genome-Wide Association Study) detected a strong connection between genetic loci and CAD, involving the examination of SNPs and their crucial role in CAD (42-44). A group of genes responsible for regulating hemodynamic function has been associated with CAD (45). Studies have shown that *eNOS* is a nominee gene, as its polymorphisms influence the activity of the enzyme linked to CAD (46). For instance, while the T786C *eNOS* polymorphism is situated in the promoter region, the 4a/b *eNOS* gene is found in the 4th intron and consists of two alleles: 4a (with 4 repeat tandems) and 4b (with 5 repeat tandems) (47). It has been investigated that a reduced *eNOS* gene expression results in low NO levels, essential for endothelial vasodilation, thereby raising the risk of cardiovascular disease and hypertension (47). Yet, studies regarding these two mutations seem to be contradicted in terms of location. As far as we are aware, the relationship between CAD and *eNOS* gene polymorphisms in Lebanon has not been previously established. Our work aims to unravel the insertion/deletion (4a/b) and the T786C *eNOS* polymorphisms. Moreover, it aims to look for an additive effect of both polymorphisms with the coronary artery disease. This is a case-control study that examines the allele frequencies and the genotype distribution of the 4a/b and T786C *eNOS* gene polymorphisms between healthy individuals and CAD patients. Furthermore, it investigates the association between these two polymorphisms and the risk of CAD on Lebanese population (> 35 years). The prevalence of the genotypes and alleles, in our sample, for the 4a/b *eNOS* gene polymorphism is 2.4% (4a/a), 23.6% (4a/b), 74% (4b/b), 14.17% (4a) and 85.83% (4b), similar to results obtained in Turkish, Australian Caucasian and Slovene populations. (35, 48, 49) The occurrence of the genotypes and alleles, in our sample, for the T786C *eNOS* gene polymorphism (CC: 7.4%, TC: 40.4%, TT: 52.2%, C: 27.7% and T: 72.3%) is different than the results of an Australian Caucasian population and in an Italian population (34, 35). Although Table 3 shows no significant results ($p > 0.05$) when examining the impact of the 4a/b polymorphism on CAD, but the Odds Ratio of 4a/b (1.108) shows a greater risk than the homozygous mutant form 4a/a (OR=0.806). Therefore, the heterozygous mutant form displays promising results (for further investigations) regarding the

effect of its presence on CAD in the studied population, especially that the risk of 4a allele (OR=1.033) seems to be slightly greater than the risk of 4b allele (reference). In comparison to literature, a positive link between 4a/b polymorphism and CAD has been demonstrated in Turkey (48-50), while in Germany and Slovenia no correlation between the 4a/b *eNOS* polymorphism and CAD was investigated (28, 49). As for the investigation of the risk of the T786C polymorphism on CAD, no significant results have arisen in Table 3 (p-value > 0.05). However, even though the possible risk of the C allele on CAD is reflected in its OR (1.040) that is slightly bigger than that of the reference T allele (OR=1), it was impossible to calculate the OR of the homozygous mutant CC due to its absence in the control group although 10% of the CADs carried the CC genotype. This implies that the T786C polymorphism might be very risky in its homozygous form and that might need extra examination. Regarding previous studies done in Italy, a positive correlation between T786C polymorphism and CAD have been showed, likewise in Iranian, in African-Brazilians, and in Chinese population. (31-34) While no association was displayed between T786C polymorphism and CAD in Australian-Caucasian population (35). Taking into consideration the additive effect of both polymorphisms (4a/b and T786C) on CAD, the distributions shown in Table 4 show no significant results after calculating the different possible odds ratios (p-value > 0.05). However, it is noticeable that the OR of the existence of 4a allele and the T allele (4a, T) is quite high (1.936). This could be due to the fact that, in our sample, the number of individuals, with both mutations, seems to be low; this issue seems to be a matter of further investigation. In North Lebanese population, as in many geographic and ethnic populations, different mutations, whether in the *eNOS* gene or in other genes, may elevate the risk of CAD. For instance, it has been recently demonstrated that a change in the cholesteryl ester transfer protein (CETP) gene could result in higher levels of CETP protein, which subsequently raises the risk of coronary artery disease (CAD) (51). On the other hand, T786C and 4a/b polymorphisms could simply not be linked to CAD, but it may contribute to the development of other emerging diseases. For example, investigators have noted that T786C is linked to diabetes, kidney diseases, Alzheimer's, pre-eclampsia and migraines (52). Moreover, studies showed that the 4a/b polymorphism may contribute to the development of breast cancer by influencing the expression of the *eNOS* gene, resulting in elevated levels of ROS and NO production, which promote tumor growth through the activation of oncogenes (9). Furthermore, the *eNOS* 4a/b polymorphism is linked to Avascular Necrosis of the Femoral Head (ANFH) through a reduction in *eNOS* activity (53). Similarly, this polymorphism is associated with Hypertension by altering the expression and enzymatic activity of the *eNOS* gene (10). Contradictory studies can occur even within the same country. Our research focuses on the North Lebanese population, which has a mixed-race origin due to its historical diversity and strategic location between Europe and Africa. Thus, results may vary across different regions of Lebanon, moreover resampling might change any biases. Moreover, CAD is not always inherited; it can develop over a person's lifetime. Even with the existence of mutations, it is possible to lower and control the risk of CAD through maintaining a wholesome lifestyle. Further studies are needed on polymorphisms, including factors like physical activity, diet, age, and gender. Recent research shows that nitroglycerin enhances *eNOS* production, essential for vasodilation and CVD treatment. Thus, personalized medicine should investigate how polymorphisms affect drug sensitivity and resistance, linking *eNOS* gene variations to drug efficacy, which is crucial for CAD treatment and underscores the importance of pharmacogenetics.

Abbreviations

ANFH: Avascular Necrosis of the Femoral Head, BMI: Body Mass Index, CAD: Coronary Artery Disease, CETP: Cholesteryl Ester Transfer Protein, cGMP: Cyclic Guanosine Monophosphate, CI: Confidence Interval, CVD: Cardiovascular Disease, DNA: Deoxyribonucleic Acid, eNOS: Endothelial Nitric Oxide Synthase, MI: Myocardial Infarction, mRNA: Messenger Ribonucleic Acid , NO: Nitric Oxide, OR: Odds Ratio, PCR: Polymerase Chain Reaction, RFLP: Restriction Fragment Length Polymorphism, siRNA: Small Interfering Ribonucleic Acid , SMCs: Smooth Muscle Cells, SNP: Single-Nucleotide Polymorphism, VNTR: Variable Number of Tandem Repeats.

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Author Contributions

Azza DIB: She has contributed significantly to the conception, design, execution, and interpretation of the research. More specifically, she has contributed in sample collection, project planning and design, laboratory supervision, result interpretation and paper writing and editing. Hanan HIJAZI: She has contributed significantly to the design, execution, and interpretation of the research. More specifically, she has contributed in the statistical study planning, result interpretation, paper writing and editing. Hana HAMMOUD: She has contributed significantly in sample collection, laboratory work and literature review. Yasmeen OBEID: She has contributed significantly in sample collection, laboratory work and literature review.

Conflict Of Interest

There are no apparent or actual conflicts of interest among the authors regarding this manuscript. Each author has reviewed and endorsed the final version of the paper and consents to its submission.

Ethics Approval

The study protocol was approved by the institutional ethics committee, and all participants gave informed consent in accordance with the committee's requirements. For reference: Professor Ghazi Tadmouri, Head of the Ethical Committee, Faculty of Public Health, Jinan University, Lebanon, acad.dean@jinan.edu.lb

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