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eNOS Gene Variants and Their Genetic Susceptibility Associated with Coronary Heart Disease

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Abstract:

Objective: Gene variations in the gene encoding endothelial nitric oxide synthase (eNOS) may impact the initiation of coronary heart disease (CHD). Insufficient production of nitric oxide (NO) is the most obvious cause of endothelial dysfunction. The aim of this study was to investigate polymorphisms in the eNOS genes G894T and T786C that influence the development of CHD.

Material and Methods: A total of 91 angiographically proven CHD subjects at the Department of Cardiology and Medicine and 91 controls at master health check, in the age group of 30–45 years were evaluated in this cross-sectional study. After overnight fasting blood samples were collected for evaluation of lipid profile by using Auto analyzer AU 480 and NO by Griess reaction, using Enzyme linked Immunosorbent assay. Polymerase Chain Reaction and Restriction Fragment Length Polymerization were used to amplify the eNOS gene, T786C, and G894T, respectively.

Results: A significant decrease in the serum level of NO was observed in CHD subjects compared to controls. In eNOS T786C polymorphism, the distribution of TC genotype (p-value=0.017) odds ratio (OR)=2.1 and minor C allele frequency (p-value=0.001). Additionally, for eNOS G894T polymorphism, the distribution of GT genotype (p-value=0.014) OR=2.03 and minor T allele frequency (p-value=0.001).

Conclusion: This study concludes that polymorphisms of the eNOS genes G894T and T786C could increase the risk of CHD.

Keywords: coronary heart disease, endothelial nitric oxide synthase, gene polymorphism, nitric oxide

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Introduction

Genetic variables are more likely to impact young patients with coronary heart disease (CHD) than older subjects. Some of the variables that contribute to the prevalence of CHD are: atherosclerosis, hypertension, valvular disease, diabetes, smoking, and others. Atherosclerosis in the coronary arteries as well as a family history of the condition are associated with premature CHD.[1] Premature CHD is defined by signs of the disease between the ages of 30 and 56.[2] Premature CHD affects around 10% of all CHD patients.[4] Almost 5.9 million individuals died as a result of early CHD in 2013, and the globe is expected to confront 7.8 million premature CHD deaths by 2025.[3]

Due to the dysfunction in the endothelium, CHD is involved in numerous pathogenic processes, with subsequent development of atheromatous plaques. As a mediator of endothelial function, the endothelial nitric oxide synthase (eNOS) enzyme synthesizes nitric oxide (NO) from L-arginine. Nitric oxide regulates cerebral blood flow and thrombogenesis.[4] NO increases vascular smooth muscle cell growth and relaxation, inhibiting platelet adhesion to endothelium, and preventing platelet adhesion to endothelium; thereby, helping to prevent atherosclerosis.[5] NO inhibits atherogenic low-density lipoprotein (LDL) from becoming oxidized LDL. Several studies have shown that eNOS gene polymorphisms are associated with CHD as a result of the pleiotropic effects of NO.[6]

Numerous mutations in the eNOS encoding gene have been found in recent years. Two identified mutations in the eNOS gene, namely G894T (G-T) dislocation of guanine at nucleotide 894 position with thymine, and T786C (T-C) due to conversion of thymidine to cytosine at nucleotide 786 positioned within the promoter region, which significantly decreases eNOS promoter gene action accompanying with increased exposure to CHD.[7] Addiionally, the latter has been more intensely investigated and it had been found that these two mutations diminish the release of NO, which promotes the development of CHD.[8]

Material and Methods

This observational cross-sectional study was conducted on subjects attending the Cardiology and Medicine unit at SRM Medical College Hospital and Research Centre, Chennai, Tamil Nadu, India; from November 2019 to March 2020. This study included 182 subjects who were in the age group of 30-45 years. A total of 91 subjects were selected as CHD cases; whereas, 91 control subjects were selected as healthy individuals. The institutional ethical committee approved the study protocol (ECN: 1513/IEC/2018).

Inclusion criteria: We selected CHD subjects based on more than 50% stenosis on coronary angiography, chest pain lasting longer than 30 minutes, and elevated Creatine Kinase to peak levels that were above the normal range of at least 2-fold.

The control group was included with no cardiovascular disease (CVD) based on the angiogram report and no electrocardiogram evidence of CHD, smoking, hypertension, or DM.

Exclusion criteria: CVD other than CHD, acute/chronic infection subjects, pregnant women, thyroid, and subjects with cancer were excluded from the study.

After approval from the Institutional Ethical Committee, the consent form was obtained from all CHD subjects and controls. Anthropometric measurements including: height, weight, and BMI were noted.

Measurement of laboratory parameters

A blood sample (5 ml) was collected in a plain vacutainer, under aseptic precautions. A 2 ml sample was taken to measure the lipid profile by using Beckman Coulter Auto Analyzer (AU480). After centrifuging at 5,000

RPM for 10 minutes with the Griess Method, the remaining 3 ml of blood was tested for NO using an ELISA kit. Deoxyribonucleic acid (DNA) was extracted from whole blood and stored at −20 °C after it has been stored in ethylenediamine tetraacetic acid (EDTA) containers for DNA extraction.

Measurement of NO

NO levels were measured by Griess reagent as nitrite/nitrate in CHD subjects and controls. The two-step procedure involves converting nitrate to nitrite first, which allows nitrate reductase to work. In the second step, Griess Reagents are used to transform nitrite into a deep purple azo compound. This deep purple azo compound, known as the azo chromophore, provides a measure of NO concentration. The measurement is taken at 540 nm.

Molecular analysis

DNA isolation

One milliliter peripheral blood samples were obtained for DNA isolation from human whole blood in all CHD subjects and controls, which were analyzed by using the QIAGEN DNA Extraction Kit (Catalogue number: 51104). The isolated DNA was quantified to check for the purity of DNA present in the sample. The integrity of isolated DNA was confirmed by 2% of Agarose Gel Electrophoresis computed by Ultra Violet (UV) Spectroscopy, and then the isolated DNA was stored at −20 °C.

Analysis of T786C and G894T eNOS gene polymorphism

Polymorphism of eNOS T786C and G894T genes were analyzed by PCR (Qiagen⁻ Rotor-Gene Q (Two-Plex) machine), in the Department of Medical Research. Denaturation at 95 °C for 2 minutes (30 cycles), Annealing at 58 °C for 30 seconds, Elongation at 72 °C for 90 seconds, and final elongation at 70 °C for 10 minutes were the thermal cycling conditions.

Primer sequence for eNOS T786C

Forward primer

[5'- GTCTCTCAGCTTCCGTTTCTT-3']

Reverse primer

[5'- CCTTGAGTCTGACATTAGGGTATC-3']

Primer sequence for eNOS G894T:

Forward primer

[5'- GAC CCT GGA GAT GAA GGC AGG AGA -3']

Reverse primer

[5'- ACC TCC AGG ATG TTG TAG CGG TGA -3']

Statistical analysis

The data were statistically analyzed using social science statistical software (SPSS 16). To examine the allele distribution in genotypes, the chi-square test was used to assess the connection between groups for qualitative factors. The odds ratio was used to calculate the hazards of genotypes and alleles. For quantitative variables, Student's t-tests and Pearson correlations were used. The "p-value" of =0.001 was regarded as statistically significant.

Results

A total of 182 participants were enrolled in the study: 91 CHD subjects (84 males and 7 females) and 91 Healthy Control subjects (79 males and 12 females), whose average age was 39.8±2.7. CHD participants were more in the 35-45 year age group compared to control subjects.

The association between the demographics and biochemical parameters of CHD subjects shows a significant difference in CHD subjects compared to controls. Higher BMI, waist circumference, waist-hip ratio, fasting blood sugar, total cholesterol, triglyceride, and LDL-C levels were observed in this present study. Among the two groups, the mean levels of HDL-C did not differ significantly (Table 1). CHD subjects had a significantly lower mean level of serum NO (12.97±1.20) compared to controls (19.08±4.74).

Polymorphisms of eNOS T786C and G894T genes were significantly different between CHD and control subjects (p-value=0.001). We also found a significant difference in TT, TC, and CC genotype distributions and stability of the T/C allele between controls and CHD individuals (Table 2). Table 2 summarizes the results of the polymorphism analysis of eNOS, which showed a significant difference between CHD and control subjects due to the presence of the TC genotype (p-value=0.017), the CC genotype (p-value=0.011), and the C allele (p-value=0.001). The polymorphism of eNOS T786C significantly increased the risk of CHD by 2.15 fold and 2.92 fold, respectively. The risk of CHD was greater in C allele compared to T allele carrying subjects. Polymorphism of the eNOS T786C gene in CHD and control subjects differed statistically (p-value=0.001). All samples were amplified for a specific eNOS T786C gene with a size of 458bp. The TT genotype produced single fragments of 600bp, while the TC genotype produced three fragments of 600bp, 400bp, 250bp, and the CC genotype produced two fragments of 600bp, 400bp (Figure 1).

Distribution of genotype GT, GG, TT, and the G/T allele genotype consistency was associated with the eNOS G894T variant between control and CHD subjects. The risk for the presence of CHD was found in the GT genotype and TT genotype. G894T polymorphism in the eNOS gene had been shown to increase the risk of CHD in T allele subjects compared to G allele carriers (p-value=0.001) (Table 3).

For eNOS G894T gene products were amplified in size of 517bp 458bp and the polymorphism of the eNOS G894T gene showed a significant difference between CHD and control subjects (p-value=0.001). Single fragments of 517bp were observed in the GG genotype, while three fragments of 517bp, 346bp, and 171bp were observed in the GT genotype and two fragments of 346bp and 171bp were found in the TT genotype (Figure 2).

Table 1 Demographics and biochemical characteristics coronary heart disease subjects and healthy controls

Variables	Controls (n=91)	CHD subjects (n=91)	p-value
Mean age	39.81±2.73	40.33±4.52	0.2138
Male sex, n (%)	79 (86.8)	84 (92.3)	-
Female sex, n (%)	12 (13.1)	7 (7.6)	-
Height (cm)	170.57±2.46	170.81±3.44	0.4853
Weight (kg)	65.09±3.26	71.75±5.73	<0.001
BMI (kg/m^2)	21.91±0.37	24.47±1.61	<0.001
WC (cm)	84.77±3.26	90.51±4.45	<0.001
HC (cm)	99.54±3.11	98.8±5.23	0.2475
W/H ratio	0.84±0.02	0.90±0.04	<0.001
Smoking, n (%)	-	49 (57.1)	-
Family history of CHD, n (%)	-	44 (37.6)	-
FBS (mg/dl)	90.24±4.18	98.29±6.98	NS
Total cholesterol (mg/dl)	168.82±16.34	219±41.42	<0.001
Triglyceride (mg/dl)	84.63±30.56	159.74±69.21	<0.001
HDL-C (mg/dl)	46.21±9.05	34.16±7.08	NS
LDL-C (mg/dl)	106.47±12.59	189.4±27.46	<0.001
VLDL (mg/dl)	17.26±8.77	28.06±12.14	<0.001
Nitric oxide ((µMol/L)	19.08±4.74	11.97±1.20	<0.001

CHD=coronary heart disease, BMI=body mass index, WC=waist circumference, WHR=waist-hip ratio, FBS=fasting blood sugar, HDL-C=high density lipoprotein, LDL-C=low density lipoprotein, VLDL=very low density lipoprotein, NO=nitric oxide

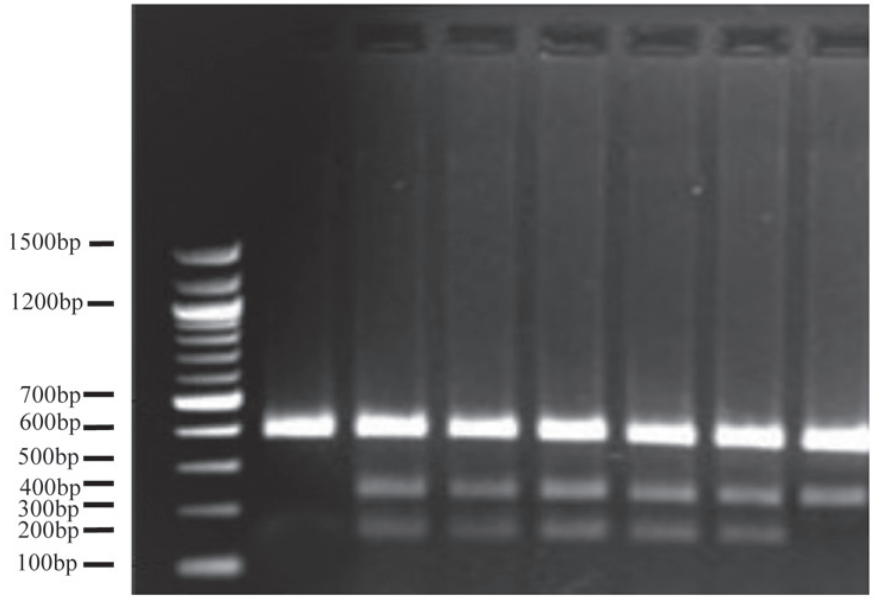
A (p-value=0.001) is regarded as significant

NS-not statistically significant

Table 2 Genotype frequency of eNOS T786C gene polymorphisms between control and CHD subjects

Genotypes	Controls n=91 (%)	CHD subjects n=91 (%)	Relative risk Odds ratio	95% confidence interval	p-value
Heterozygous variant (TC)	34 (37.3)	59 (64.8)	2.47	1.13–3.80	0.017
Homozygous variant (CC)	5 (5.4)	12 (13.1)	2.92	1.34–10.44	0.011
Homozygous wild type (TT)	52 (57.1)	20 (21.9)	–	–	–
Allele frequency C allele	44 (24.1)	83 (45.6)	1.76	1.34–3.27	0.001

CHD=coronary heart disease, TT=wild type, TC=heterozygote, CC=homozygote
p-value=0.001 is regarded as statistically significant



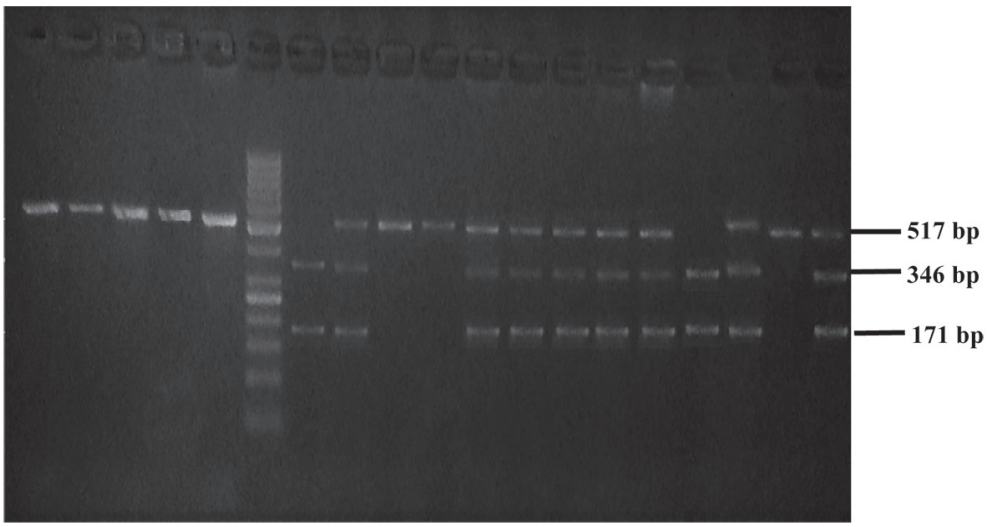
From the RFLP analysis,
Sample 2, 3, 4, 5, 6, shows genotype TC (Heterozygous)
M – 100bp Marker
Banding pattern for TT Genotype – 600bp.
Banding pattern for TC Genotype – 600bp, 400bp, 250bp
Banding pattern for CC Genotype – 600bp, 400bp
PCR-RFLP=polymerase chain reaction-restriction fragment length polymerization, eNOS=endothelial NO synthase

Figure 1 PCR-RFLP report of eNOS T786C gene polymorphism

Table 3 Genotype frequency of eNOS G894T gene polymorphisms between control and CHD subjects

Genotypes	Control n=91 (%)	CHD subjects n=91 (%)	Odds ratio	95% confidence interval	p-value
Heterozygous variant (GT)	30 (32.9)	47 (51.6)	2.03	1.07–3.81	0.014
Homozygous variant (TT)	10 (10.9)	8 (8.7)	0.43	0.23–0.82	0.005
Homozygous wild type (GG)	51 (56.1)	36 (39.5)	–	–	–
Allele frequency					
T allele	50 (27.4)	63 (34.6)	0.86	0.602–1.537	0.001

CHD=coronary heart disease, GG=wild type, GT=heterozygote, TT=homozygote
p-value=0.001 is regarded as statistically significant



Lanes (A, B, C, D, E): 517bp eNOS G-894T PCR product
From the RFLP analysis, the sample 2, 5, 6, 7, 8, 9, 11, and 13 shows GT genotype (Heterozygous)
Lane 1 to 10 Samples, M – 100bp Marker, Product size – 517bp.
Banding pattern for GG Genotype – 517bp
Banding pattern for GT Genotype – 517bp, 346bp, and 171bp
Banding pattern for TT Genotype – 346bp and 171bp
PCR-RFLP=polymerase chain reaction-restriction fragment length polymerization, eNOS=endothelial NO synthase

Figure 2 PCR-RFLP report of eNOS G894T gene polymorphism

Discussion

Growing research suggests that genetic variants may be connected to the occurrence of the illness and its effects. Polymorphisms in eNOS, which produces endothelial NO; an arterial vasodilator influences pathogenesis and propensity to atherosclerosis. Several prospective and case-control studies have shown a correlation between family history and CHD. Although, the criteria of family history and the outcome measure differed between researchers, the results were extremely similar. Prospective studies have indicated that a parent's history of CHD or myocardial infarction (MI) is related to a 1.3-2 fold increase in the risk of CHD or MI.[9] Case-control research revealed that CHD risk is raised in people with a family history; especially, when afflicted relatives are young.[10]

The findings of this study corroborate that family history is a good proxy for genetic vulnerability; these findings are comparable to those of Marenberg et al., who demonstrated that premature CHD is more genetically determined than late-onset variants of the illness.[11] Although, the link between family history and CHD is well recognized, it gives little information regarding the processes through which it contributes to diseased risk.

In the arteries, endothelial-derived NO suppresses endothelial adhesion, increases endothelial vasodilation, and prevents atherogenesis by inhibiting the formation of oxidized LDL. It has been found that endothelial dysfunction is related to atherosclerosis risk factors and that increased reactive oxygen species (ROS), or oxidative stress, contribute to cardiovascular morbidity and mortality; including, hyperlipidemia, hypertension, diabetes, cigarette smoking, etc.[12]

The biochemical basis of the relationship between CHD and numerous NOS3 gene polymorphisms has been investigated. Most investigations concluded that all of these NOS3 gene SNPs Glu298Asp, and T786C impact blood NO concentrations in humans via diverse mechanisms. This imbalance in NO concentrations over time develops in "endothelial dysfunction," which eventually blossoms into an overt form of CHD.

Endothelial dysfunction is a typical finding in cardiovascular disease that arises in response to a variety of cardiovascular risk factors and precedes the development of atherosclerosis. NO, an essential relaxing factor and atheroprotective agent, is a key regulator of vascular endothelial cell activity.[13] NO also suppresses platelet activation, platelet and leukocyte adhesion, adhesion molecule and chemokine production, inflammatory cell infiltration, and smooth muscle cell migration and proliferation, which are all important processes in atherosclerosis.

This study found that serum concentrations of NO are decreased in subjects who had CHD compared with control subjects. Reduced NO availability results in initiation, progression, and complications of atherosclerosis. Lack of NO bioavailability in the coronary and peripheral artery has been regarded as a prospective cardiovascular event.

Lower NO levels in CHD patients suggested vascular inflammation, which leads to lipid oxidation and the production of foam cells in the coronary artery. Some studies reported an increase in NO levels in CHD subjects who receive angiotensin-converting enzyme (ACE) inhibitors that may contribute to an elevation in the level of NO. Additionally, this may be explained as a compensatory mechanism against CHD that causes an increased secretion of NO from the vascular endothelium.[14] So, in subjects receiving ACE inhibitors, the serum NO levels might increase as evidence of improved endothelial function. However, decreased NO levels observed in this study are in response to increased oxidative stress.

NO, catalysed by eNOS, can cause artery dilation in vascular endothelial cells by activating G-protein kinase in vascular smooth muscle cells. The major cause of hyperlipidemia is believed to be a decrease in NO. Long-term presence of LDL in the circulatory system causes it to

oxidize, inhibiting the production of eNOS in hyperlipidemic patients. Hyperlipidemia also reduces NO by generating oxygen-free radicals, because of the complex interplay between eNOS, NO, the cardiovascular system, and lipid metabolism.[15]

In hyperlipidemia, oxidized LDL causes endothelial dysfunction by uncoupling the eNOS, which increases the generation of superoxide anions (O2-). This superoxide interacts spontaneously with NO to generate peroxynitrite anion - (ONOO-), which is extremely reactive and cytotoxic; causing lipid peroxidation and endothelial dysfunction.[16]

eNOS genetic deficit or pharmacological suppression can result in diminished endothelial-dependent vasodilation, leading to an increase in vascular resistance. NO regulates vascular tone as well as suppresses vascular smooth muscle growth, inhibits platelet adherence to the vascular endothelium, and interferes with leukocyte-endothelial cell interaction. Endothelial function is altered due to an imbalance between endothelial vasoprotective factors; such as, NO, endothelial-dependent hyperpolarization, oxidative stress state, and produced vasoconstrictors.[17]

Polymorphism of the eNOS T786C gene genotype frequency was 64.8 percent, 21.9 percent, and 13.1 percent in CHD participants, and 37.3 percent, 57.1 percent, and 5.4 percent in the control group. When compared to the TT genotype, the TC genotype had a 2.4-fold (p-value=0.017) and the CC genotype had a 2.92-fold (p-value=0.011) increased chance of developing CHD.

According to the findings of Nakayama and Wang et al., decreased promoter activity of the eNOS gene was related to the incidence of a point mutation at nucleotide 786 bp in the eNOS gene's 5'-flanking region, which was thought to be implicated in coronary spasm.[18,19] The data in the literature suggests that the mutant C allele of the T786C variation is linked to decreased NO production and an increased risk of CHD illness.[20]

Miyamoto et al. found that binding of replication protein A1 to the -786C nucleotide, but not the -786T wild allele, in the promoter region of the eNOS gene reduced transcription, resulting in a significant reduction in blood NO levels among -786T/C carriers.[21]

In the Spanish population, Alvarez et al. revealed that the -786T/C variation increases the risk of early CHD.[22] Similarly, Zigra et al. found that T786C and G894T polymorphisms are attributed to an elevated risk of MI in those under the age of 35.[23]

An Italian study found that the eNOS T786C polymorphism was related with the severity of CHD.[24] Elakkad et al. found that a T allele at the –786 location of the eNOS gene promotes enzyme gene expression, but a CC genotype at the same region may be a risk factor for CVD, due to eNOS downregulation.[25]

According to Kim et al., the T786C polymorphism is closely connected to CHD and MI.[26] Salimi et al. discovered that SNPs in the promoter region of T786C differed substantially between CHD and control patients. As well, CHD subjects had a significant increase in the C allele being observed in CHD subjects than controls.[27]

In this study, polymorphism of eNOS G-894T genotype frequencies was GG (54.7%), GT (36%), and TT (9.3%) in the CHD group and GG (53.8%), GT (32.9%), and TT (10.9%) in the control group, showing a significant difference.

The evidence in the literature has suggested the T allele to be associated with reduced NO production and increased susceptibility to CHD.[28] In accordance with this study, Rai et al. found a significant difference in frequency distribution between CHD and the control groups for genotypes GG, GT, and TT.[29] According to Syed et al., genotypic distributions in the G894T show significant differences between controls and cases (p-value=0.001).[30]

According to the study conducted by Idrissi et al., the polymorphism of the G894T eNOS gene was significantly correlated with MI risk in Moroccans.[31] Ben Ali et al. assessed both dominant and additive models to determine the relationship between eNOS polymorphism in the G894T gene and CHD in the Tunisian population.[32]

This study's results showed that Polymorphism of the G894T eNOS gene was significantly related to CHD between the genotypes of GT and TT (ORs=1.09 and 0.78) and these results coincided with the findings attained by Angeline et al.[33], in India.

Joshi et al. observed a lack of interaction between the 298Asp eNOS protein (TT, GT genotypes) and caveolin-1, which is required for the eNOS protein's localization and activation in caveolae, inhibits the eNOS enzyme's native endothelium cell function.[34] Several studies have supported our findings. According to Abdel-Aziz et al., the TT genotype of this variation represents an independent risk factor for early CHD.[35] Furthermore, Salas et al. revealed the Glu298Asp polymorphism to be an independent risk factor for early ST-elevation myocardial infarction (STEMI) in Mexican people under the age of 45.[36]

Endothelial dysfunction and atherosclerosis may be associated with alterations in the NO pathway. This current study's findings also revealed a lower amount of NO in CHD participants. This might be due to a higher prevalence of the C allele of T786C and the T allele of G894T in CHD patients compared to controls.

Oxidative stress in CHD may produce decreased NO levels in atherosclerotic arteries. Oxidative stress causes the formation of ROS that scavenge NO, lowering its bioavailability.[37] As a result, poor endothelial function induced by low NO levels in CHD patients might contribute to the etiology of cardiovascular events. Furthermore, this study found that eNOS T786C and G894T polymorphism is correlated to lower NO plasma concentrations in CHD patients, which is consistent with earlier research that found lower NO bioavailability in eNOS gene polymorphism carriers.[38]

The preferential proteolytic cleavage in endothelial cells and vascular tissues may be the mechanism by which the C allele of T786C and T allele of G894T leads in lower NO levels in the body. The resulting cleaved fragments, particularly among carriers of homozygous mutants of this polymorphism, result in reduced enzyme activity and NO biosynthesis.[39]

Conclusion

Genetic polymorphisms in eNOS G894T and T786C may contribute to a genetic predisposition leading to reduce NO bioavailability, and further leading to CHD because of their association with genetic factors; including, oxidative stress, smoking, and hyperlipidemia.

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Conflict of interest

None

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