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The *eNOS* T786C polymorphism is not related to atherosclerosis and cofactors in a Brazilian population

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ABSTRACT. Atherosclerosis is a chronic multifactorial inflammatory disease that evolves in response to aggression of the endothelium, causing plaque formation in large or medium-sized arteries. Various co-factors can accelerate the evolution of this pathology, such as systemic arterial hypertension, dyslipidemia and diabetes mellitus. Given that nitrous oxide is a potent vasodilator and that its synthesis depends on the activity of the endothelial nitric oxide synthase (*eNOS*), we examined a possible association of the *eNOS*-T786C polymorphism (which reduces nitrous oxide synthesis) in atherosclerotic patients who have symptoms of hypertension, dyslipidemia or diabetes. We made a case-control study of two groups of 100 atherosclerotic patients, one with at least one of the above symptoms and the other a control group with atherosclerosis, but with none of the above symptoms. The samples were submitted to DNA extraction, then to PCR and analyzed on agarose gels. We found no significant association of the T786C (*eNOS*) polymorphism with the cofactors hypertension, dyslipidemia or diabetes. In addition, no

association was found between smoking and *eNOS* T786C polymorphism. As for drinking, there was also no association with this polymorphism and or with cofactors.

Key words: *eNOS*; Atherosclerosis; Dyslipidemia; Diabetes; Hypertension

INTRODUCTION

Arterial endothelium dysfunction is an early abnormality during atherogenesis and is an indication of arterial damage since it precedes the formation of atheromatous plaques (Pizzi et al., 2013). Endothelium-dependent vascular response analysis is clinically important for the study of endothelial function. Moreover, physiological and pharmacological stimuli that promote the release of nitric oxide (NO) in the endothelium and other vasoactive compounds directly influence blood pressure (Gorenne et al., 2006).

The endothelium is a complex system, influenced by various chemical mediators. NO stands out as a potent vasodilator with beneficial effects. NO synthesis depends on the activity of the endothelial nitric oxide synthase (*eNOS*) (Zago et al., 2013). The T786C polymorphism promotes the change of a thymine (T) to a cytosine (C) at position 786. The genotypes TT, TC and CC can be prevalent in certain diseases such as atherosclerosis. This single nucleotide polymorphism (SNP) is capable of decreasing the promoter function of the *eNOS* gene by approximately 50%, which highlights the physiological importance of this polymorphism (Zhao et al., 2006).

Low bioavailability and continuous decreases in NO production are associated with events that accelerate the development of atherosclerosis, such as thrombocyte aggregation, vasoconstriction, oxidized low-density lipoprotein (LDL) and migration of monocytes to the vascular wall (Ciftçi et al., 2008). *eNOS* regulates the activated or quiescent endothelium phenotype by playing a central role in endothelial homeostasis. Polymorphisms and biochemical mechanisms are able to alter nuclear chromatin remodeling, phosphorylation of transcription factors, protease activation and gene transcription during the synthesis of the *eNOS* protein and consequently reduce its bioavailability (Gorenne et al., 2006). The study of *eNOS* SNPs helps to understand how these mechanisms may favor the development of atherogenesis and its consequent clinical effects.

There are several risk factors, acquired and genetic, acting together to determine the occurrence of atherosclerosis in more than 50% of the adult population worldwide and with high incidence in many populations around the world (Freitas et al., 2008; Han et al., 2010). Risk factors for atherosclerosis can be modifiable and non-modifiable. The former comprises smoking, physical inactivity, obesity, stress, hyperlipidemia and hypertension, and the latter includes diabetes, familial hypertension, thrombophilia, gender, age and genetic inheritance. Dyslipidemia and arterial hypertension are among the main factors for the development of the atherosclerosis pathology (Locatelli et al., 2009). Systemic arterial hypertension (SAH) is a multifactorial clinical condition characterized by elevated blood pressure levels. SAH is associated with functional and structural changes in organs such as the heart, brain, kidneys and blood vessels, and metabolic changes, which may lead to an increased risk of cardiovascular anomalies (Buckley and Ramji 2015). Regarding dyslipidemia, its occurrence is due to changes in lipid synthesis or catabolism, genetic and environmental factors, inadequate diet or sedentary lifestyle (Sanidas and Grassos 2018). In addition, studies have been trying to find new diagnostic and therapeutic approaches in order to improve the quality of life of patients (Silva et al., 2011).

Hypercholesterolemia also reduces NO bioavailability, leads to a greater production of free radicals through the action of NADPH oxidase and consequently may inactivate NO molecules. A higher concentration of free radicals and reactive oxygen species (ROS) increases oxidation of LDL that could contribute to atheromatous plaque formation. ROS can interfere with nuclear transcription of the enzyme *eNOS*, reducing the intracellular stability of mRNA responsible for the encoding of this enzyme and NO production (Gaggini et al., 2013). Another atherosclerosis risk factor is diabetes. The disease is associated with several cardiovascular risk factors, such as SAH, obesity, insulin resistance, lipid and lipoprotein serum changes, triglyceride elevation, and high-density lipoproteins (HDL) reduction. The association between these risk factors has been termed as metabolic syndrome or dysmetabolic syndrome X, which is attributed to the relationship between hyperglycemia and cardiovascular diseases (Gurgenian et al., 2014). In diabetic patients the manifestation of atherosclerosis may be accelerated, however, the underlying reasons are not yet fully established. The toxic effects of glucose on vasculature and insulin resistance may be related to the early onset of atherosclerosis (Vedantham et al., 2011).

We analyzed the T786C polymorphism of the *eNOS* gene in patients with atherosclerosis associated with cofactors, including diabetes mellitus, systemic arterial hypertension and dyslipidemia.

MATERIAL AND METHODS

Peripheral blood samples were collected from 100 patients with atherosclerosis at Angiogyn clinic in the city of Goiânia. Individuals were 38 years of age or older. The control group consisted of 100 atherosclerotic patients who were non-hypertensive, non-diabetic and had a normal lipidemic profile. Patients were diagnosed with atherosclerotic disease from imaging examinations and clinical criteria. The case group consisted of atherosclerotic patients who had cofactors such as diabetes, hypertension or dyslipidemia. Within the case group, 31 patients had diabetes, 92 had systemic arterial hypertension and 52 had dyslipidemia. All the individuals that participated in this research signed an Informed Consent Form. The research was approved by the Ethics and Research Committee of the Pontifical Catholic University of Goiás (number 35321614.30000.0037).

We performed DNA extraction from blood samples using a Genomic DNA Purification Kaswi kit® (Kaswi, Curitiba, Brazil). DNA quantification was performed using a NanoVue™ Plus spectrophotometer (GE, Cambridge, UK), according to the manufacturer's instructions. The samples were considered adequate when the DNA quantification was higher than 5 ng/μL. The DNA samples obtained were submitted to PCR (Table 1) for the *eNOS* gene (T786C). We used the ZFX/ZFY primers as a internal control. PCR reactions were performed with a final volume of 25 μl according to a published protocol (Silva and Moura, 2016).

Table 1. Analysis of the T786C polymorphism of the *eNOS* gene through ARMS-PCR in blood samples from patients of case and control groups.

ARMS-PCR	C0: 5' TTT CTC CAG CCC CTC AGA TG 3'	387 bp
	2684C: 3' GGC AGA GGC AGG GTC AGA CG 5'	250 bp
T786C	2684T: 5' CAT CAA GCT CTT CCC TGT CT 3'	
	T0: 3' AGG CCC AGC AAG GAT GTA GT 5'	176 bp

The PCR products were subjected to 1.5% agarose gel electrophoresis in 1x Tris-borate EDTA solution (TBE) in a 10 V/cm electric field. The gels were stained with ethidium bromide (5 μg/mL) and visual recording was performed on the BIORAD Photodocumentator (Bio-Rad, California, USA. Statistical analysis of the data was performed using the BioEstat 5.3 software. Results with a P value less than 0.05 were considered relevant.

RESULTS

The genotypic frequencies for the genotypes of the T786C polymorphism of the *eNOS* gene in the group of diabetic atherosclerotic patients did not differ significantly from that in the control group (Table 2).

Table 2. Genotypic frequency of the T786C *eNOS* polymorphism in atherosclerotic patients with (case) or without diabetes (control).

DM/*eNOS*	**TT**	**TC**	**CC**	**TOTAL**	
	n	**n**	**n**	**n**	**P**
Case	3	18	10	31	0.66*
Control	5	62	33	100	

*G Test; DM: diabetes mellitus

We did not find the TT genotype in the group of diabetic atherosclerotic patients with a smoking habit (Table 3). In the comparison with individuals who do not smoke, there was no significant difference in genotype frequencies (Table 3). In the control group smokers and nonsmokers also did not have significantly different frequencies in the TT and CC genotypes (Table 3).

Among the patients in the case group with drinking habits, the frequencies of genotypes also did not differ significantly from those in the group that did not drink (Table 3). The same was true for the control group with and without drinking habits (Table 3).

Table 3. Genotypic frequency of the T786C polymorphism of the *eNOS* gene in the atherosclerotic patients with (case) or without diabetes (control) according to smoking and drinking habits.

DM CASE/*eNOS*	**Smoking**			**Drinking**		
	Yes	**No**		**Yes**	**No**	
	n	**n**	**P**	**n**	**n**	**P**
DM TT	0	3		3	0	
DM TC	2	16	0.53*	17	1	0.37*
DM CC	2	8		8	2	
TOTAL	4	27		28	3	
CONTROL						
TT	0	5		1	4	
TC	12	50	0.27*	12	50	0.87*
CC	4	29		5	28	
TOTAL	16	84		18	82	

*G Test; DM: diabetes mellitus

The genotypic frequency for the T786C polymorphism of the *eNOS* gene in the group of hypertensive atherosclerotic patients did not differ significantly from that in the control group (Table 4). The genotypic frequency for the T786C polymorphism of the *eNOS* gene in the group of hypertensive atherosclerotic patients with smoking habits did not differ significantly from those who did not smoke in this group (Table 5). The same lack of significance was fond in the control group in the comparison of smokers and non-smokers (Table 5).

Table 4. Genotypic frequency of the T786C polymorphism of the *eNOS* gene in atherosclerotic patients with (case) or without hypertension (control).

SAH/*eNOS*	**TT**	**TC**	**CC**	**TOTAL**	
	n	**n**	**n**	**n**	**P**
Case	9	54	29	92	0.44*
Control	5	62	33	100	

*Chi-square test; SAH: Systemic Arterial Hypertension

Among hypertensive atherosclerotic patients with drinking habits, TT, TC and CC frequencies did not differ significantly from those of the group of patients without drinking habits (Table 5). The same was true for control patients with and without drinking habits (Table 5).

Table 5. Genotypic frequency of the T786C polymorphism of the *eNOS* gene in atherosclerotic patients with (case) or without (control) hypertension divided according to smoking and drinking habits.

SAH CASE/*eNOS*	**Smoking**			**Drinking**		
	Yes	**No**		**Yes**	**No**	
	n	**n**	**P**	**n**	**n**	**P**
SAH TT	1	8		3	6	
SAH TC	7	47		3	51	
SAH CC	6	23	0.62*	4	25	0.07*
TOTAL	14	78		10	82	
CONTROL						
TT	0	5		1	4	
TC	12	50	0.27*	12	50	0.87*
CC	4	29		5	28	
TOTAL	16	84		18	82	

*G Test; SAH: Systemic Arterial Hypertension

The genotypic frequencies for the T786C polymorphism of the *eNOS* gene in the group of dyslipidemic and atherosclerotic patients did not differ significantly from those in the control group (Table 6).

Table 6. Genotypic frequency of the T786C polymorphism of the *eNOS* gene in the atherosclerotic patients with (case) or without (control) dyslipidemia.

DISLIPIDEMIA/*eNOS*	**TT**	**TC**	**CC**	**TOTAL**	
	n	**n**	**n**	**n**	**P**
Case	3	34	15	52	0.86*
Control	5	62	33	100	

*G Test

The genotypic frequencies for the T786C polymorphism of the *eNOS* gene in the group of dyslipidemic and atherosclerotic patients with smoking habits did not differ significantly from those of nonsmokers in the same group (Table 7). The same lack of significance was found for the control group in the comparison of genotype frequencies between smokers and non-smokers (Table 7).

Among dyslipidemic and atherosclerotic patients with drinking habits, the TT, TC and CC genotype frequencies did not differ significantly that those of patients who did not drink (Table 7). Patients with drinking habits in the control group also did not significantly different genotype frequencies from those who did not drink (Table 7).

Table 7. Genotypic frequency of the T786C polymorphism of the *eNOS* gene in the atherosclerotic patients with (case) or without (control) dyslipidemia divided according to smoking and drinking habits.

DYSLIPIDEMIA CASE/*eNOS*	**Smoking**			**Drinking**		
	Yes	**No**		**Yes**	**No**	
	n	**n**	**P**	**n**	**n**	**P**
Dyslipidemia TT	1	2		0	3	
Dyslipidemia TC	3	31		1	33	
Dyslipidemia CC	3	12	0.37*	0	15	0.65*
TOTAL	7	45		1	51	
CONTROL						
TT	0	5		1	4	
TC	12	50	0.27*	12	50	0.87*
CC	4	29		5	28	
TOTAL	16	84		18	82	

*G Test

DISCUSSIONS

The presence of a SNP (Single Nucleotide Polymorphism) affects the activation or inhibition of gene transcription and the efficiency of transcript factors. Several mutations in the *eNOS* gene have been described in the promoter region (Biros et al., 2008), but also in introns (Tanaka et al., 2005) and exons (Liu et al., 2018). Usually, a polymorphic allele is considered as a SNP when its frequency is at least 1% in a certain population (Yousefi et al., 2018). As we found in our study, Shoji et al. (2000) and Tsujita et al. (2001), reported no association between polymorphism of position 786 and the comorbidity hypertension, in a Japanese population. Benjafield & Morris (2000) also showed in his study that there was no association of this polymorphism with arterial hypertension.

The T786C polymorphism of the *eNOS* gene was found to be associated with severe coronary artery disease independently of common cardiovascular risk factors in Caucasian patients (Rossi et al., 2003). A meta-analysis involving 69,235 individuals confirmed the association of T786C polymorphism and coronary artery disease in various ethnic populations (Rai et al., 2014).

In our study, we did not find an association between the T786C polymorphism and smoking. Other studies had similar findings and did not found significant associations between smoking, the T786C polymorphism and atherosclerosis (Colombo et al., 2003; Kosior-Jarecka et al., 2016). A positive relationship between the T786C polymorphism and smoking habits has been found (Rossi et al., 2003). The NO produced by endothelial cells

inhibits the oxidation of LDL molecules, prevents platelet aggregation, and plays an important protective role in atherosclerosis. The antioxidant action (which is concentration dependent) is the mechanism by which NO inhibits the formation of the LDL molecule, preventing the formation of superoxide anions (free radicals), and consequent formation of the LDL cholesterol molecule (Moro et al., 1996). Regarding drinking habits, we did not find association between this social habit and the development of atherosclerosis. Recently, this relationship was found to be positive in patients with atherosclerosis and other cardiovascular diseases in other populations studied elsewhere (Kelso-Chichetto et al., 2017).

There is still several controversy involving the association or not of *eNOS* polymorphism with arterial hypertension disease. Hyndman et al. (2002) demonstrated an association characterized as positive between the occurrence of hypertension and the presence of *eNOS* gene polymorphism at position -786. Studies evaluating vascular response have shown that individuals with this polymorphism in question (*eNOS* for position -786) present a decrease in the vasodilator response that depends on the endothelium (ROSSI et al., 2003).

Research conducted on animals has shown that a complete shutdown of the *eNOS* system causes formation of atherosclerotic lesions rich in lipids, severe diet-induced dyslipidemia, and sudden cardiac death in mice in vivo by upregulation of the hepatic LDL receptor (Yatera et al., 2010). In a different evaluation, sedentary, non-smokers, non-diabetic (fasting glycemia <100 mg/dL), non-hormone-treated, postmenopausal and fit to practice exercises were studied by Esposti et al. (2011). These researchers found that the presence of polymorphism of the *eNOS* gene at the -786T> C position caused a reduction in cholesterol levels in response to physical exercise, suggesting a correlation between this polymorphism and lipid alterations, in contrast to what we found here.

The T786C polymorphism of the *eNOS* gene is related to insulin resistance in Japanese non-diabetic patients as in type II diabetic patients (Ohtoshi et al., 2002). The in vivo study with rats in two experimental models of diabetes (induced model and insulin resistant model) found that relief of *eNOS* occurs when the Cav-1 inhibitory clamp is initiated. This closure generates a marked decrease in atherosclerosis. In general, it is well accepted that endothelial dysfunction with its reduced bioavailability of nitric oxide contributes to the formation of early atherogenesis in diabetes. Predominantly, this early formation occurred by the modulation of oxidative stress and leukocyte-endothelial interactions (Sharma et al., 2015).

In diabetics, the decoupling of *eNOS* is so deep in the endothelium that the formation of a superoxide derived from *eNOS* occurs. This causes increased dysfunction and vascular remodeling. Especially in diabetics, it strengthens in this way, the knowledge that the regulation of *eNOS* becomes is critical for protection against atherosclerosis (Bonomini et al., 2008). In our previous work, our research group showed that the presence of multiple risk factors increased the deleterious effects of the C allele regarding the *eNOS* (T786C) gene polymorphism (Barbosa et al., 2017).

CONCLUSIONS

The population under study had a greater frequency of *eNOS* (T786C) heterozygotes (CT), both in the control group (patients with atherosclerosis but without

diabetes, hypertension or dyslipidemia) and in the control group (patients with atherosclerosis and suffering from diabetes, hypertension or dyslipidemia). We did not find significant differences in genotype frequencies for the cofactors in this population. Further studies are needed in order to better understand the complex relationship between this polymorphism, the disease and susceptibility cofactors of atherosclerosis.

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