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Polymorphisms of the genes *eNOS*, *GSTT1* and *GSTM1* are significantly associated with atherosclerotic disease in hypertensive patients

M.P. Moraes[1,2], K.S.F. e Silva[1,3], M.H. Lagares[1,2], A.M. Barbosa[1,2], J.V.M. Martins[1,2], F.L. Campedelli[1,2], I.R. da Costa[1,2], D.A. Rodrigues[1,2] and K.K.V.O. Moura[1,2]

[1] Núcleo de Pesquisas Replicon, Departamento de Biologia, Pontifícia Universidade Católica de Goiás, Goiânia, GO, Brasil
[2] Departamento de Biomedicina, Pontifícia Universidade Católica de Goiás, Goiânia, GO, Brasil
[3] Instituto de Ciências Biológicas, Universidade Federal de Goiás, Goiânia, GO, Brasil

Corresponding author: K.S.F. e Silva
E-mail: smallbinho@hotmail.com

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ABSTRACT. Atherosclerosis is a multifactorial chronic inflammatory disease that occurs in response to endothelial aggression. Systemic arterial hypertension is the main risk factor for the formation of atheromas, increasing the risk of cardiovascular diseases. Several genes are involved in atherogenesis and hypertension. We analyzed polymorphisms of candidate genes that potentially participate in processes related to this pathology, including *G894T* and *T786C* of *eNOS*, as well as *GSTT1* and *GSTM1* in 167 hypertensive patients and 100 controls. Blood samples were from patients attended at the Angiogenesis/Vascular Surgery and Cardiology Department of the Angiogyn clinic in Goiania. There was significant prevalence of the genotype GT (76%) and the mutant allele T (56%) of the T786C (*eNOS)* polymorphism in the patients. For the polymorphism T786C (*eNOS)*, the heterozygote genotype (TC) was found in 58% of the samples; allele C was found in 61%, but there was no significant difference compared to controls. The

GSTT1 genotype was found in 84% and *GSTM1* was found in 73%; for both their predominance was significant. There are many possible explanations for how these polymorphisms affect the development of atherosclerosis and hypertension, but more studies are necessary for their elucidation.

Key words: Atherosclerosis; Hypertension; *eNOS*; *GSTT1; GSTM1*

INTRODUCTION

Atherosclerosis is the clogging of an artery due to fat accumulation (Keele, 1973). It has been found in paleopathological studies of ancient and medieval mummies. The first finding of atherosclerosis confirmed by morphological evidence was made in Egyptian mummies thousands of years old at the beginning of the 20th century (Kim et al., 2015).

Atherosclerosis is characterized by atheroma plaques, which are lesions projecting into the vascular lumen, where they can cause obstruction and other complications, such as peripheral vascular disease, stroke, ischemic heart disease, acute myocardial infarction and sudden death. These complications are among the leading causes of mortality (Murray and Lopez, 2013).

Systemic arterial hypertension, increased LDL, family history of coronary artery disease (CAD), diabetes mellitus, dyslipidemia, obesity, smoking, sedentary lifestyle, alcoholism and history of stroke are the most common risk factors for atherosclerosis. Elderly, postmenopausal women and individuals with any family history of coronary diseases are more likely to develop complications from atherosclerosis. Development of atherosclerosis depends on genetic characteristics and environmental influences; thus it is a multifactorial disease (Lopez et al, 2006).

Systemic arterial hypertension is the main risk factor for the development of cardiovascular diseases. Formation of atheromas can be increased by a decrease in NO due to endothelial dysfunction. Approximately 29% of the causes of death in Brazil are due to CAD and 12.8% of those are caused by systemic arterial hypertension (Carvalho et al., 2001). Blood pressure became a target of genetic studies in the 1980s. Currently, new technologies enable the detection of genetic variants of candidate genes related to the regulation of blood pressure. Blood pressure is an inherited trait and it is suggested that 15-60% can be attributed to genetic factors (Norton et al., 2010). Moreover, the LDL fraction in hypertensive patients is more susceptible to oxidation than LDL in normotensive patients. Oxidized LDL alters endothelial and vascular function; thus hypertension strongly influences atherogenesis. Several polymorphisms of candidate genes such as *eNOS, GSTT1, GSTM1* are related to arterial hypertension and atherogenesis onset (Keidar and Attias, 1997).

Nitric oxide (NO) is produced by the endothelial enzymes nitric oxide synthase (*eNOS*), neuronal (*nNOS*) and inducible (*iNOS*). The *eNOS* enzyme is the main source of NO generated in the vascular system. It is synthesized from L-arginine and has an important role in cardiovascular control, especially for vasodilation (Rush et al., 2005). NO derived from the endothelium influences the relaxation and proliferation of vascular smooth muscle cells, in addition to limiting oxidation of LDL (Channon and Guzik, 2002).

The human *eNOS* gene is located on chromosome 7 (7q35-36), which is composed of 26 exons and 25 introns, with a length of 21 kb; it is constitutively expressed in vascular endothelial cells (Marsden et al., 1993). Altered activity of the *eNOS* gene, due to polymorphisms, may lead to NO deficiency and consequently to hypertension, myocardial infarction, coronary artery disease and heart failure, in addition to being associated with increased risk of cardiovascular complications. Several polymorphisms have been identified for the *eNOS* gene, and G894T and T786C are the most common (Vallance et al., 1989).

GSTs are found in virtually all eukaryotic species (Strange et al., 2001). The most common variants of GSTs are the *GSTM1* and *GSTT1* genes. A homozygous deletion characterizes the null genotypes, which have been associated with loss of enzyme activity and increased susceptibility to cytogenetic damage. These enzymes metabolize xenobiotics, which have a protective effect against endogenous oxidative stress and potential exogenous toxins. (Hayes et al., 2000).

In order to determine whether variations in these genes affect the development of arterial disease, we examined the relationship of atherosclerotic disease in hypertensive patients with polymorphisms of the *eNOS*, *GSTT1* and *GSTM1* genes.

MATERIAL AND METHODS

The research was approved by the National Ethics Commission in Research involving human beings CEP/PUC Goiás (No. 35321614.3.0000.0037). Peripheral blood samples were collected at the Angiogenesis/Vascular Surgery and Cardiology Department of the Angiogyn clinic in the city of Goiânia. The sample consisted of 267 individuals divided into two groups. A control group formed by 100 subjects and a case group formed by 167 subjects. Exceptionally, for the *eNOS* T786C polymorphism there were 165 subjects. The average age of the participants in the case group and control group was 62 and 50 years, respectively. Diagnosis of atherosclerosis was based on clinical examination and confirmed by imaging methods, including echo Doppler, angiotomography and/or digital angiography, angiotomography and/or cineangiocoronariography. The inclusion criterion was subjects who were on medication for hypertension.

In order to investigate *eNOS* (G894T and T786C), *GSTT1* and *GSTM1* gene polymorphisms, we performed PCRs in duplicate with a final volume of 25 μL. The primers are described in Table 1. The amplicons were subjected to agarose gel electrophoresis with EDTA tris-borate solution (TBE) at 1x. The gels were stained with ethidium bromide (5 g/mL) and visualized on a VDS® Video Master Documentation System (Amersham Pharmacia Biotech, USA). The data was analyzed with the Chi-square test the Bioestat software (version 5.0; biocis-tron.blogspot.com).

Table 1. Nucleotide sequence of *eNOS* (G894T and T786C), *GSTT1* and *GSTM1* primers.

G894T (Tajehmiri et al, 2013)	FC: 5'AAGGCAGGAGAGACAGTGGATG 3' R N: 5'TGAAGGAAGAGTTCTGGTGGC 3' RM: 5'TGAAGGAAGAGTTCTGGTGGA 3'	181 bp 171 bp
T786C (Fernandes, 2016)	C0: 5' TTT CTC CAG CCC CTC AGA TG 3' 2684C: 5' GGC AGA GGC AGG GTC AGA CG 3' 2684T: 5' CAT CAA GCT CTT CCC TGT CT 3' T0: 5' AGG CCC AGC AAG GAT GTA GT 3'	387 bp 250 bp 176 bp
GSTT1 (Martins, 2016)	F: 5' TTCCTTACTGGTCCTCACATCTC 3' R: 5' TCACCGGATGGCCAGCA 3'	480 bp
GSTM1 (Rodrigues, 2016)	F: 5' GAACTCCCTGAAAAGCTAAAGC 3' R: 5' GTTGGGCTAAATATACGGTGG 3'	215 bp

RESULTS

Regarding the genotypes of the G894T *eNOS* polymorphism (Figure 1), we found 13% GG homozygotes, 76% GT heterozygotes and 14% TT homozygotes in the case group and 2% GG homozygotes, 85% of GT and 13% TT homozygotes in the control group. The GG genotype was 6.5 times more frequent in the case group (P = 0.03; Table 2). The G894T *eNOS* polymorphism had an allelic frequency of 48% for the wild-type allele (G) and 52% for the T allele in the case group, whereas the G and T alleles frequencies were 44.5% and 55.5%, respectively, in the control group (Table 2).

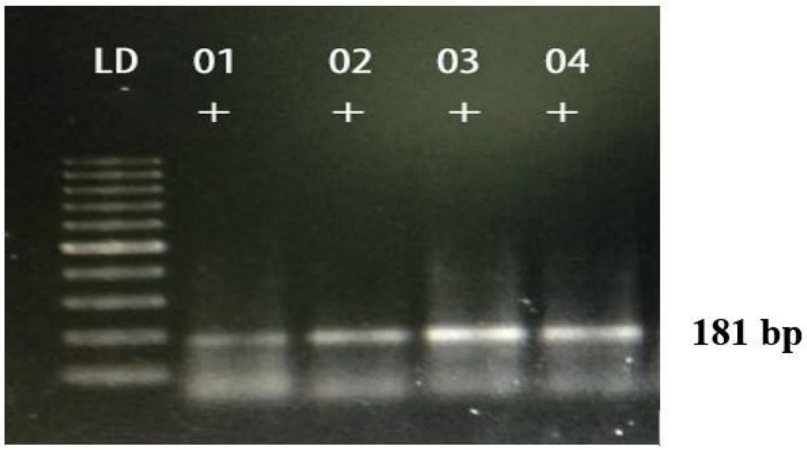

Figure 1. A 2% agarose gel stained with ethidium bromide, indicating the presence or absence of the wild-type (G) allele of the G894T polymorphism of the *eNOS* gene. The ladder (LD) confirms that the amplified fragments consist of 181 bp. Columns 1 through 4 shows the amplification of these fragments.

Table 2. Genotypic and allelic distribution of the G894T *eNOS* polymorphism in case and control groups.

	GG	GT	TT	P*	G	T	P*
	%	%	%		%	%	
Case	12	76	14	0.03	48.0	52.0	0.4
Control	2	85	13		44.5	55.5	

*Chi-square test

The genotype frequency of the T786C *eNOS* polymorphism was 32% for the CC genotype, 58% for the TC heterozygotes and 10% for the TT genotype in the case group. We found 31% individuals with CC genotype, 64% TC and 5% homozygous TT in the control group. Although the p value was greater than 0.05, TT was twice as frequent in the case group (Table 3). The frequency of alleles T and C was 39% and 61%, respectively, in the case group and 32% and 68% in the control group. There was no significant difference between the groups (Table 3).

Table 3. Genotypic and allelic distribution of the T786C *eNOS* polymorphism in case and control groups.

	CC	TC	TT	P*	T	C	P*
	%	%	%		%	%	
Case	32	58	10	0.30	39	61	0.08
Control	31	64	5		32	62	

*Chi-square test

GSTT1 was present in 84% within the case group and 65% within the control group. Thus, *GSTT1* was 1.3 times more frequent in the hypertensive atherosclerotic group than in the control group (P = 0.0003; Table 4). The *GSTM1* gene was detected in 73% of patients and 60% in the control. *GSTM1* was 1.2 times more frequent in the case group than in the control group (P = 0.02; Table 4).

Table 4. Distribution of *GSTT1* and *GSTM1* polymorphisms in the case and control groups (in %).

		Case	Control	P*
GSTT1	Present	84	65	0.0003
	Null	16	35	
GSTM1	Present	73	60	0.02
	Null	27	40	

*Chi-square test

DISCUSSION

The analysis of the G894T of the *eNOS* polymorphism showed more frequent homozygotes (GG) in patients with atherosclerosis and hypertension. The GG homozygote was 6.5 times more frequent in patients than in controls. Lajer et al. (2009), in Denmark, found 64% of atherosclerotic patients with GG in the case group and 57% in the control group; Cai et al. (1998) found 55.6% GG in the case group and 52.11% in the control group.

Most patients presented the heterozygote genotype of the G894T *eNOS* polymorphism. A study associating this same polymorphism with heart failure found a higher frequency of heterozygotes in the case group in Rio de Janeiro (Tardin et al., 2013). Fatini et al. (2005) found a higher frequency of the GT genotype in patients with abdominal aortic aneurysm, an atherosclerosis associated disease, where 74% of the case group consisted of hypertensive patients, similar to what we found.

Some studies related to the G894T *eNOS* polymorphism found a prevalence of homozygous GG in the study population. Saini et al. (2011) investigated the association of endothelial dysfunction and the G894T polymorphic variation in patients with a confirmed history of CAD. According to their results, the GG frequency was higher in both case and control groups compared to the heterozygote GT genotype, but it was higher for the control group (88%).

Hillermann et al. (2005) in South Africa found a low frequency of the TT genotype (2.4%) of the G894T *eNOS* polymorphism. Some studies have reported absence of the TT genotype in their populations (Kato et al., 1999; Moon et al., 2002; Nishevitha et al., 2009; Saini et al., 2011).

In our investigation, analysis of the distribution of the G and T alleles of the G894T *eNOS* polymorphism in the case and control groups showed no significant difference. A study carried out by Hinz et al. (2013) that evaluated the influence of G894T polymorphism in the early clinical evolution of patients submitted to cardiac surgery found no significant difference between case and control groups. Regarding hypertension and G894T polymorphism, Niu et al. (2011) analyzed 19,284 patients in the case group and 26,003 in the control group. They showed that the T allele increased the risk of hypertension by 16% (P = 0.001).

The T786C *eNOS* polymorphism showed a higher frequency of homozygous TT genotype in patients with atherosclerosis and hypertension; it was two times more frequent in patients from the case group than in the control group; however, this difference was not

significant. Kosior-Jarecka et al. (2016) analyzed the T786C *eNOS* polymorphism and found no significant difference. Similar to our results, Colombo et al. (2008) found predominance of the CT genotype of *eNOS* (T786C) in patients with cardiovascular damage and hypertension. Colombo et al. (2003) found a higher prevalence of the TC genotype in two populations with genotype frequencies of 52.5% in the case group and 51.4% in the control group, which is not statistically significant. Similarly, Khurana et al. (2003) found a higher frequency of the TC genotype in an American population, with a frequency of 51% in the control group and 67% in the case group.

In our study the frequencies of the CC genotype of the T786C *eNOS* polymorphism were similar when the case and control groups were compared; Colomba et al. (2008) found similar results. Regarding the T and C allelic distribution of the T786C polymorphism, the C allele was present in 61% of the case group. Colombo (2003), with a similar result, found 51% for the C allele in the case group, while AliReza (2012) detected a frequency of 8% of the C allele in the case group. *In vitro* research has shown that the C allele reduces the activity of the *eNOS* promoter about 50% and consequently decreases the production of nitric oxide (Nakayama et al., 1999).

For the *GSTT1* gene, we found a higher frequency in the case group, suggesting its association with atherosclerosis and hypertension. Moreover, the *GSTT1* present genotype was 1.3 times more frequent in the case group. Bazo et al. (2011), in Brazil, investigated patients undergoing angiography with a diagnosis of CAD and observed prevalence of the *GSTT1* genotype. Another study in Brazil found the *GSTT1* null genotype to be more frequent (Maciel et al., 2009). In India, the *GSTT1* genotype was related to CAD, with 92.34% of the patients with this genotype (Girisha et al., 2004). In Turkey, Turkanoglu et al. (2010) found a higher frequency of the *GSTT1* genotype in ischemic patients and in Serbia, Zivković et al. (2014) found that the *GSTT1* genotype was prevalent among atherosclerotic patients and 88.9% of them had hypertension.

Analysis of the *GSTM1* gene polymorphism showed a significant prevalence of this genotype in control and case groups. The frequency of the *GSTM1* genotype was 1.2 times higher in the case group. The proportion of individuals with the *GSTM1* null genotype in the control group (40%) was similar to frequencies reported in case control studies conducted in Brazil (45.7%; Burim et al., 2004) and in Europe (46.9%; D'alo et al., 2004).

Grignoli et al. (2009) analyzed polymorphism of *GSTM1* and its relation to various pathologies, among them atherosclerosis. They reported that the *GSTM1* genotype was more prevalent (57.0%) than the *GSTM1* null genotype (43.0%). Maciel et al. (2009) analyzed 1577 individuals from the general population and found a higher prevalence of the *GSTM1* genotype. Taspinar et al. (2012) detected *GSTM1* in 58.2% of the case group and 53.5% of the control group. Wilson et al. (2000) found a higher frequency of the *GSTM1* genotype in the case group (52.0%), and a lower prevalence of this genotype in the control group (42.8%).

Several research groups have found different results regarding polymorphisms of *GSTM1* and *GSTT1* and their relation to atherosclerosis and other vascular diseases. Hussain et al. (2012) concluded that the *GSTT1* and *GSTM1* deletion may be considered a risk factor for hypertension. Wang et al. (2012), in China, concluded that the null polymorphism of *GSTT1* and *GSTM1* can interact synergistically with hypertension, diabetes mellitus and smoking and increases the risk of ischemic stroke. Türkanoğlu et al.

(2010) showed that GSTT1 null and GSTM1 null genotypes play a significant role in hypertension and in the pathogenesis of ischemic stroke.

The discrepancy in results may be due to differences in the ethnic composition of the Brazilian population (Arruda et al., 1998) and also differences in the exclusion and inclusion criteria of the study groups that can be related to other vascular pathologies. Further studies will help to clarify the association of these polymorphisms with atherosclerosis and hypertension.

Atherosclerosis and systemic arterial hypertension are multifactorial pathologies and consequently a challenge for molecular genetics. Here we showed that the distribution of the G894T *eNOS* polymorphism in the case and control groups differed significantly; the G allele was 6.5 times more frequent in patients. The GG patients could be more susceptible to atherosclerotic disease and systemic arterial hypertension. However, the genotypic frequency of the *eNOS* T786C polymorphism in the case and control groups did not differ significantly. The *GSTT1* gene was 1.3 times more frequent in the hypertensive atherosclerotic participants than in the control group. The frequency of of the *GSTM1* present genotype in the hypertensive atherosclerotic patients was 1.2 times higher when compared to the control group.

CONFLICTS OF INTERESTS

The authors state that they have no conflict of interest regarding this research.

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