Atherosclerosis: analysis of the eNOS (T786C) gene polymorphism

A.M. Barbosa1,2, K.S.F. Silva1, M.H. Lagares1,2, D.A. Rodrigues1,2, I.R. da Costa1,2, M.P. Morais1,2, J.V.M. Martins1,2, R.S. Mascarenhas1, F.L. Campedelli1,2 and K.K.V.O. Moura1,2

1Departamento de Biologia, Núcleo de Pesquisas Replicon, Pontifícia Universidade Católica de Goiás, Goiânia, GO, Brasil
2Departamento de Biomedicina, Pontifícia Universidade Católica de Goiás, Goiânia, GO, Brasil

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

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ABSTRACT. The coronary arteriosclerotic disease is the most common cardiovascular disease. Atherosclerosis affects large- and medium-sized arteries leading to severe thrombosis or artery stenosis that could evolve to myocardial infarction, ischemic stroke, ischemic injury of kidneys and intestines, and several other life-threatening clinical manifestations. Nitric oxide has been shown to be a promising therapeutic agent against cardiovascular diseases. The eNOS gene assumes several important functions, including regulation of vascular tone and regional blood flow, the suppression of vascular smooth muscle cell proliferation, and modulation of leukocyte-endothelium interactions. The T786C polymorphism is an important point mutation, where thymine is changed to cytosine. T786C significantly reduces the activity of the eNOS promoter gene. Two hundred and ninety-seven peripheral blood samples were collected from patients with the previous diagnosis of atherosclerotic disease based on clinical examination and
confirmed by imaging methods. Results were compared using the chi-square test and the G-test. In the present study, the TC genotype was more frequent in both case and control groups with no statistically significant difference. Comparing the relation TC/TT and CC genotypes in the case and control groups, there was no statistically significant difference. No significant difference was found when genotypes were analyzed regarding gender and smoking. Our results suggest a strong tendency of the T allele, in single or double dose, to be associated with atherosclerosis that was not confirmed by the scientific data.

**Key words:** Atherosclerotic lesion; eNOS; Polymorphism; Genotype

**INTRODUCTION**

Coronary arteriosclerotic disease (CAD) is the most common cardiovascular disease. CAD is a multifactorial disorder with complex etiology and influenced by genetic and environmental components (Nanni et al., 2006).

The increased incidence of cardiovascular diseases is likely the result of a high prevalence of non-traditional risk factors such as inflammation, oxidative stress, infectious agents, and traditional risk factors such as hypertension, dyslipidemia (Balagopal et al., 2011), smoking, and increased glycemia that contribute to the disorder chronicity or super stimulation of the vascular endothelium. These factors together, synergistically stimulate the production of inflammatory cytokines, lipid peroxidation, and oxidative stress, which increase the adhesive surface of the endothelium and decrease the production of relaxing factors and anticoagulants derived from endothelium (Féletou and Vanhoutte, 2006).

Atherosclerosis affects large- and medium-sized arteries leading to severe thrombosis or artery stenosis that could evolve to myocardial infarction, ischemic stroke, ischemic injury of kidneys and intestines, and several other life-threatening clinical manifestations.

Before it becomes clinically evident, the atherosclerotic process progresses silently for years. The progression of atherosclerosis is characterized by the development of plaques within the arteries, leading to a reduced supply of oxygen-rich blood to the organs and other parts of the body (Puddu et al., 1995). The evolution of CAD, from an initial lesion to a plaque rupture, is due to many cellular and molecular events that lead to an inflammatory stage (Robert, 2005). Recent advances have established a crucial role for inflammation in all phases of atherosclerosis, including initiation, progression, and complicated advanced injury since inflammation has emerged as an important driving force in the initiation and progression of atherosclerotic lesion formation (Libby, 2013).

Common predisposing conditions for atherosclerosis such as hypercholesterolemia, hypertension, diabetes, and smoking are associated with endothelial dysfunction, leading to a proinflammatory and prothrombotic endothelial phenotype (Konsola et al., 2016). Advanced understanding of the pathobiology of atherosclerosis suggests that such alterations of endothelial function may play a key role in the development and progression of atherosclerosis and its clinical complications (Landmesser et al., 2004). Endothelial dysfunction is faced as an early marker for the initiation and progression of atherosclerosis and a predictor of future cardiovascular events (Giannotti and Landmesser, 2007). Moreover, atherosclerosis is associated with a great change in the endothelial phenotype, and the assessment of endothelium-
dependent vasodilator function of the peripheral arteries has emerged as an accessible indicator of endothelial health (Charakida et al., 2010).

Endothelial dysfunction is an early and important event that initiates atherogenesis, and this is mainly mediated through the impaired regulation of endothelial nitric oxide synthase (eNOS) with a decrease in vasoprotective nitric oxide (NO) and increased production of reactive oxygen species that promote vascular injury (Gorene et al., 2006).

Cardiovascular diseases such as hypertension, atherosclerosis, and diabetes mellitus are associated with decreased NO bioactivity due to a reduced NO production by eNOS or an increase in NO inactivation after the reaction with superoxide (Li and Förstermann, 2009; Konsola et al., 2016). eNOS and NO derivatives have several important functions, including regulation of vascular tone and regional blood flow, suppression of vascular smooth muscle cell proliferation, and modulation of leukocyte-endothelium interactions (Davignon and Ganz, 2004). NO has been shown to be a promising therapeutic agent. It is synthesized from L-arginine by NOS, which has three isoforms: neuronal, inducible, and eNOS. eNOS is regulated by estrogen and can be altered by drugs, including cigarettes, and by several diseases, such as hypercholesterolemia, diabetes, and hypertension (Li et al., 2002). eNOS is a potent oxidant produced by endothelial cells and macrophages that exert protective and atherogenic effects. The NO produced by eNOS has vasodilator functions and besides being potentially atheroprotective (Li and Förstermann, 2009).

Genetic polymorphisms of eNOS were shown to have a significant effect on NO levels in plasma lipids and were associated with diabetes mellitus (Monti et al., 2003), cardiac insufficiency (McNamara et al., 2003), coronary spasm (Lüscher and Noll, 1999), atherosclerosis (Paradossi et al., 2004), myocardial infarction, hypertension (Yoshimura et al., 2003), and intra-stent coronary restenosis (Gomma et al., 2002).

Due to pleiotropic effects of NO, several studies have investigated the link between eNOS polymorphisms and the development of coronary events. Among the many polymorphisms reported regarding the eNOS gene, two polymorphisms, namely Glu298Asp (G:T) located within exon 7 and T-786C within the promoter, have received much interest in the possible association between such polymorphisms and CAD (Zeng et al., 2017), and the latter has been more intensely investigated (Miyamoto et al., 2000; Nakayama et al., 2000). It involves a cytosine (C) substitution of the thymine nucleotide (T) at the 786 locus of the eNOS gene and is associated with increased susceptibility to coronary vasospasm in homozygotes (C/C) and heterozygotes (T/C), that is individuals expressing the mutant allele (C allele) (Lüscher and Noll, 1999; Nakayama et al., 1999).

Health care professionals should not only treat clinically atherosclerotic cardiovascular disease manifestations but also identify targets for prevention and early treatment in apparently healthy individuals. Both tasks require an understanding of the pathogenesis of atherosclerosis. In the present study, we analyzed the T786C polymorphism of the eNOS gene in a group of individuals diagnosed with atherosclerosis and in a control group.

MATERIAL AND METHODS

The research was approved by the National Ethics Committee in Research/ National Information System on Ethics in Research Involving Human Beings CEP/PUC Goiás (Number: 35321614.3.0000.0037). All patients signed the informed consent term and agreed to participate in the research. Inclusion criteria were patients over 38 years of age, diagnosed
with atherosclerosis under medical treatment and/or submitted to interventional vascular procedures and who agree to respond to the questionnaire and signed the free and informed consent form. Exclusion criteria were patients under 38 years of age and who did not agree to participate in the study. For the control group, inclusion criteria were age over 38 years who did not present a diagnosis of atherosclerotic disease based on clinical criteria and/or imaging tests. Exclusion criteria were patients under 38 years of age who did not agree to participate in the study.

The SNP ID numbers and sequence information used in the present study are available publicly (http://www.ncbi.nlm.nih.gov/SNP/). We selected for the present study the SNP T-786C (rs 2070744).

We collected peripheral blood samples from 197 patients with the previous diagnosis of atherosclerotic disease confirmed by image methods. We also collected 100 blood samples from patients, based on clinical manifestations and noninvasive imaging methods, to compose the control group. The samples were from patients of the Cardiology and Peripheral Vascular Surgery Department of the Angiogyn Clinic in the city of Goiânia in the period from October 2014 to February 2015. The peripheral blood samples were collected and subjected to molecular tests to detect the eNOS (T786C) gene polymorphism.

Regarding smoking, the case and control groups were arranged into three groups: current smokers, non-smokers, and ex-smokers. According to the guidelines of the Brazilian Medical Association in 2013, a smoker refers to a person who makes regular use of at least one of the products of tobacco regardless the period the product is consumed; ex-smoker refers to the individuals who in the past have made use of tobacco products, do not smoke currently, and has stopped smoking for a period greater than or equal to 15 years.

DNA extraction was performed according to Kaswi® (Genomic DNA Purification Kit) instructions. After extraction, the samples were quantified in the NanoVue™ Plus Spectrophotometer (GE, Cambridge, UK) according to the manufacturer’s instructions, with relevance only to samples whose quantification results concerning DNA concentration were higher than 5 ng/μL. The 260/280 nm ratio was used to estimate the purity of the DNA samples. DNA was stored at -20°C until amplification by polymerase chain reaction (PCR) method.

Samples were subjected to PCR amplification to detect the polymorphism of the eNOS gene (T786C). The final volume of the reaction was 25 μL according to the protocol proposed by Frare et al. (2013). All analyses were performed in duplicate. The resultant fragments were subjected to 1.5% agarose gel electrophoresis in 1x EDTA Tris-borate solution in an electric field of 10 V/cm. The gels were stained with ethidium bromide (5 μg/mL) and then visualized on the Bio-Rad Photo Documentation (Bio-Rad, Hercules, CA, USA).

For genotyping of the eNOS (T786C) gene, we performed ARMS-PCR (amplification refractory mutation system), which is referred to as the allele-specific oligonucleotide of PCR and is a technique originally designated by Newton et al. (1989) for the detection of known sequence polymorphisms. According to this technique, two primer pairs in a single-PCR tube can simultaneously amplify both mutant and normal alleles as well as allow the amplification of an internal DNA control (Wang et al., 2014).

The results of the eNOS (T786C) gene polymorphism were organized into Excel spreadsheets, composing a database. We performed the statistical analysis based on the G-test and the chi-square (χ²) test to analyze the relationship between polymorphism and atherosclerotic disease. The value of P < 0.05 was considered statistically significant. The statistical tests were carried out using BioEstat® 5.0 (Ayres et al., 2007).
RESULTS

The genotype frequencies found in the eNOS (T786C) polymorphism in the case group were 10.15% (20/197) for the homozygous TT genotype, 59.39% (117/197) for the heterozygous TC genotype, and 30.46% (60/197) for CC. In the control group, the genotype frequencies were 5.00% (5/100) TT, 64.00% (64/100) TC, and 31.00% (31/100) CC. There was a greater prevalence of the TC allele in both case and control groups. There was no statistical difference between the case and control groups regarding the genotypic distribution (P = 0.3120) (Table 1).

<table>
<thead>
<tr>
<th>Case</th>
<th>TT (N %)</th>
<th>TC (N %)</th>
<th>CC (N %)</th>
<th>Total (N %)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20</td>
<td>117</td>
<td>60</td>
<td>197</td>
<td>0.3120</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>64</td>
<td>31</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

*Chi-square test.

When the TC and TT genotypes were analyzed simultaneously, a frequency of 30.46% (60/197) was found for the CC genotype and 69.54% (137/197) for the TC/TT in the case group. In the control group, the frequencies were 31.00% (31/100) CC and 69.00% (69/100) TC/TT. There was no significant difference (P = 0.9236) (Table 2).

<table>
<thead>
<tr>
<th>Case</th>
<th>CC (N %)</th>
<th>TC/TT (N %)</th>
<th>Total (N %)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60</td>
<td>137</td>
<td>197</td>
<td>0.9236</td>
</tr>
<tr>
<td>Control</td>
<td>31</td>
<td>69</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

*Chi-square test.

For the male patients in the case group, 7.86% (7/89) presented the TT genotype, 58.43% (52/89) the TC genotype, and 33.71% (30/89) the CC genotype. In the control group, 3.78% (2/53) presented the TT genotype, 67.92% (36/53) the TC genotype, and 28.30% (15/53) the CC genotype. The difference between the genotypes of the eNOS gene (T786C) of both groups was not statistically significant (P = 0.4196) (Table 3).

For the female patients in the case group, the frequency of TT, TC, and CC genotypes were 12.04% (13/108), 60.18% (65/108), and 27.78% (30/108), respectively. Among the female patients in the control group, 6.38% (3/47) had the TT genotype, 59.58% (28/47) had the TC genotype, and 34.04% (16/47) had the CC genotype. This difference was not statistically significant (P = 0.4642) (Table 3).

About the distribution of the gene polymorphism regarding the gender of the patients, in the control group of both genders, the presence of the wild-type homozygous (TT) genotype was lower, being 3.78% in men and 6.38% in women.

Regarding the smoking habit, 94 patients (32.30%) declared to be smokers, 145 patients (49.83%) were non-smokers, and 52 patients (17.87%) declared to be ex-smokers, totaling 291 patients. The inconsistency in the total number or patients is because a patient in the case group (CC) did not state whether he smokes or not; 2 patients in the case group with the TC genotype and 1 with the CC genotype declared to be ex-smokers but did not state how long they had stopped smoking, and 2 patients in the control group (TC and CC) declared themselves to be ex-smokers but did not say how long they had stopped smoking.

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Analyzing the eNOS (T786C) polymorphism of the case group, in the individuals who stated themselves as smokers, 11.59% (8/69) of patients had the TT genotype, 59.42% (41/69) the TC genotype, and 28.99% (20/69) had CC. From the 83 atherosclerotic patients who stated to be non-smokers, 12.05% (10/83) had the TT genotype, 62.65% (52/83) had the TC genotype, and 25.30% (21/83) had the CC genotype. Among ex-smokers patients, that is, those who stopped smoking in the period of 15 years or more, we observed that the frequency of the TT genotype was 4.88% (2/41), TC genotype was 53.66% (22/41), and CC was 41.46% (17/41). This difference was not statistically significant (P = 0.3487) (Table 4).

For the control group, 64.00% (16/25) of patients with a smoking habit presented the TC genotype and 36.00% (9/25), the CC genotype. No individual with the TT genotype declared to be a smoker. Regarding the patients who never smoked, 8.06% (5/62) presented the TT genotype, 64.52% (40/62) the TC genotype, and 27.42% (17/62) the CC genotype. Among ex-smokers, the TC genotype frequency was 63.64% (7/11), and the CC genotype frequency was 36.36% (4/11). No individual with the TT genotype was ex-smoker. There was no statistically significant difference (P = 0.2742) (Table 4).

### Table 3. Distribution of the eNOS (T786C) gene polymorphism concerning gender in the case and control groups.

<table>
<thead>
<tr>
<th>Gender</th>
<th>TT (N %)</th>
<th>TC (N %)</th>
<th>CC (N %)</th>
<th>Total</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case</td>
<td>7</td>
<td>52</td>
<td>30</td>
<td>89</td>
<td>0.4196</td>
</tr>
<tr>
<td>Control</td>
<td>2</td>
<td>36</td>
<td>15</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case</td>
<td>13</td>
<td>65</td>
<td>30</td>
<td>108</td>
<td>0.4642</td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>28</td>
<td>16</td>
<td>47</td>
<td></td>
</tr>
</tbody>
</table>

*G-test.

### Table 4. Association of smoking with the eNOS gene polymorphism (T786C) in the case and control groups (≥15 years).

<table>
<thead>
<tr>
<th>Groups</th>
<th>TT (N %)</th>
<th>TC (N %)</th>
<th>CC (N %)</th>
<th>Total</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smokers</td>
<td>8</td>
<td>41</td>
<td>20</td>
<td>69</td>
<td>0.3487</td>
</tr>
<tr>
<td>Non-smokers</td>
<td>10</td>
<td>52</td>
<td>21</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>Ex-smokers</td>
<td>2</td>
<td>22</td>
<td>17</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smokers</td>
<td>0</td>
<td>16</td>
<td>9</td>
<td>25</td>
<td>0.2742</td>
</tr>
<tr>
<td>Non-smokers</td>
<td>5</td>
<td>40</td>
<td>17</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>Ex-smokers</td>
<td>0</td>
<td>7</td>
<td>4</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

*G-test.

**DISCUSSION**

In the present study, we found a 2.3-fold higher prevalence of TC/TT genotypes regarding the wild genotype (CC), both for the case and control groups. This result is similar to that of Piccoli et al. (2012), where they analyzed a South-Brazilian population and found a greater prevalence of the TC genotype in patients with acute coronary syndrome (ACS) and controls. Ragia et al. (2010) found that the TC genotype (55.2% in the case group and 54.8% in control) had a higher prevalence concerning the other genotypes and they also did not find a statistically significant difference for patients who underwent myocardial revascularization surgery. A study conducted by Ghilardi et al. (2002) in Italian patients found that the genotypic distribution in the controls was 54 TT (41%), 61 CT (46%), and 18 CC (13%), corroborating the studies in the present research.

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However, the study conducted by the Florence Nightingale Hospital Group (Istanbul, Turkey) on the \textit{T-786C} polymorphism of the \textit{eNOS} gene found that the \textit{CC} genotype frequency was the most prevalent in the ACS group compared to the CAD and control groups. TT was the genotype most frequently observed in both patients with CAD (Ciftçi et al., 2008). Similarly, Dosenko et al. (2005) studying a group of patients with ACS in the Ukrainian population, found the C polymorphism of the 5'-flanking region of the \textit{eNOS} gene 2.7-fold more frequently in patients with ACS than in controls. The authors suggested that this allelic polymorphism may be considered as one of the genetic risk factors for the development of ACS. Similarly, Gluba et al. (2009) found that in the young Polish population, the \textit{T786C} polymorphism does not increase the risk of myocardial infarction. Jaramillo et al. (2010), studying the \textit{T786C} polymorphism in Chilean patients with a diagnosis of CAD found a higher frequency of the TT genotype and the distribution of that genotype was not significantly different between the individuals with CAD and control.

We found a higher prevalence of the TC genotype in the polymorphism of the \textit{eNOS} gene in both genders. Agema et al. (2004) conducted a study on male patients diagnosed with CAD that corroborates our results. A higher prevalence of the TC genotype was observed both in the case and control groups, but the result was not statistically significant. Alp et al. (2009), in Turkey, reported a higher prevalence of the TT genotype in male patients with CAD (50.6%) contradicting the present study.

According to our results on the smoking habit, a higher prevalence of the TC genotype was found, but the results were not statistically significant. Nasreen et al. (2002) evaluated genotypic frequencies in patients with a smoking habit and did not find significant differences between the groups. It is well known that smoking induces oxidative stress, which is a potent suppressor of \textit{eNOS} activity (Ota et al., 1997), and alternatively, such oxidative stress may promote the degradation of NO (Kitiyakara and Wilcox, 1998). Nakayama et al. (1999) showed that the risk of coronary spasm in patients with C-786 allele was higher in smokers than in non-smokers.

Agema et al. (2004) analyzed patients with a history of myocardial infarction and the relation with smoking. Smokers and non-smokers showed a higher prevalence of the TT genotype, but the results were not statistically significant. Alp et al. (2009) also found a higher prevalence of the TT genotype (54.8%) in patients who have the habit of smoking; non-smoking patients presented the TC + CC genotype with higher prevalence. These results contradict the present study.

Thus, it is likely that in a condition with increased oxidative stress, such as arterial hypertension, the genetic predisposition to generate less NO may become apparent and, in the long run, may be detrimental, resulting in a susceptibility to atherogenesis. Rossi et al. (2003) found that the combination of at least one major cardiovascular risk factor increased the risk associated with the C allele.

In conclusion, the presence of multiple risk factors increased the deleterious effects of the C allele. Moreover, most conditions involving oxidative stress, such as aging, smoking, hypercholesterolemia, male gender (lack of estrogen), and overweight or obesity lead to a genetic predisposition that generates less NO, which is associated with the C allele, contribute to decreasing NO bioavailability, and finally, the onset of coronary atherogenesis.

**Conflicts of interest**

The authors declare no conflict of interest.
ACKNOWLEDGMENTS

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