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# *GSTM1* polymorphism in patients with clinical manifestations of atherosclerosis

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**ABSTRACT.** Atherosclerosis is characterized by lesions, called atheroma or atheromatous plaques, in the inner layer of blood vessels, which block the vascular lumen and weaken the underlying tunica media. Several modifiable and non-modifiable risk factors for the development of atherosclerosis exist. The modifiable risk factors include hypertension, smoking, obesity, high LDL and low HDL cholesterol levels, sedentary lifestyle, and stress; the non-modifiable factors include diabetes mellitus, family history of hypertension and heart disease, thrombophilia, sex, age, and genetic factors. The association of polymorphisms in *GST* with coronary artery disease has been studied since the polymorphisms can affect enzyme activity and

contribute to the onset of atherosclerosis. We analyzed polymorphisms in *GSTM1* in individuals diagnosed with atherosclerosis as well as in healthy individuals (control group). The frequency of the *GSTM1* present genotype in the atherosclerosis group was 1.2 times higher than that observed in the control group. We found no sex- or alcohol-consumption-dependent differences between the occurrences of the present and null genotypes. However, the *GSTM1* present genotype occurred in 52.6% individuals with atherosclerosis who reported smoking 20 or more cigarettes per day and in 60% individuals who smoked 10 to 20 cigarettes per day (P = 0.0035). In addition, the *GSTM1* present genotype was more frequent in individuals who reported being former smokers - 45.5% in individuals with atherosclerosis who smoked for more than 20 years and 50% each for individuals in the control group who smoked for less than 10 years or for 10 to 20 years, respectively (P = 0.0240).

**Key words:** Atherosclerosis; Polymorphism; *GSTM1*; PCR

## INTRODUCTION

Ancient reports documenting the history and pathogenesis of atherosclerosis exist. Atherosclerosis has been described in artifacts pertaining to Egyptian mummies, and the first clinical symptom of angina pectoris was reported by Hippocrates (460-370 B.C.) (Favarato and Luz, 2003; Kumar et al., 2005; Martelli, 2014).

In 1904, the pathologist Felix Marchand used the term atherosclerosis to describe medium and large lesions of arteries with deposits of cholesterol- and lipoid matter-containing yellow plaques in the intima. However, it was Herrick who made the first correlation of pain syndrome with atherosclerosis in a pathological study in 1912 (Kumar et al., 2005; Gottlieb et al., 2005).

Eradication of infectious diseases and changes in lifestyle have converted atherosclerosis into a significant health problem, and a greater understanding of the disease and related cardiovascular disorders is essential (Kumar et al., 2005; Martelli, 2014).

According to the Brazilian guidelines on dyslipidemia and prevention of atherosclerosis in 2013, cardiovascular diseases comprise an important cause of death in developed and underdeveloped countries. Studies have shown that the atherosclerotic process is present from intrauterine life to death and may be a cause or merely an adjuvant (Higuchi et al., 2002; Pellanda, 2014).

The word atherosclerosis comes from the Greek words “athero”, meaning gruel or paste, and “sclerosis”, meaning hardness. The disease is characterized by lesions in the inner layer of blood vessels, called atheroma or atheromatous plaques, which block the vascular lumen and weaken the underlying tunica media (Gottlieb et al., 2005; Martelli, 2014).

The deposition of plaques in the arterial wall is the decisive factor for the onset of atherogenesis. Although any artery can be affected, the deposition of plaques mainly affects the aorta, and the coronary and cerebral arteries. However, the main consequences are myocardial infarction, cerebral ischemia, and aortic aneurysm (Kumar et al., 2005; Gottlieb et al., 2005; Martelli, 2014).

Dyslipidemia increases the risk of coronary artery disease, which is closely linked to

the concentration of lipoproteins in the bloodstream (Correia and Leal, 2010; Cymbron, 2011).

The risk factors for the development of atherosclerosis can be modifiable or non-modifiable. The former includes hypertension, smoking, obesity, high levels of low density lipoprotein (LDL) and low levels of high density lipoprotein (HDL) cholesterol, sedentary lifestyle, and stress, whereas the latter includes diabetes mellitus, family history of hypertension, thrombophilia, sex, age, genetic factors, and premature family history of heart disease (Santos et al., 2008; Martelli, 2014).

Smoking is among the most important risk factors for the development of atherosclerosis. Besides being a determinant for low birth-weight, placental abruption, and pulmonary diseases, smoking decreases concentration of HDL in the blood, leading to significant endothelial dysfunction. It is also associated with prevalence of advanced atherosclerotic lesions, as has been observed in the abdominal aorta of autopsied young individuals (Rabelo, 2001; Santos et al., 2008).

Genetic traits associated with atherosclerosis have been investigated since ancient times. Interestingly, the methodologies followed and the interpretation of results of these ancient studies connected certain characteristics of the disease to its pathogenesis (Mansur, 2000).

Genetic factors such as polymorphisms may also contribute to the pathogenesis of atherosclerosis. To understand the role of polymorphism in the development of atherosclerosis, polymorphisms in multiple genes encoding proteins involved in various molecular etiopathogenesis of cardiovascular diseases (CVD) have to be analyzed (Bourbon, 2008).

The association of polymorphisms in the glutathione S-transferase (*GST*) gene with coronary artery disease (CAD) has been the focus of investigation since polymorphisms in *GST* may affect enzyme activity and contribute to the onset of atherosclerosis (Girisha et al., 2004).

The metabolism and detoxification of xenobiotics related to inheritance of polymorphisms of genes, either with life or cell death, have a relevant role regarding susceptibility to diseases (Koch et al., 2010; Pinheiro et al., 2012). For example, free radicals are continuously generated as a result of the body's antioxidative defense mechanism. Conditions that perturb the natural balance of free radicals can damage lipids, proteins, and nucleic acids, which subsequently cause cellular dysfunction, as has been observed in the pathophysiology of diseases like atherosclerosis, cancer, and diabetes (West, 2000; Rahman, 2007).

The *GST* family contains multiple genes, whose products are classified based on different loci isoelectric point, similarity of amino acid sequence and substrate specificity. There are 8 known classes of human cytosolic GSTs: α (GSTA), μ (GSTM), θ (GSTT), π (GSTP), σ (GSTS), κ (GSTK), ω (GSTO), and ζ (GSTZ) (McIlwain et al., 2006).

Studies on the identification of genetic susceptibility to atherosclerosis revealed that polymorphisms of *GSTM1* are critical for regulating the detoxification of products generated by oxidative stress. (Girisha et al., 2004; Silva, 2011; Zi et al., 2014).

*GSTM1* consists of 8 exons, which are 36-112 bp in length, while the introns vary in length from 87-2641 bp. *GSTM1* is "embedded" in a region of extensive homologies, being flanked by two regions with identical 4.2 kb stretches (Manfredi et al., 2009).

Studies suggest that active GSTM1 is responsible for the detoxification of polycyclic aromatic hydrocarbons (PAC) present in cigarettes and in solvents such as benzene. The absence of GSTM1 may enhance susceptibility to cancer due to the reduced efficiency of detoxification of carcinogens (Marinković et al., 2013; Zi et al., 2014).

This study aimed to analyze the relationship between polymorphisms of *GSTM1* in individuals from Goiânia and clinical manifestations of atherosclerosis.

## MATERIAL AND METHODS

The present research was approved by a formally constituted research ethics committee from the Pontifice Catholic University of Goiás, protocol No. 35321614.3.0000.0037.

Two hundred patients with an average age of 61.1 years who were previously diagnosed with atherosclerotic disease based on clinical examination and results of diagnostic tests such as eco-color Doppler, angiography, digital angiography, computed tomography angiography, and/or cine-angiography, from October 2014 to February 2015, consisted the case group. The control group consisted of 100 individuals of average age 50.2 years, who had no clinical manifestation of atherosclerosis and/or were negative for atheromatous plaque or myointimal thickening (intima-media <1 mm) in carotid eco-color Doppler imaging test. Sex and ethnicity were not matched due to differences in sample size; however, the age matched but the analysis regarding age did not show any statistically significant difference.

Regarding smoking habits, both case (atherosclerotic) and control groups were classified into three sub-groups: smoker, ex-smoker, and non-smoker. The smoker group consisted of people who regularly used tobacco products regardless of the time they started smoking. The ex-smoker group consisted of individuals who had used tobacco products in the past but have not smoked for at least 15 years in the present. The last group consisted of individuals who had never smoked. This classification is according to the Guidelines of the Brazilian Medical Association, 2013.

Samples of peripheral blood (15 mL) were collected and genomic DNA was extracted using the Kaswi® kit (Genomic DNA Purification Kit). DNA was subsequently analyzed by polymerase chain reaction (PCR) to detect *GSTM1* polymorphism (Table 1). As a positive control in PCR, we used DNA with known polymorphisms in *GSTM1*.

**Table 1.** *GSTM1* primer sequence.

| Primer | Sequences | Size |
|---|---|---|
| *GSTM1* | F: 5'-GAACTCCCTGAAAAGCTAAAGC-3' | 215 bp |
| | R: 5'-GTTGGGCTAAATATACGGTGG-3' | |

Simoni et al., 2004.

The whole procedure was conducted in a laminar flow hood to minimize contamination. The final volume of the PCR was 25 μL according to the protocol proposed by Frare et al. (2013).

Polymorphic deletion in *GSTM1* (Figure 1) accounts for normal enzyme levels when there is no deletion of alleles or a heterozygous deletion or the absence of enzyme when homozygous deletion take place. Experiments were performed in duplicates. A genotype was considered to be "null" after obtaining identical results thrice.

The PCR product was electrophoresed on 2% agarose gel in 1X Tris-borate EDTA solution (TBE). Gels were stained with ethidium bromide (5 mg/mL) and visualized in the Video System VDS Documentation® (Image Master VD® - Amersham Pharmacia Biotech, EUA).

### Statistical analysis

The results of *GSTM1* polymorphism were tabulated in Microsoft Excel spreadsheets. We used odds ratio, F test and chi-square test to analyze the relationship between polymorphism and atherosclerotic disease. $P < 0.05$ was considered statistically significant. The statistical tests were performed with BioEstat software version 5.3.

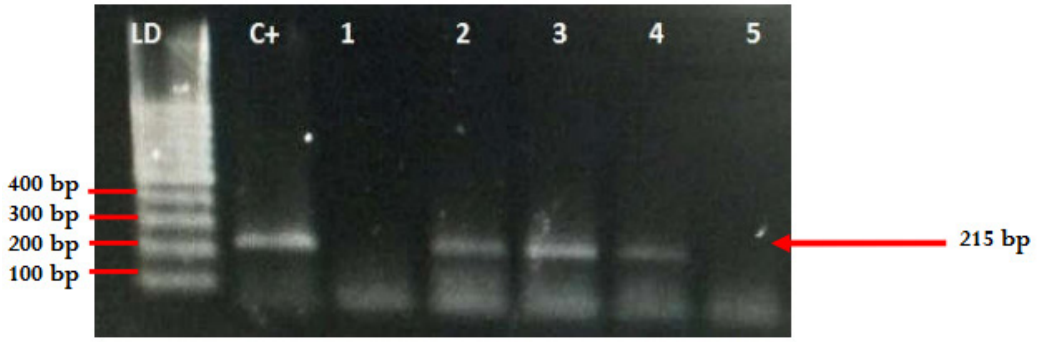


**Figure 1.** Agarose gel stained with 2% ethidium bromide showing the amplicons obtained using *GSTM1* primers (215 bp). LD, ladder; C+, positive control; *lanes 1* and *5*, *GSTM1* null; *lanes 2*, *3*, and *4*, *GSTM1* present.

## RESULTS

The case group consisted of 200 individuals with an average age of 61.1 years and the control group consisted of 100 subjects with an average age of 50.2 years.

We analyzed the relationship of *GSTM1* polymorphism and atherosclerosis among individuals of both groups. We found that the occurrence of "*GSTM1* present" genotype was 1.2 times higher in the case group (72.5%, 145/200) compared to that in the control group (60%, 60/100) (P = 0.0282) (Table 2).

**Table 2.** Distribution of *GSTM1* Polymorphism in the case and control groups.

| | Present [N (%)] | Null [N (%)] | Total [N (%)] | P* |
|---|---|---|---|---|
| Case | 145 (72.5) | 55 (27.5) | 200 (100.0) | |
| Control | 60 (60.0) | 40 (40.0) | 100 (100.0) | 0.0282 |

*Chi-square test.

The frequency of *GSTM1* present genotype was analyzed in smokers and ex-smokers (≥15 years) in both the case and control groups (Table 3). Among individuals who carried the *GSTM1* present genotype within the smoker sub-group of the case group, 13.2% (5/38) reported consuming 5 to 10 tobacco products, 34.2% (13/38) consumed 10 to 20 products and 52.6% (20/38) consumed 20 or more products. In the control group individuals who carried the *GSTM1* present genotype, it was observed that no individual reported using 5 to 10 tobacco products, while 90% (9/10) reported the use of 10 to 20 and 10% (1/10) 20 or more tobacco products, respectively (P = 0.0035) (Table 3). Analysis of the relationship between amount of tobacco consumption and the *GSTM1* present genotype in ex-smokers revealed that there was no significant difference between the case and control groups (P = 0.3915).

Table 4 shows the association between smoking habits and the time that an individual has been smoking or has quit smoking (ex-smokers) with the *GSMT1* genotypes in the case and control groups. We found no significant difference between the case and control groups regarding smoking habits and the *GSTM1* present genotype (P = 0.4869) (Table 4). Among patients from the case group who were ex-smokers (³15 years) and had the *GSTM1* present genotype, 15.1% (5/33) claimed to have smoked less for than 10 years, 39.4% (13/33) for 10-20 years, and 45.5% (15/33) for more than 20 years (P = 0.0240) (Table 4).

**Table 3.** Association of smoking history with *GSTM1* present genotype in case and control groups.

| Groups | *GSTM1* present | | | | |
|---|---|---|---|---|---|
| | Smoker group | | | | P* |
| | Case | | Control | | |
| | N | % | N | % | |
| 5-10 | 05 | 13.2 | 00 | 0.0 | |
| 10-20 | 13 | 34.2 | 09 | 90.0 | 0.0035 |
| 20 or more | 20 | 52.6 | 01 | 10.0 | |
| Total | 38 | 100.0 | 10 | 100.0 | |
| | Ex-smoker group | | | | |
| 5-10 | 11 | 34.4 | 01 | 20.0 | |
| 10-20 | 09 | 28.1 | 03 | 60.0 | 0.3915 |
| 20 or more | 12 | 37.5 | 01 | 20.0 | |
| Total | 32 | 100.0 | 05 | 100.0 | |

*Chi-square test.

**Table 4.** Association of smoking with GSTM1 present genotype in case and control groups associated with time of smoking.

| Groups | *GSTM1* present | | | | |
|---|---|---|---|---|---|
| | Smoker group | | | | P* |
| | Case | | Control | | |
| | N | % | N | % | |
| <10 years | 02 | 4.0 | 01 | 7.7 | |
| 10 to 20 years | 03 | 6.0 | 02 | 15.4 | 0.4869 |
| > 20 years | 45 | 90.0 | 10 | 76.9 | |
| Total | 50 | 100.0 | 13 | 100.0 | |
| | Ex-smoker group | | | | |
| <10 years | 05 | 15.1 | 03 | 50.0 | |
| 10 a 20 years | 13 | 39.4 | 03 | 50.0 | 0.0240 |
| >20 years | 15 | 45.5 | 00 | 00.0 | |
| Total | 33 | 100.0 | 06 | 100.0 | |

*Chi-square test; **Teste G.

## DISCUSSION

Genetic polymorphisms, which occur at a frequency of more than 1% in the general population, is characterized by variations in DNA sequence that results in the production of proteins with altered activities. Polymorphisms can explain certain differences in clinical outcomes and therapeutic responses among patients with the same condition and receiving identical pharmacological treatments (Willard, 2000). The limitation of the present study is related to the heterogeneity of the population under study due to ethnic admixture. The allelic frequency may be considerably divergent among these ethnic groups, which hinders the ability to detect disease association of polymorphisms and leads to stratification bias.

Atherosclerosis is a multifactorial disease, and genetic factors act as determinants of risk for its onset and development. More than 400 genes may be involved in the regulation of processes related to endothelial function, carbohydrate metabolism, inflammation, coagulation, and metabolism of amino acids and lipids (Doevendans et al., 2001).

Polymorphisms in *GST*s, which encode enzymes with detoxification functions, alter the sensitivity of an organism to inflammatory processes involved in the development of atherosclerosis (Wilson et al., 2000; Izzotti et al., 2001; Hayes et al., 2005*;* Marinković et al., 2013).

A study of *GSTM1* polymorphism in a population of northern India revealed that the present/null genotype is related to CAD. In the case and control groups, the *GSTM1* present genotype was 76.65 and 79.29%, respectively (Girisha et al., 2004). Our results corroborate this

finding. We found 1.2 times higher percentage of *GSTM1* present genotype in the case group (72.5%) compared to that in the control group (60%) (P = 0.0282). Similar observations were made by Taspinar et al. (2012), who showed a higher prevalence of *GSTM1* present genotype (58.2% for the case group and 53.5% in the control group) within the Turkish population. Studies conducted by Wilson et al. (2000) in the UK showed a higher proportion (52%) of the *GSTM1* present genotype in the atherosclerosis group than in the control group (42.8%).

Grignoli et al. (2009) analyzed the relationship of polymorphisms in *GSTM1* with different pathologies, including atherosclerosis, in the population of the Araras region of Brazil, and reported that the *GSTM1* present genotype was more prevalent (57%) in individuals with atherosclerosis than the *GSTM1* null genotype (43%), which corroborated the results of our study.

In the present study, we have assessed individuals who were diagnosed with atherosclerosis. In contrast, the case group was composed of Caucasian individuals who had suffered from ischemic stroke in the study by Türkanoğlu et al. (2010), who found an overall lower prevalence of the *GSTM1* present genotype (49.4% (N = 172) and 43.8% (N = 105) for the case and control groups, respectively). In addition, there was no significant differences between case and control groups.

Zhang and Zhang (2014) performed a meta-analysis to investigate the association between *GSTM1* null genotype and CAD. Their results, which are statistically significant, suggest that this genotype is a risk factor for CAD.

Manfredi et al. (2009) found that in a part of the Italian population, 41.4% patients and 54.7% individuals harbored the *GSTM1* present genotype in the case and control groups, respectively.

In the present work, we identified a significant association between smoking habit, namely, the amount of tobacco consumed, and the *GSTM1* present genotype. The frequency of this genotype was higher in individuals with atherosclerosis who reported smoking more than 20 cigarettes a day, while in the control group it was higher for individuals who reported smoking 10-20 cigarettes per day (P = 0.0035). Similar observations were made by Olshan et al. (2003) who suggested an association between the *GSTT1* polymorphism and heavy smoking. These results imply that a better understanding of the relationship between *GSTM1* polymorphism and smoking habits may improve our understanding about the development of atherosclerosis.

Pan et al. (2011) investigated the association of polymorphisms in *GSTM1* with family history of chronic diseases such as CAD within the Hakka population of south China. They found no significant differences between the *GSTM1* present/null genotypes and the amount of tobacco consumed (<10 cigarettes, 10-20 cigarettes, and >20 cigarettes per day). Individuals who smoked at least one cigarette per day for 6 months was considered as smokers. They observed that there was a higher frequency of occurrence of the *GSTM1* present phenotype in individuals of the case group who reported smoking for more than 20 years; in the control group, the highest frequencies were found both for individuals who reported smoking in the period of less than 10 years and between 10 to 20 years. They found statistically significant difference for individuals who were ex-smokers (P = 0.0240). Olshan et al. (2003) found no correlation between smoking habit (≥20 years) and the genotypes of *GSTM1* in the American population.

Grignoli et al. (2009) found that the *GSTM1* null genotype was more common (58%) in individuals who smoked for more than 10 years, leading to deficiency in detoxification processes of the body and accumulation of mutagenic substances, which consequently modulated the susceptibility to the development of cancer and other diseases such as atherosclerosis.

Xenobiotics present in tobacco are metabolized by an enzyme complex that gathers activating enzymes of phase I and detoxifying enzymes of phase II. In phase II of the biotransformation, enzymes combine hydrophilic compounds such as acetyl or glutathione, which increases the solubility of xenobiotics in water and facilitates their elimination from the body (Marinković et al., 2013).

*GSTM1*, a member of the *GST* family, encodes a phase II enzyme and exhibits polymorphisms in the human population. The main role of *GSTM1* lies in detoxification of xenobiotic compounds such as benzo-(a)-pyrene-diol-epoxide (BPED), electrophilic compounds, hydrocarbons, PAC, and other mutagens that are highly reactive and possess the ability to form covalent bonds with the DNA (Wilson et al, 2000; Bourbon, 2008).

In summary, we found no significant difference between the associations of the *GSTM1* present and null genotypes with variable risk factors such as sex and alcohol consumption (tested but data not shown). A significant difference was observed between the groups (atherosclerosis versus control) regarding the occurrence of *GSTM1* polymorphism. The frequency of the *GSTM1* present genotype in atherosclerosis group was 1.2 times higher than in the control group. However, when we compared the associations of the present and null genotypes with smoking habits, we found that the *GSTM1* present genotype was more frequent, and this difference was statistically significant. The results presented here add to the current knowledge regarding *GSTM1* polymorphism in atherosclerosis and suggest that polymorphisms may play important roles as risk factors for the onset of atherosclerosis.

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