# GST polymorphisms and early-onset coronary artery disease in young South African Indians

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*Background.* Glutathione S-transferases (GSTs) detoxify environmental agents which influence the onset and progression of disease. Dysfunctional detoxification enzymes are responsible for prolonged exposure to reactive molecules and can contribute to endothelial damage, an underlying factor in coronary artery disease (CAD).

*Objectives.* We aimed to assess 2 common polymorphic variant isoforms in GSTM1 and GSTP1 of GST in young CAD patients.

*Methods.* All patients (*N*=102) were South Africans of Indian ancestry, a population associated with high CAD risk. A corresponding age-, sex- and race-matched control group (*N*=100) was also recruited. Frequency of the GSTM1 +/0 (v. +/0 and 0/0) and GSTP1 $A_{105}/G_{105}$ (v. wild-type $A_{105}/A_{105}$) genotypes was assessed by differential polymerase chain reaction (PCR) and PCR restriction fragment length polymorphism (PCR-RFLP), respectively.

*Results.* The GSTM1 0/0 and GSTP1 $A_{105}/A_{105}$ genotypes occurred at higher frequencies in CAD patients compared with the control group (36% v. 18% and 65% v. 48%, respectively). A significant association with CAD was observed in GSTM1 0/0 (odds ratio (OR)=2.593; 95% confidence interval (CI) 1.353 - 4.971; $p$=0.0043) and GSTP1 $A_{105}/A_{105}$ OR=0.6011; 95% CI 0.3803 - 0.9503; $p$=0.0377). We found a significant association between smoking and CAD; the presence of either of the respective genotypes together with smoking increased the CAD risk (GSTP1 $A_{105}$ relative risk (RR)=1.382; 95% CI 0.958 - 1.994; $p$=0.0987 and GSTM1 null RR=1.725; 95% CI 1.044 - 2.851; $p$=0.0221).

*Conclusion.* Our findings support the association of genotypes GSTM1 0/0 and GSTP1 $A_{105}/A_{105}$ and smoking with CAD.

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Atherosclerosis and thrombosis are the 2 major contributing mechanisms in coronary artery disease (CAD), a major health problem and leading cause of death worldwide. The formation of atherosclerotic lesions is augmented by elevated levels of reactive oxygen species (ROS).[1]

Endothelial cell injury is initiated by increased shear stress, caused by the combination of high blood viscosity, hypertension and vasoconstriction which may be induced by smoking. The toxic chemicals present in tobacco smoke affect the endothelium and enhance smooth-muscle cell proliferation by inducing platelet adherence to damaged cells.[2] Nicotine is a strong activator of the sympathetic nervous system, which releases catecholamines that interfere with haemodynamics, resulting in increased myocardial oxygen demand.[3] Cigarette smoke contains a complex mixture of approximately 4 000 chemical species and plentiful oxygen-, nitrogen- and carbon-centred free radicals which increase ROS generation and promote oxidative damage.[4]

Cells produce ROS through normal metabolic processes and disruption of the mitochondrial electron transport chain.[5] Cell-intrinsic mechanisms, such as the glutathione (GSH) antioxidant defence system, act to combat ROS. Disturbances in the endogenous antioxidant defence mechanism or ROS overproduction, however, lead to accumulation and availability of free radicals which induce oxidative damage to biomolecules by lipid peroxidation, protein nitration and DNA adduct formation.

Cellular detoxification systems protect against endo- and exogenous harmful substances. Of particular interest is the super-family of glutathione S-transferases (GSTs), which modulate prostaglandin signalling pathways and oxidative stress.[6] GSTs function to detoxify electrophilic toxicants, including those found in cigarette smoke, by facilitating conjugation to GSH.[7] However, GST function is influenced by genotypic differences from single nucleotide polymorphisms (SNPs).[8]

GST genes contain several polymorphic variants occurring at high frequency. In the GSTP1 $A_{105}/G_{105}$ variant, a single nucleotide change from adenine (A) to guanine (G) at codon 105 results in an isoleucine (Ile) to valine (Val) amino acid change. In the GSTM1 null (0/0) polymorphism, complete deletion of the gene encoding this isoform results in abolished enzymatic activity.[9]

Several studies assessing genetic susceptibility in response to air pollution and cardiovascular outcomes show that GSTP1 and GSTM1 play a role in ROS modulation.[10] Others found an association of GSTM1 0/0 with heart failure.[11,12] Black carbon and particulate matter exposure was shown to be associated with increased intracellular and vascular cell adhesion molecules, which promoted atherosclerosis; this association was modified by the presence of the GSTM1 polymorphism.[13] GSTP1 and GSTM1 polymorphisms have been associated with the biomarker of oxidative DNA damage, 8-hydroxy-2'-deoxyguanosine and increased susceptibility to smoking-related CAD.[14,15-17]

The high incidence of CAD in South Africans of Indian ancestry in Durban was reported in 1969.[18] More than 40 years later, epidemiological data in South Africa (SA) showed Indians to have the highest mortality rates for CAD, followed by mixed race, white and black.[19] Recent studies show a strong familial link with the history of diabetes mellitus type 2, hypertension and CAD, supporting a genetic basis for development.[20,21]

In light of the evidence that variants of GSTP1 and GSTM1 influence disease risk, we assessed the association of GST polymorphisms in young smoking and non-smoking South African Indian CAD patients.

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## Methods

A total of 102 young Indian CAD patients and 100 age-, race- and sex-matched controls were enrolled following institutional ethical approval (BE154/010). A full pathology report was compiled. The inclusion criterion for CAD patients were: Indian ancestry and unrelated, adults aged <45 years, and stable CAD confirmed at angiography. The exclusion criteria for controls included an acute coronary syndrome/revascularisation procedure in the preceding 3 months, chronic renal or liver disease, malignancy and known active inflammatory or infectious disease.

Genomic DNA was extracted from whole blood. Cells were transferred to 500 µl lysis buffer (0.5% sodium dodecyl sulphate (SDS), 150 mM NaCl, 10 mM ethylenediaminetetra-acetic acid (EDTA), 10 mM Tris-HCl (pH 8.0)). RNase A (100 µg/ml; DNase-free) was added and the mixture incubated (37°C, 1 h). Subsequently, proteinase K (200 µg/ml) was added and incubated (3 h, 50°C) and a 0.1% volume 5 mM potassium acetate was added before centrifugation (5000 xg; 15 min). Supernatants containing genomic DNA were transferred to fresh tubes and extracted with 100% isopropanol and washed with 70% ethanol. DNA samples were solubilised in 10 mM Tris and 0.1 mM EDTA (pH 7.4, 4°C). DNA concentration was determined spectrophotometrically.

Differential polymerase chain reaction (PCR) was performed to assess the GSTM1 polymorphism: 268 base pair (bp) (β-globin) and 215 bp (GSTM1) PCR products were amplified using 30 pmol of primers for the GSTM1 gene and 10 pmol primers for the β-globin gene in a 25 µl reaction (200 µM of each deoxyribonucleotide (dNTP), 3.3 mM $MgCl_2$, 1x Green GoTaq Flexi buffer, 1 U GoTaq DNA polymerase (Promega), 100 ng genomic DNA template). Primer sequences: GSTM1-Fwd 5'-GAACTCCCTGAAAAGCTAAAGC-3'; GSTM1-Rev 5'-GTTGGGCTCAAATATACGGTGG-3'; β-globin-Fwd 5'-CAACTTCATCCACGTTCACC-3'; β-globin-Rev 5'-GAAGAGCCAAGGACAGGTAC-3'. Following denaturation (96°C, 5 min), amplification was carried out with 25 cycles of denaturation (96°C, 30 sec), annealing (57°C, 30 sec) and extension (72°C, 30 sec), followed by a final extension (72°C, 5 min). Amplification products were electrophoresed on an agarose gel (4%, 0.5 mg/ml ethidium bromide) and visualised. The presence of a single 268 bp product was indicative of the homozygous null genotype. The amplification of a 215 bp product was indicative of the presence of the gene in either a homozygous positive or heterozygous state.

PCR-restriction fragment length polymorphism (PCR-RFLP) was used to determine the GSTP1 genotype. A 176 bp PCR product was amplified using 15 pmol of each primer (forward 5'-ACCCCAGGGCTCTATGGGAA-3' and reverse 5'-TGAGGGCACAAGAAGCCCCT-3') in a 25 µl reaction containing 200 µM of each dNTP, 2.5 mM $MgCl_2$, 1x Green GoTaq Flexi buffer, 0.5U GoTaq DNA polymerase and 100 ng genomic DNA template. Following initial denaturation (96°C, 5 min), amplification was carried out with 30 cycles of denaturation (96°C, 30 sec), annealing (55°C, 30 sec) and extension (72°C, 30 sec). This was followed by a final extension (72°C, 5 min). Presence of the polymorphic restriction site was analysed by restriction endonuclease digestion of the PCR amplicon. Overnight digestion (37°C) was performed in 25 µl reactions: 15 µl PCR product, 4.5 µl Buffer-R and 0.5 µl (5 U) *Bsm*AI (Fermentas). Amplicons homozygous for the $G_{105}$ allele were completely digested and resulted in 2 restriction fragments (91 bp and 85 bp). Restriction fragments were electrophoresed on an agarose gel (3%, 0.5 mg/ml ethidium bromide) and visualised.

Statistical analyses were performed with GraphPad Prism software (version 5.0).

## Results

Among the clinical parameters assessed between the groups, body mass index (BMI), hypertension, diabetes, triglycerides, fasting glucose and $HbA_{1c}$ were higher in patients than in controls (Table 1). Single nucleotide changes at codon 105 of the GSTP1 gene were investigated using RFLP-PCR. The genotype frequencies observed did not deviate from those predicted by Hardy-Weinberg statistics (GSTP1: $p$=0.294 in CAD patients, $p$=0.413 in controls; GSTM1 $p$=0.083 in CAD patients, $p$=0.64 in controls; chi-square test).

A significant skew towards frequency of the $A_{105}/A_{105}$ genotype was observed in CAD patients compared with the controls. In the control group, the homozygous $A_{105}/A_{105}$ and heterozygous $A_{105}/G_{105}$ genotypes were observed at frequencies of 48% and 45%, respectively. The frequency of the $A_{105}/A_{105}$ genotype was significantly higher in CAD patients (65% v. 48%, respectively; odds ratio (OR)=0.6011, 95% confidence interval (CI) 0.3803 - 0.9503, $p$=0.0377; Table 2). GSTM1 0/0 was also significantly more frequent in CAD patients (36% v. 18%; OR=2.593, $p$=0.0043; Table 3) compared with +/+ and +/0 genotypes.

A higher frequency of the GSTP1 $A_{105}$ and GSTM1 null alleles was observed in patients; therefore, we investigated whether there were genotypic differences in relative CAD risk in respect of these loci. Our findings suggest that the presence of either of these genotypes confers a significant CAD risk (Table 2). We stratified the groups according to smoking history: the number of smokers in the patient group was much higher than in the control, with a significant association between smoking and CAD ($p$<0.0001, OR=0.2245, 95% CI 0.1062 - 0.4746). Most smokers presented with GSTP1 $A_{105}/A_{105}$ ($p$=0.0987, OR=0.5667, 95% CI 0.2952 - 1.088; Table 4) and GSTM1 0/0 ($p$=0.0221, OR=2.386 95% CI .137 - 5.009; Table 4). The higher relative risk of CAD associated with these genotypes is increased with smoking.

## Discussion

The INTERHEART study ranked smoking as the second highest risk factor for myocardial infarction (OR=2.87, 99% CI).[22] The Systemic Coronary Risk Evaluation project estimated the 10-year fatal cardiovascular risk to be twice as high for smokers v. non smokers for any given age, systolic BP and cholesterol level.[23] The number of smokers worldwide is expected to reach 1.7 billion by 2025.[24,25] With current trends, smoking-related deaths are expected to rise from 4.8 million in 2000 to 8 million in 2030.[25]

Endothelial cell damage in vasculature is caused by changes in haemodynamics and oxidative stress, leading to thrombosis and atherosclerosis. The continuous cycle of inflammation from atherosclerotic plaques and arterial wall lesions contributes to elevated levels of C-reactive protein in CAD. The low-grade inflammation, recruitment and activation of leucocytes to the atherosclerotic lesion plays an important role in ROS generation in an attempt to circumvent the spread of foreign material, while simultaneously exposing biomolecules to oxidative stress.[5]

Our data show an association of wild-type GSTP1 $A_{105}/A_{105}$ and GSTM1 0/0 with CAD. The presence of allele GSTP1 $G_{105}$ in the healthy control patients signals a possible role of this polymorphism in enhancing the efficacy of antioxidant mechanisms. GST acts as a general base catalyst, increasing the rate of GSH conjugation to hydrophobic substrates by deprotonation to $GS^-$ by an active tyrosinate. The change in codon 105 of the GST gene results in an amino acid substitution from Ile to Val. This was previously shown to cause deviations in the atomic co-ordinates of key H-site residue side-chains, thereby altering GST catalytic ability and increasing susceptibility to smoking-related CAD.[15-17] Earlier literature, however, indicates 7 times more active conjugation to diol epoxides and increased catalytic efficiency to aromatic epoxides.[8]

**Table 1. Demographics and clinical parameters***

| | Control (*N*=100) | CAD patients (*N*=102) | *p*-value |
|---|---|---|---|
| Prediabetic/diabetic, *n* (%) | 39 (39) | 59(58) | 0.0070* |
| Hypertensive, *n* (%) | 22 (22) | 44 (43) | 0.0020* |
| Age (years) | 37.5 (0.44) | 37.6 (0.40) | 0.8560 |
| BMI (kg/m$^2$) | 26.5 (0.47) | 28.2 (0.46) | 0.0110* |
| Laboratory parameters | | | |
| Total cholesterol (mmol/l) | 5.48 (0.10) | 5.36 (0.18) | 0.5830 |
| LDL-cholesterol (mmol/l) | 3.72 (0.09) | 3.42 (0.17) | 0.1150 |
| HDL-cholesterol (mmol/l) | 0.96 (0.03) | 0.93 (0.03) | 0.3580 |
| Triglycerides (mmol/l) | 1.88 (0.18) | 2.37 (0.15) | 0.0399* |
| Fasting glucose (mmol/l) | 5.45 (0.16) | 6.34 (0.27) | 0.0054* |
| Fasting insulin (uIU/ml) | 16.34 (1.26) | 16.02 (1.08) | 0.8500 |
| $HbA_{1c}$ (%) | 5.70 (0.10) | 6.61 (0.19) | <0.0001* |
| hsCRP (mg/l) | 6.69 (1.12) | 8.05 (1.15) | 0.4000 |
| Medication | | | |
| Statins (%) | 3 | 84 | |
| Aspirin (%) | 0 | 87 | |
| Beta blockers (%) | 0 | 79 | |
| Nitrates (%) | 0 | 37 | |
| ACE-Is (%) | 2 | 72 | |

BMI = body mass index; LDL = low-density lipoprotein; HDL = high-density lipoprotein; $HbA_{1c}$ = glycated haemoglobin; hsCRP = high sensitivity C-reactive protein.
Clinical parameters represented as mean ± standard error of the mean.
*$p$<0.01.

**Table 2. Genotype and allelotype GSTP1 frequency in CAD patients and controls**

| | Control *n* (%) | CAD patients *n* (%) | *p*-value |
|---|---|---|---|
| Genotype frequency | | | 0.797* |
| GSTP1 $A_{105}/A_{105}$ | 48 (48) | 66 (65) | |
| GSTP1 $A_{105}/G_{105}$ | 45 (45) | 31 (30) | |
| GSTP1 $G_{105}/G_{105}$ | 7 (7) | 5 (5) | |
| Allelotype frequency | | | 0.0377 |
| GSTP1 $A_{105}$ | 141 (70.5) | 163 (80) | |
| GSTP1 $G_{105}$ | 59 (29.5) | 41 (20) | |

*Chi-square test for heterogeneity between CAD patient and control genotype distribution. Chi-square statistic = 0.587, 1 degree of freedom.

**Table 3. Genotypic frequency of GSTM1 in CAD patients and controls**

| | Control *n* (%) | CAD patients *n* (%) | *p*-value |
|---|---|---|---|
| Genotype frequency | | | |
| GSTM1 +/+ or +/0 | 82 (82) | 65 (64) | 0.8300* |
| GSTM1 0/0 | 18 (18) | 37 (36) | 0.0043 |

*Chi-square test for heterogeneity between CAD patients and controls allele frequency. Chi-square statistic = 4.463, 1 degree of freedom.

GSTM1 0/0 results in complete absence of the enzyme isoform, which affects detoxification capacity. The strong association of this genotype in CAD patients indicates a key role in disease pathogenesis. The association between smoking and CAD in this cohort is in agreement with previous studies.[15-17] The high percentage of patients who smoked and presented with GSTP1 $A_{105}/A_{105}$ and GSTM1 0/0 supports the hypothesis that GSTs play an important role in CAD.

Study limitations included sample size, lack of exclusion criteria based on body mass index (BMI), hypertension and diabetes.

## Conclusion

This study provides evidence that the GSTP1 $A_{105}$ allele and the GSTM1 null genotype are associated with CAD in young South Africans of Indian ancestry.

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**Table 4. Summary of smokers stratified by genotypes for CAD risk identification**

| | | Control *n* (%) | CAD patients *n* (%) | |
|---|---|---|---|---|
| Non-smokers | | 35 (35) | 11 (10) | $p<0.0001$ RR=1.826 95% CI (1.427 - 2.336) |
| Smokers/ex-smokers | | 65 (65) | 91 (90) | |
| Smokers/ex-smokers | GSTP1 $A_{105}/G_{105}$ and $G_{105}/G_{105}$ | 31 (31) | 31 (30) | $p=0.0987$ RR=1.382 95% CI (0.958 - 1.994) |
| | GSTP1 $A_{105}/A_{105}$ | 34 (34) | 60 (59) | |
| | GSTM1 +/+ and +/0 | 52 (52) | 57 (56) | $p=0.0221$ RR=1.725 95% CI (1.044 - 2.851) |
| | GSTM1 0/0 | 13 (13) | 34 (33) | |

RR = relative risk; CI = confidence interval.


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