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Original article:

Smoking, genetic polymorphisms of glutathione S-transferases and biological indices of inflammation and cellular adhesion in the STANISLAS study

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ABSTRACT

A recent clinical study has focused on: 1- the interaction between genetic variants of glutathione S-transferases M1 and T1 (GSTM1 and GSTT1) and smoking on the risk of cardiovascular diseases, 2- the potential capacity of GSTM1 and T1 genotypes in modifying the effect of smoking on inflammation and endothelial function. In this study, we investigated whether carriage of these 2 polymorphisms altered the smoking impact on biological indices of inflammation and cellular adhesion. White blood cell count (WBC), albumin, C-reactive protein (CRP), interleukine-6 (IL-6), tumor necrosis factor-alpha (TNF-α), L-selectin, E-selectin, P-selectin and intracellular adhesion molecule-1 (ICAM-1) were measured in 189 non-smokers and 76 smokers (aged 20-55 years) genotyped for the GSTM1 and T1 polymorphisms. Accounting for age and sex, smokers lacking GSTM1 had a higher WBC count, CRP and ICAM-1 levels as compared to the other groups; interaction term between smoking and genotype being significant ($p \leq 0.05$). Conversely, non-smokers lacking GSTM1 had a higher levels of TNF-α; the test for interaction being significant ($p \leq 0.05$). No significant interaction was found between smoking and GSTT1 genotypes, considering the 9 biological indices. However, significantly lower levels of IL-6 were noticed for non-smokers with *GSTT1-0* null allele ($p \leq 0.05$). Our study confirms previous results showing that GSTM1 polymorphism could modulate the interrelationships between smoking and biological markers of inflammation and endothelial function.

Keywords: smoking, inflammation, adhesion molecule, glutathione S-transferase polymorphism

INTRODUCTION

Numerous genetic variants of human drug-metabolizing enzymes, involved in either the activation or detoxification of carcinogenic compounds in tobacco smoke, influence the individual susceptibility to smoking related diseases, including atherosclerosis. The glutathione S-transferases (GST) are a polymorphic supergene family that detoxifies carcinogens and pro-atherogenic compounds generated by oxidative stress, in particular those found in tobacco smoke. In addition, it has been shown that the GST enzymes are expressed in human atherosclerotic plaques (Hayes et al., 1995; Hayes et al., 2000; Ketterer, 1998). Several polymorphisms of these enzymes have been identified, including GST mu (GSTM1-1) and theta (GSTT1-1). Two functionally different alleles of GSTM1-1 and GSTT1-1 genes have been described (*GSTM1-0* or *M1-1* and *GSTT1-0* or *T1-1*). Individuals with null alleles *GSTM1-0* and *GSTT1-0* express no activity of the respective isoenzyme, and may be unable to efficiently eliminate electrophilic intermediates (Hayes et al., 1995; Hayes et al., 2000).

Recent studies have reported altered allelic frequencies of the polymorphic genes *GSTM1-1* and *T1-1* as potential risk factors for the development of cardiovascular diseases (de Waart et al., 2001; Li et al., 2000; Li et al., 2001; Masetti et al., 2003; Olshan et al., 2003). Thus, GST genetic polymorphisms may have an influence on the development of these diseases, possibly through the pivotal role of GST in the cellular metabolism of cytotoxic products generated in part by tobacco smoke constituents, including carcinogenic metabolites and reactive oxygen species (ROS) (Dusinska et al., 2001; Ketterer, 1998; Onaran et al., 2001). Cigarette smoking has been firmly established as an independent risk factor for vascular diseases. However, the pathogenic effects of tobacco smoke on the atherosclerosis development remains unclear. Recently, studies have implicated oxidative stress modifications of low density lipoprotein and DNA damage of target tissues by cigarette smoke as a fundamental step in atherogenesis (Binkova et al., 2001; de Maat et al., 2002). It is also tempting to speculate that smoking-induced pro-inflammatory markers are responsible for smooth muscle cells (SMC) proliferation and endothelial dysfunction, thereby contributing to atherosclerosis plaque formation. Recent evidence suggests that ROS may function as second messengers in cytokines or some growth factor-mediated intracellular signal transduction pathways. In particular, ROS are reported to stimulate SMC growth and proto-oncogene expression (Nishio et al., 1998).

ROS and pro-oxidants in tobacco smoke could lead to regulation responses mediated by inflammatory markers such as C-reactive protein (CRP), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), altering the physiological properties of vascular cells (Libby et al., 2002; Saadeddin et al., 2002). Because the GSTs have been shown to protect cells from the toxicity of ROS and pro-oxidant compounds, including α,β-unsaturated carbonyls (He et al., 1998; Ketterer, 1998), they have been proposed as an important defense mechanism against atherogenesis. Moreover, previous studies have identified interactions between smoking and two GST genes, GSTM1 and T1, in relation to coronary artery disease, lower extremity arterial disease and carotid artery intimal-medial thickness (de Waart et al., 2001; Li et al., 2000; Li et al., 2001; Masetti et al., 2003; Olshan et al., 2003). Recently, Miller et al. (Miller et al., 2003) showed that GSTT1 and GSTM1

could reduce the potential for oxidative damage and therefore modify inflammatory response to oxidative stress in the ARIC study. Smokers carrying *GSTM1-0* null allele had the highest level of CRP, fibrinogen, von Willebrand factors, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). Conversely, smokers with *GSTT1-1* functional allele had the highest level of CRP and fibrinogen.

Thus, the purpose of the present study was to assess the interactions between genetic variation in two GST and smoking on 9 biomarkers of inflammation and cell adhesion (WBC, albumin, CRP, IL-6, TNF-α, L-selectin, E-selectin, P-selectin and ICAM-1) in a sub-sample of healthy French individuals of the STANISLAS cohort.

MATERIALS AND METHODS

Subjects

The sample population including 189 non-smokers and 76 smokers (males and females, aged 20-55 years) was taken from the Stanislas cohort, a longitudinal family study recruited at 1994-95 and having a 5 years check up (Siest et al., 1998). Individuals included in this study had to be born of French parents. Moreover, they had to live in the east of France (particularly in two administrative departments: Vosges and Meurthe et Moselle). For this specific study, subjects were selected with the following criteria: to be present at the second (1999-2000) health screening; free from serious and/or chronic illnesses, especially diabetes mellitus or cardiovascular, hepatic or renal failure; without treatment with lipid-lowering drugs or antidiabetic agent. Volunteers having aspartate aminotransferase (AST), alanine aminotransferase (ALT) or gamma-glutamyltransferase (GGT) activities > 200 U/L, orosomucoid or haptoglobin > 3 g/L, cholesterol or triglyceride > 10 mmol/L, CRP > 30 mg/L, or glucose > 7 mmol/L were excluded. This study was approved by the Local Ethics Committee of Nancy (France), and each subject gave a written informed consent.

Blood sample and data collection

Venous blood samples were collected by venipuncture after an overnight fast. Blood samples were centrifuged (1,500 g for 15 min at 4°C) within 2h after collection, and resulting EDTA plasma samples for IL-6 and TNF-α determination, and serum samples for L-selectin, E-selectin, P-selectin and ICAM-1 level determination were promptly frozen at -196°C in liquid nitrogen until analysis. Aliquots and biological samples were stored in the Biobank of the Centre de Médecine Préventive (Siest et al., 1998; Visvikis et al., 1998). Data collection included measurements of basic blood constituents, functional tests, physical examination, questionnaires on life-style and personal medical history. Medication was assessed by patient interview during the blood sampling. Information on alcohol and smoking consumption were collected by self-administered questionnaires.

Analytical methods

White blood cell counts were determined on a MAX'M analyzer (Beckman-Coulter, Roissy, France). Serum concentrations of albumin, glucose, total cholesterol, triglycerides, and activities of AST, ALT and GGT were measured with commercially available kits on an AU5021 apparatus (all from Merck, Darmstadt, Germany) on fresh aliquots within 2 hours. Serum CRP, orosomucoid and haptoglobin were measured by immunonephelometry on a Behring Nephelometer Analyser (BN II, Dade-

Behring, Marburg, Germany) with Behring reagents (Reuil-Malmaison, France) within two hours after sampling. Circulating levels of ICAM-1, E-selectin, P-selectin, L-selectin, IL-6 and TNF-α were measured with commercially available enzyme-linked immunosorbant assay (R&D System, Abington UK) in serum samples stored in liquid nitrogen until use (storage period between 21 and 39 weeks, mean: 31 weeks). The intra- and inter-assay coefficients of variation for inflammatory markers were as follows: CRP: 4.0% and 6.1%, IL-6: 11.1% and 16.5%, TNF-α: 8.8% and 16.7%, L-selectin: 8.9% and 11.7%, E-selectin: 9.4% and 14.9%, P-selectin: 5.8% and 7.0%, ICAM-1: 6.6% and 8.6%, respectively.

GST genotyping

DNA extraction was performed according to the "salting out" method, after validation of the procedure and DNA stability (Visvikis et al., 1998). GSTM1 and GSTT1 genotypes were identified using an allele-specific multiplex PCR assay. Genotyping of GSTM1 and GSTT1 was carried out using a multiplex-PCR method (Abdel-Rahman et al., 1996). The multiplex-PCR protocol consisted of 4 min at 94°C; 45 cycles of 30 s min at 94°C, 30 min at 60°C, 60 s at 72°C and 5 min at 72°C. PCR products were separated on 3% agarose gel via electrophoresis, and visualized using ethidium bromide staining. The multiplex PCR method used for both GSTM1 and GSTT1 polymorphism determination allows distinguishing between carriers of at least one functional allele (homozygous functional genotype + heterozygous) and carriers of no functional allele (homozygous null genotype).

Statistical analysis

Statistical analyses were performed by using the SAS software version 8.01 (SAS Institute Inc., USA). Since the distribution of serum concentrations of CRP, Il-6, TNF-α, ICAM-1, E-selectin, P-selectin, and L-selectin were skewed, $\log_{10}$-transformations were applied for setting purpose. Levels of these markers were presented as geometric means. Smoking exposure was defined as current or non-current smokers (dichotomous); GST genotypes as 2 categories: GSTM1-1 or GSTT-1, if the subject had at least one functional allele and GSTM1-0 or GSTT1-0, if the subject had no functional allele (homozygous null genotype). Differences in biological indices between non-smokers and smokers were assessed by using 1-way analysis of variance after adjustment for age and sex. Two-way analysis of variance with interaction was used to determine whether the influence of smoking on biological analytes varied between genotype groups. The significance of difference between genotype groups in non-smokers and in smokers was assessed by ANOVA accounting for age and sex. GSTM1-GSTM1-smoking interactions were not investigated because of too few subjects in these groups. In all analyses, as we did not adjust for multiple testing, $p \leq 0.05$ was considered as statistically significant; moreover, $p \leq 0.10$ was indicated and discussed.

RESULTS

Characteristics of non-smokers and smokers are shown in table 1. Subjects were of middle age; smokers were significantly younger and included more males than non-smokers ($p \leq 0.05$). Accounting for age and sex, smokers had a significantly increased WBC count, Il-6 and ICAM-1 concentrations than non-smokers ($p \leq 0.001$, $p \leq 0.10$ (borderline significant) and $p \leq 0.001$, respectively). Levels of albumin, CRP, TNF-α, L-selectin, E-selectin and P-selectin, and

genotype frequencies for GSTM1 and GSTT1 did not significantly differ between smokers and non-smokers.

Table 1: Clinical characteristics of the 265 subjects

	Non-smokers n = 189	Smokers n = 76
Age (years)	38.3 ±11.1[1]	34.6 ± 12.0*
Male sex (%)	46.6	63.2*
Tobacco (cig/day)	0.0 ± 0.0	13.8 ± 9.7***
White blood cells (10^3/mm^3)	6.75 ± 1.46	7.80 ± 2.34***
Albumin (g/l)	46.9 ± 2.5	47.1 ± 2.4
CRP (mg/l)	0.81 (0.26-2.49)[2]	0.88 (0.30-2.59)
IL-6 (ng/l)	0.85 (0.38-1.93)	1.05 (0.40-2.72)°
TNF-α (ng/l)	1.23 (0.42-3.59)	1.06 (0.36-3.14)
L-selectin (mg/l)	1011 (717-1426)	1030 (747-1421)
E-selectin (mg/l)	44.5 (23.9-82.4)	48.9 (26.6-89.7)
P-selectin (mg/l)	125 (89-174)	132 (94-186)
ICAM-1 (mg/l)	244 (197-303)	278 (204-379)***
GSTT1-0 (%)	20.1	25.0
GSTM1-0 (%)	52.4	42.9

[1] Arithmetic means adjusted for age and sex ± SD
[2] Geometric means adjusted for age and sex (range of 1 SD)
° p≤0.10, * p≤0.05, *** p≤0.001: difference between non-smokers and smokers, ANOVA or χ^2 test.

Tables 2 and 3 show geometric means adjusted for age and sex for all analytes according to smoking status and GST genotypes (*GSTM1* and *T1*, respectively); males and females being analyzed together. Using 2-factors ANOVA, interaction terms between GSTM1 genotypes and smoking status were statistically significant for WBC count, and CRP, TNF-α and ICAM-1 concentrations (all p≤0.05, Table 2). Accounting for age and sex, smokers with the *GSTM1-0* null allele had a higher WBC count, CRP and ICAM-1 levels as compared to the other groups. In the smoker group, inter-genotype differences were significant for WBC count and CRP levels (p≤0.05) and borderline significant for ICAM-1 concentrations (p≤0.10). Conversely, in non-smokers, subjects lacking *GSTM1* had the highest levels of TNF-α (p≤0.01) and P-selectin (p≤0.05) as compared to those with *GSTM1-1* functional allele. For the 9 analytes, interaction terms between *GSTT1* genotypes and smoking status were not significantly different by using 2-factors ANOVA (Table 3). In smokers, no significant difference was noticed between *GSTT1-0* and *GSTT1-1* alleles. Conversely, in non-smokers, subjects with *GSTT1-1* functional allele had a higher Il-6 and TNF-α levels as compared to those with *GSTT1-0* null allele (p≤0.05 and p≤0.10 (borderline significant), respectively) (Table 3).

Table 2: Biological indices of inflammation and cellular adhesion according to GSTM1 genotype and smoking status

	Non-smokers		Smokers		Smoking / GSTT1 Interaction[2]
	GSTM1-0 n = 99	GSTM1-1 n = 90	GSTM1-0 N = 33	GSTM1-1 N = 43	
WBC (10^3/mm^3)	6.72 ± 1.64[1]	6.77 ± 1.25	8.41 ± 2.85	7.32 ± 1.77*	0.018
Albumin (g/l)	46.9 ± 2.6	46.9 ± 2.4	47.7 ± 2.8	46.7 ± 2.0	0.098
CRP (mg/l)	0.76 (0.25-2.24)[3]	0.87 (0.27-2.77)	1.16 (0.34-4.00)	0.71 (0.28-1.74)*	0.035
IL-6 (ng/l)	0.90 (0.40-2.04)	0.80 (0.35-1.82)	1.14 (0.44-2.95)	0.86 (0.32-2.27)	0.878
TNF-α (ng/l)	1.55 (0.57-4.23)	0.96 (0.34-2.71)**	1.01 (0.33-3.09)	1.11 (0.35-3.58)	0.043
ICAM1, mg/l	241 (197-296)	248 (198-312)	297 (209-422)	265 (202-347)°	0.033
L-selectin, mg/l	990 (672-1460)	1035 (776-1379)	1094 (810-1478)	984 (705-1373)	0.097
E-selectin, mg/l	45.2 (23.8-85.6)	44.1 (24.3-80.0)	54.9 (27.6-109.2)	45.1 (26.5-76.6)	0.284
P-selectin, mg/l	131 (99-173)	119 (82-173)*	130 (91-186)	135 (96-189)	0.134

[1] arithmetic means adjusted for age and sex ± SD
[2] test of smoking/GSTM1 genotype interaction p values
[3] geometric means adjusted for age and sex (range of 1 SD)
° p≤0.10, * p≤0.05 ** p≤0.01: difference between GSTM1 genotype in non smokers and smokers, ANOVA test on values adjusted for age and sex.

Table 3: Biological indices of inflammation and cellular adhesion according to GSTT1 genotype and smoking status

	Non-smokers		Smokers		Smoking / GSTT1 Interaction[2]
	GSTT1-0 N = 38	GSTT1-1 N = 151	GSTT1-0 N = 19	GSTT1-1 N = 57	
WBC (10^3/mm^3)	6.61 ± 1.44[1]	6.78 ± 1.47	7.32 ± 1.90	7.95 ± 2.48	0.416
Albumin (g/l)	46.4 ± 2.6	47.1 ± 2.4	46.6 ± 2.7	47.4 ± 2.3	0.877
CRP (mg/l)	0.77 (0.32-1.85)[3]	0.82 (0.25-2.65)	0.82 (0.32-2.08)	0.89 (0.28-2.78)	0.948
IL-6 (ng/l)	0.66 (0.30-1.44)	0.91 (0.40-2.07)*	0.97 (0.39-2.38)	1.07 (0.41-2.85)	0.413
TNF-α (ng/l)	0.94 (0.35-2.52)	1.32 (0.45-3.89)°	1.03 (0.32-3.26)	1.07 (0.37-3.12)	0.383
ICAM1 (mg/l)	1003 (675-1491)	1013 (728-1409)	1051 (702-1572)	1024 (765-1369)	0.744
L-selectin (mg/l)	44.2 (25.1-77.8)	44.7 (23.8-84.2)	51.5 (26.1-101.2)	48.3 (26.8-87.0)	0.697
E-selectin (mg/l)	123 (83-181)	125 (91-173)	143 (104-194)	130 (91-185)	0.289
P-selectin (mg/l)	251 (204-309)	243 (196-302)	266 (183-385)	283 (212-378)	0.243

[1] arithmetic means adjusted for age and sex ± SD
[2] test of smoking/GSTT1 genotype interaction, p values
[3] geometric means adjusted for age and sex (range of 1 SD)
° p≤0.10, * p≤0.05: difference between GSTT1 genotype in non smokers and smokers, ANOVA test on values adjusted for age and sex.

DISCUSSION

This study provides limited evidence that GSTM1 polymorphism might modify the effect of smoking on biological indices of inflammation and cellular adhesion. We observed that smokers lacking *GSTM1* had increased levels of CRP and ICAM-1, and a higher WBC count compared to smokers with *GSTM1-1* functional allele and non-smokers. A significant interaction was found between smoking and *GSTM1* genotypes for WBC, CRP, TNF-α and ICAM-1 concentrations, suggesting that: 1- the effects of smoking and *GSTM1* genotypes were dependant and 2- the changing levels of inflammatory proteins were closely linked to smoking. Cigarette

smoking is a major risk factor for developing coronary artery disease, causing not only an endothelial dysfunction but also enhancing ROS production (Cai et al., 2000). Consequently, increased levels of inflammatory markers such as cytokines and cell adhesion molecules were currently found in smokers (Butkiewicz et al., 2000; Cai et al., 2000; Saadeddin et al., 2002; Zhang et al., 2002). Patterns of expression of inflammatory proteins were intimately related to generalized inflammatory response of vascular cells to smoking.

One interesting result of our study was the *GSTM1* genotype differences in TNF-α and P-selectin levels observed in non-smokers: subjects carrying *GSTM1-1* functional allele had lower TNF-α and P-selectin levels compared to those with *GSTM1-0* null allele. In order to explain this difference, we postulate that subjects with *GSTM1-0* null allele were less protected against oxidative stress than those with *GSTM1-1* functional allele. Indeed, the GSTM1 enzyme has been shown to protect cells against endogenous compounds of oxidative stress, including epoxides, redox-cycling products and carbonyls (Hayes et al., 1995; He et al., 1998; Ketterer, 1998). Several studies provide evidences that DNA adducts levels were consistently increased in individuals lacking GSTM1 (Butkiewicz et al., 2000; Godschalk et al., 2001; Izzotti et al., 2001; Scarpato et al., 1997), suggesting that *GSTM1* genotypes might influence the individual susceptibility to oxidative damages (Dusinska et al., 2001; Onaran et al., 2001). One of the consequences of oxidative stress is the activation of the transcriptional factor complex NF-κB, which leads to the production of inflammatory proteins as TNF-α and P-selectin. Expression of inflammatory proteins could be modulated by the glutathione system (Rahman et al., 2000).

The biological indices of inflammation and cell adhesion were slightly higher in smokers than in non-smokers, regardless of the GSTT1 genotypes. As previously mentioned, cigarette smoking increases oxidative stress which participates in vascular dysfunction, enhancing inflammatory markers expression. When stratified by GSTT1 genotypes, no difference in inflammatory proteins levels was found between smokers. Surprisingly, we observed a significant association between GSTT1 genotypes and pro-inflammatory cytokines in non-smokers. Subjects with the *GSTT1-1* functional allele had higher levels of Il-6 and TNF-α than those carrying the *GSTT1-0* null allele, suggesting that the non-expression of *GSTT1* was associated with an attenuated inflammatory profile. No interaction was found between *GSTT1* genotypes and smoking, suggesting that the *GSTT1*-genotype differencies observed in non-smokers would be closely related to biological effect of GSTT1. A recent case-control study showed that individuals expressing GSTT1 have an elevated risk of smoking- and occupational-related diseases (Buzio et al., 2003; de Waart et al., 2001; Li et al., 2000; Li et al., 2001; Masetti et al., 2003; Olshan et al., 2003). This finding was consistent with the hypothesis that GSTT1 catalyses the bioactivation of some substrates to cyto- and geno-toxic metabolites, in combination with ROS production (Hayes et al., 1995; Ketterer, 1998; Thier et al., 1993). Therefore it was not surprising to find the increasing levels of IL-6 and TNF-α in subjects expressing GSTT1, which might reflect the compensatory biological responses of vascular cells to deleterious effects of oxidative stress.

As mentioned by Miller et al. (Miller et al., 2003), some limiting factors could influence the results of this study and their interpretation such as cross-sectional design, diet, infection, pre-analytical and analytical factors, and unidentified confounding factors. Given the multitude of biological variables studied, these associations found between GST genotypes and inflammatory proteins may have been obtained by chance. However, we did not use the Bonferroni adjustments, and we decided simply to describe the data found and to discuss possible interpretations (Perneger, 1998). Despite the small size of our sample population, our results were in agreement with those found by Miller et al. (Miller et al., 2003) and provided evidence that GSTM1 and GSTT1 influence markers of inflammation and endothelial function in combination with smoking. We also examined the effects of smoking and combined genotypes (i.e *GSTM1-0/GSTT1-0* and *GSTM1-0/GSTT1-1*) on biological indices of inflammation and cell adhesion. However, these analyses and their interpretation are limited by two factors: 1- the power of study was severely decreased due to small number of individuals with both "at risk" genotypes and 2- *GSTM1* and *GSTT1* genotypes exhibit different patterns of interaction based on this study and the earlier studies of Li R team (Li et al., 2000; Li et al., 2001; Miller et al., 2003; Olshan et al., 2003). Therefore, the most appropriate and logical way to combine genotypes is not obvious.

Recent epidemiological studies provide evidences that *GSTM1* and *GSTT1* genotypes were associated with an increased risk of smoking-related diseases, including coronary artery disease, lower extremity arterial disease and carotid artery intimal-medial thickness (de Waart et al., 2001; Li et al., 2000; Li et al., 2001; Masetti et al., 2003; Olshan et al., 2003). Indeed, Li R et al. (Li et al., 2000; Li et al., 2001) were the first studies providing evidences that risk of coronary artery diseases outcome is increased in lifelong smokers with *GSTM1-0* null allele and *GSTT1-1* functional allele. De Waart et al. (de Waart et al., 2001) reported that the 2-year progression of common carotid intima-media thickness was clearly more increased in lifelong smokers with *GSTM1-0* null allele. Moreover, studies (Butkiewicz et al., 2000; Dusinska et al., 2001; Godschalk et al., 2001; Izzotti et al., 2001; Onaran et al., 2001; Scarpato et al., 1997; Thier et al., 1993) showed that oxidative damages and DNA alterations in atherosclerotic lesions were increased in individuals with the *GSTM1-0* null allele and *GSTT1-1* functional allele, suggesting that smokers with these *GST* genotypes develop progression of atherosclerosis at an increased rate.

Data from epidemiological studies suggest a protective role of *GSTT1-0* allele and deleterious effect of *GSTT1-1* functional allele, and were in line with our findings. Thus, *GST* genetic polymorphisms could modulate the individual susceptibility to develop the inflammatory-related diseases such as cardiovascular diseases. Consequently, individual differences in *GST* genetic polymorphisms and expression could be of considerable importance for inflammatory processes. This opens an interesting research field concerning the relevance of GST polymorphisms for inflammation.

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