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Influence of glutathione *S*-transferase polymorphisms on genotoxic effects induced by tobacco smoke

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Abstract

Genotoxicity of tobacco smoke has long been investigated and tobacco smoke is considered to be one of the principal human carcinogens. Although its role in DNA-damage induction and cancer development has been documented, the mechanisms by which this happens are not well understood. Many chemical constituents of tobacco smoke are enzymatically metabolized by phase-I and phase-II enzymes, but modifications in coding and regulating sequences of these genes could influence their ability to detoxify these compounds.

In this work, we studied several enzymes involved in the metabolism of xenobiotics, viz. the glutathione *S*-transferases (GST) M1, T1, P1 and A1, with respect to their influence on the genotoxic effects induced by cigarette smoking. We assessed the genotoxic effects of tobacco smoke on peripheral blood lymphocytes of 72 healthy caucasians by use of the chromosomal aberration (CA) assay and the micronucleus (MN) test.

Genotypes of GST M1, T1, P1 and A1 were determined by means of the polymerase chain reaction and methods based on restriction fragment length polymorphism (RFLP).

We found that smoke and gender are the two variables that most influence the DNA damage. In particular, we observed that female smokers seem to be more sensitive than male smokers, having a significantly higher frequency of CAs. Moreover, a significant increase in frequency of micronuclei in bi-nucleated cells (BNMN) was found in smokers, but not in non-smokers. This increase seems to be influenced not only by age and gender, but also by genetic constitution. Subjects carrying *GSTM1-null* genotype seemed to have an higher susceptibility to DNA damage induced by tobacco smoke than *GSTM1*-positive ones. When considering a combination of GST genotypes, we found a lower BNMN frequency in subjects with *GSTP1* variant allele plus *GSTM1*-positive genotypes, while the most damaged cells are found in subjects bearing *GSTM1-null* plus *GSTP1*-wild type.

Our results suggest that investigation of the association between several gene polymorphisms and important endpoints of DNA damage could contribute to better understanding the role of gene–gene interaction.

Keywords: Tobacco smoke; Gene polymorphisms; Glutathione *S*-transferase; Chromosome aberrations; Micronuclei; Comet assay

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1. Introduction

Tobacco smoking is one of the major public health problems in developed and developing countries. In 2002, the World Health Organization has estimated that over a billion people are users of tobacco [1]. Tobacco smoke is made up of a vapour phase including gases, carbon dioxide, nitrogen and oxygen, and a particulate phase that contains at least 3500 chemical compounds [2]. Among these chemicals there are at least 55 agents that have been characterized as carcinogens by the IARC, such as polycyclic aromatic hydrocarbons (PAHs), *N*-nitrosamines, aromatic amines, aldehydes, etc. [3]. The smoking habit is considered to be an important means of exposure to genotoxins for humans [4,5] and it is associated with a higher incidence of various types of cancer [6].

The carcinogenic effects of tobacco smoke depend on the activity and efficiency of metabolic and detoxification pathways; in fact, only 20% of smokers develop lung, bladder or head and neck cancer [7]. This suggests the involvement of host-related factors in cancer outcome. Currently, epidemiological interest has been focused on the role of genetic polymorphisms in enzymes involved in the metabolism of xenobiotics, as a substantial component of individual susceptibility or resistance to cancer. This observation arises from the hypothesis that variations in the coding and regulating sequences of these genes could affect their expression and the activity pattern of the corresponding enzymes. In this way, genetic differences in the ability to activate and inactivate xenobiotics could account for inter-individual susceptibilities to smoking-related biological effects [4], which could influence the results of genotoxicity tests used in human biomonitoring studies [8,9] and the outcome of tobacco-related tumorigenesis [10].

Human glutathione *S*-transferases (GSTs) belong to a multi-gene family of four different classes of detoxification isozymes (α, μ, π and θ) [11], which are involved in detoxification of xenobiotics by conjugating a wide range of different chemicals with reduced glutathione (GSH). GST M1 (μ, mu class), T1 (θ, theta), P1 (π, pi) and A1 (α, alpha) are known to be polymorphic.

The *GSTM1* gene is involved in the detoxification of diol epoxides derived from the metabolism of hydrocarbons (PAH) in cigarette smoke. There is evidence that a deletion in both alleles, affecting 50% of Caucasians and causing a total lack of enzyme activity, is weakly associated with lung cancer in smokers [12,13] and that the risk could be higher in *GSTM1-null* females than in males [14]. However, recent meta-analyses and pooled analyses did not confirm these results [15,16].

A similar deletion polymorphism is also present in the *GSTT1* gene, and recent studies indicated that the *GSTT1*-null genotype was associated with an increased risk for pancreatic [17] and prostate [18] cancer among smokers. A recent pooled analysis [19] indicated that *GSTT1*-null subjects have lower micronucleus frequencies than their *GSTT1*-positive counterparts and revealed a significant increase overall in MN frequency with age and gender, with females having higher MN frequencies than males.

Two other glutathione *S*-transferases, GSTP1 (pi class) and GSTA1 (alpha class), which are abundant in lung and liver, respectively [20,21], are important catalysts for glutathione conjugation with benzo(*a*)pyrene diol epoxide (BPDE) and other PAH-derived diol epoxides. The *GSTP1* gene has two different single-nucleotide polymorphisms (SNPs) in the coding region, which produce four different alleles: the wild-type allele *A (105Ile, 114Ala) and the variant alleles *B (105Val, 114Ala), *C (105Val, 114Val) and *D (105Ile, 114Val) [22]. These isoforms have different efficiencies in conjugating and metabolizing tobacco-smoke substrates, with GSTP1*C and *B being the most efficient [23,24].

The polymorphism of the *GSTA1* gene contains three linked base-substitutions in the promoter region, at positions −567, −69 and −52, which results in a differential expression [25] with a lower transcriptional activation reported for the *GSTA1* variant allele than for the *GSTA1* wild-type allele [11,26]. Recently, Ahn et al. [27] found that different *GSTA1* genotypes are related to breast cancer among current smokers.

Experimental evidence has demonstrated that tobacco smoke has clastogenic and aneugenic effects [5] leading to structural and numerical chromosome aberrations (CA) [28] and micronuclei (MN) [29] in many mammalian cell types (e.g., bone-marrow cells, peripheral blood erythrocytes, lymphocytes and lung cells).

Most of the biomonitoring studies on large populations that evaluated the DNA damage induced by tobacco smoking did not report any association between micronucleus formation and smoking [30]. A pooled re-analysis from the HUMN International Collaborative Project showed the necessity to discriminate between light smokers (<20 cigarettes per day) and heavy smokers (>30 cigarettes per day): only in the latter group there was an overall increase in micronucleus frequency [30]. However, an increased frequency of MN has been found in the tracheo-bronchial epithelium of smokers [31] and also in B-lymphocytes and suppressor/cytotoxic

T8-lymphocytes of smokers when compared with non-smokers [32]. Similarly, analyses with conventional and molecular techniques in a large population showed an increase of chromosome aberrations induced by tobacco smoke [33–36]. However, other groups have not confirmed such effects [37,38].

In the present work we studied the effects of tobacco smoke on DNA-damage induction in a population of 72 subjects of Caucasian ethnicity, and the correlation between the induced damage and some polymorphisms in genes that encode metabolic enzymes. We used two different cytogenetic assays, the MN and the CA assays. The combination of these techniques was intended to provide a broad analysis of DNA damage and possible aneugenic mechanisms; this approach is recommended for monitoring populations chronically exposed to genotoxic agents [39].

CA and MN are two useful biomarkers of genotoxicity [40], which give information on the persistent damage at the chromatid or chromosome level, identifying irreversible DNA damage. Many studies demonstrated that CA are involved in tumorigenic processes and that CA frequencies could be predictive of a potential cancer risk [41–43].

2. Materials and methods

2.1. Selection of subjects

Seventy-two healthy volunteers (25 smokers and 47 non-smokers, mean age 38.9 ± 8.7 and 34.3 ± 8.1 years, respectively) of Caucasian ethnicity were selected for the study. Among the smokers, 13 were females and 12 males; among the non-smokers, there were 30 females and 17 males. All subjects were individually interviewed by filling in the "personal health questionnaire" [44] for the evaluation of "lifestyle confounding factors". Individuals having potential confounding factors such as drug or alcohol consumption, recent radiodiagnostic exposure and major illnesses were excluded. All smokers reported to smoke more than 20 cigarettes per day.

2.2. Lymphocyte cultures

Peripheral blood samples were collected by venipuncture into heparinized tubes and lymphocyte cultures were initiated immediately for the CA and MN assay. For the lymphocyte culture, 0.5 ml whole blood was added to 4.5 ml RPMI 1640 medium (MP Biomedicals, Solon, OH), supplemented with 10% heat-inactivated foetal calf serum (Gibco-Invitrogen, Carlsbad, CA), 2% phytohaemagglutinin (Murex, Pomezia, Italy), 1.5% penicillin–streptomycin (5000 IU/ml and 5000 mg/ml, respectively) (Sigma, St. Louis, MO) and 1% L-glutamine (Sigma, St. Louis, MO). Cultures were grown at 37 °C.

2.3. Cytogenetic analyses

For the CA assay, cell cultures were fixed at 48 h according to standard methods, after a 90-min incubation with 0.2 μg/ml colcemid (Sigma). Air-dried metaphase spreads were stained by the conventional unbanded Giemsa method. A total of 200 well-spread metaphases containing (46 ± 1) centromeres were examined for each culture, on coded slides. Chromosome-type and chromatid-type aberrations were recorded.

For the MN study, cultures were incubated at 37 °C for 72 h. After 44 h of incubation, cytochalasin B (Cyt-B; Sigma) was added at a final concentration of 6 μg/ml to arrest cytokinesis. Air-dried preparations were stained by the conventional Giemsa method. The presence of micronuclei was evaluated by scoring a total of 1000 bi-nucleated (BN) cells with well-preserved cytoplasm for each donor. In addition, another 1000 lymphocytes were scored to evaluate the percentage of cells with 1, 2, 3, and 4 or more nuclei. The nuclear division index (NDI) was used for measuring cell-proliferation kinetics [45].

For each subject, all slides were scored in double-blind-coded fashion by two observers to mitigate technician variability, before the determination of the genotype.

2.4. DNA isolation and genotyping

Genomic DNA was extracted from 200 μl whole peripheral blood with the MasterPureTM Complete DNA Purification kit (Epicentre, Madison, WI), following the manufacturer's instructions. Genetic polymorphism analysis for the *GSTM1* and *GSTT1* genes was conducted with PCR-based assays, according to published methods [46–48].

GSTP1 and *GSTA1* genotype analysis was performed with an RFLP-PCR-based method. *GSTP1*B* polymorphism was determined according to the method described by Harries et al. [49], while *GSTA1* polymorphism was determined by modifying the method described by Coles et al. [25].

All genotype analyses were performed on at least two separate occasions with appropriate positive controls, and only genotypes that showed consistent results were accepted.

2.5. Statistical analysis

The Kolmogoroff–Smirnoff test showed significant departure from the normal distribution for all the biomarker parameters. We used the Chi-squared test to verify the Hardy–Weinberg equilibrium of the polymorphic alleles analysed.

The significance of differences between the two groups – smokers and non-smokers – was performed by means of multivariate analysis considering age, gender and smoke exposure as independent variables. Moreover, the same test was used to analyse the association of each biomarker with all genotypes considered.

To test the association between biomarkers and each single genotype, the Mann–Whitney *U*-test (unpaired) was

employed. Then, we tested the association of BNMN levels with a particular genotype in two-gene combinations, by means of the Mann–Whitney *U*-test (unpaired) to minimize random significances. Combinations of genes were the following: *GSTM1-GSTT1*, *GSTM1-GSTP1*, *GSTM1-GSTA1*, *GSTT1-GSTP1*, *GSTT1-GSTA1*, and *GSTP1-GSTA1*.

All statistical analyses were performed using the GraphPad InStat version 3.00, GraphPad Software, San Diego, CA, USA.

3. Results

Mean frequencies of chromatid aberrations (CtAs), chromosome aberrations (CsAs), and micronuclei in bi-nucleated cells (BNMN) were measured in peripheral blood lymphocytes from smokers and non-smokers. Results are reported in Tables 1 and 2, respectively.

Table 3 shows the distribution and the related frequencies of GSTs genotypes in the entire population and in different subgroups. The frequencies of the analysed polymorphic alleles were in Hardy–Weinberg equilibrium ($P > 0.1$ with Chi-squared test for each genotype) and consistent with literature data [27,16].

Multivariate analysis (Table 4) on the whole population showed that among the biomarkers considered in this study, the chromatid aberrations, the total chromosome aberrations and the BNMN were affected by smoke and gender, while a significant influence of age was noted only for the BNMN frequency.

Table 5 summarizes the mean values of chromosomal damage in the entire population, subdivided according to smoking habit and gender. Smokers showed a significant increase in micronuclei compared with non-smokers ($P < 0.05$, Mann–Whitney *U*-test). Comparing female smokers with male smokers, we found significantly higher values in females for all chromosome-aberration parameters. In contrast, no difference between females and males was observed among non-smokers. Furthermore, we found that female smokers showed significantly higher values of all chromosome-aberration types compared with females who did not smoke.

Within the smoker population, we found a significant difference in MN frequency between female and male subjects, with females having the highest value (Table 5). Moreover, female smokers showed an

Table 1
Chromosome aberrations and micronuclei frequencies in smokers

Subject	Gender	Age	CsA (%)	CtA (%)	CAtot (%)	BNMN (‰)	NDI
S1	M	45	0	1	1	3	1.6
S2	F	37	4	5	9	10	1.9
S3	F	35	0	9	9	5	1.8
S4	F	26	0	3	3	12	1.7
S5	M	35	1	0.5	1.5	4	1.6
S6	M	43	0.5	1	1.5	9	1.6
S7	M	40	0	2	2	3	1.9
S8	F	24	1	1	2	18	1.6
S9	F	52	2	1	3	14	2.1
S10	F	25	1	2	3	2	1.6
S11	M	42	0	1	1	7	1.7
S12	M	43	1.5	0.5	2	3	1.7
S13	F	31	1	14	15	15	2.2
S14	M	47	0.5	0	0.5	8	1.8
S15	M	41	1	0	1	9	1.5
S16	M	50	0	0.5	0.5	1	1.7
S17	M	40	0.5	1.5	2	10	1.8
S18	F	29	1	4	5	8	1.6
S19	F	44	2	10	12	12	1.8
S20	M	37	0	1.5	1.5	3	1.5
S21	F	40	3	6	9	11	1.7
S22	F	56	3	1	4	10	1.6
S23	M	50	0.5	1	1.5	8	1.8
S24	F	33	3	5	8	11	1.6
S25	F	28	1	4	5	11	1.9
Mean (S.D.)		38.92 (8.7)	1.1 (1.1)	3 (3.5)	4.1 (3.9)	8.3 (4.4)	1.7 (0.1)

CsA: chromosome-type aberration; CtA: chromatid-type aberration; CAtot: total chromosome aberrations; BNMN: bi-nucleated cells with micronuclei; NDI: nuclear division index; (S.D.): standard deviation.

Table 2
Chromosome aberrations and micronuclei frequencies in non-smokers

Subject	Gender	Age	CsA (%)	CtA (%)	CAtot (%)	BNMN (‰)	NDI
NS1	F	34	0	0.5	0.5	4	1.7
NS2	F	41	1.5	1	2.5	9	1.6
NS3	F	45	0	0	0	17	1.7
NS4	F	46	0.5	0.5	1	3	2
NS5	F	25	2	3	5	6	1.6
NS6	F	31	0	0	0	6	1.8
NS7	F	43	0.5	0.5	1	7	1.3
NS8	F	43	1.5	1.5	3	7	1.7
NS9	F	25	0.5	0.5	1	2	2.1
NS10	F	25	0.5	0	0.5	3	1.4
NS11	F	40	2	2	4	8	1.8
NS12	F	29	0.5	1	1.5	5	2.1
NS13	F	25	0	1	1	4	1.8
NS14	F	25	0	4	4	4	1.9
NS15	F	25	0	2	2	5	1.7
NS16	F	28	0	2	2	6	1.8
NS17	F	44	0	1	1	15	1.6
NS18	F	42	1	5	6	14	1.8
NS19	F	45	0	3	3	11	1.6
NS20	F	40	0	1	1	6	1.8
NS21	F	24	1	3	4	8	2
NS22	F	40	2	2	4	11	1.7
NS23	F	28	1	2	3	8	2
NS24	F	29	2	3	5	3	1.8
NS25	F	29	2	4	6	8	1.7
NS26	F	40	4	1	5	2	1.7
NS27	F	35	0	3	3	8	1.6
NS28	F	23	2	1	3	4	1.8
NS29	F	30	1	1	2	7	1.7
NS30	F	56	0	2	2	12	1.5
NS31	M	29	1	1.5	2.5	6	1.5
NS32	M	31	0	1	1	4	1.7
NS33	M	25	0.5	0	0.5	2	1.6
NS34	M	24	0	0	0	1	1.6
NS35	M	39	0.5	0.5	1	3	1.8
NS36	M	26	0.5	2	2.5	2	1.6
NS37	M	40	1.5	2	3.5	2	1.3
NS38	M	37	2.5	1.5	4	8	1.8
NS39	M	30	1	0.5	1.5	11	1.9
NS40	M	43	0	2.5	2.5	3	1.4
NS41	M	32	1.5	1.5	3	2	1.8
NS42	M	32	1	0.5	1.5	1	1.9
NS43	M	43	0.5	1	1.5	6	1.9
NS44	M	32	1.5	0.5	2	5	1.7
NS45	M	44	2.5	1	3.5	2	1.7
NS46	M	30	1	1	2	3	1.4
NS47	M	43	5.5	0.5	6	5	1.3
Mean (S.D.)		34.36 (8.07)	1 (1.1)	1.5 (1.2)	2.5 (1.6)	5.9 (3.8)	1.7 (0.2)

CsA: chromosome-type aberration; CtA: chromatid-type aberration; CAtot: total chromosome aberrations; BNMN: bi-nucleated cells with micronuclei; NDI: nuclear division index; (S.D.): standard deviation.

increased micronucleus frequency compared with non-smokers.

As far as the influence of the genotype on chromosomal damage is concerned, a significant correlation was found only between the BNMN frequency, in smokers, and *GSTM1* and *GSTP1* genotypes (Table 4). Fig. 1 shows the correlation found between the mean BNMN value and *GSTM1* and *GSTP1* polymorphism in smok-

Table 3
Distribution of *GSTM1*, *GSTT1*, *GSTP1* and *GSTA1* genotypes

	GSTM1			*GSTT1*			*GSTP1*				*GSTA1*			
	Null	Present	Null frequency	Null	Present	Null frequency	I105I	I105V	V105V	V105V frequency	C-69C	C-69T	T-69T	T-69T frequency
All ($n = 72$)	39	33	0.54	20	52	0.27	37	31	4	0.27	30	33	9	0.35
Non-smokers ($n = 47$)	24	23	0.51	13	34	0.27	23	22	2	0.27	20	21	6	0.35
Smokers ($n = 25$)	15	10	0.60	7	18	0.28	14	9	2	0.26	10	12	3	0.36
Females ($n = 43$)	24	19	0.56	11	32	0.25	24	17	2	0.24	18	19	6	0.36
Males ($n = 29$)	15	14	0.52	9	20	0.31	13	14	2	0.31	12	14	3	0.34

Table 4
Multivariate analysis in the study population

Population	Biomarker[a]	Independent variables[b]	Partial R^2	R^2	P-Value
All ($n=72$)	CtA	Gender	0.0012		**0.0048**
		Smoking habit	0.0381		**0.0003**
				0.30	0.0002*
	CAtot	Gender	0.0012		**0.0054**
		Smoking habit	0.0381		**0.0001**
				0.32	0.0001*
	BNMN	Age	0.0391		**0.0063**
		Gender	0.0012		**<0.0001**
		Smoking habit	0.0381		**0.0198**
				0.40	<0.0001*
Non-smokers ($n=47$)	BNMN	Age	0.1248		**<0.0001**
		Gender	0.1550		**0.0057**
				0.55	<0.0001*
Smokers ($n=25$)	BNMN	Gender	0.2635		**0.0287**
		GSTM1^{null}	0.2944		**0.0469**
		GSTP1^{I105V}	0.1531		**0.0383**
				0.63	0.0338*

[a] Biomarkers used as dependent variables: CtA, CsA, CAtot, BNMN.
[b] Independent variables used in the model: age, gender, smoking habit, *GSTM1^{null}*, *GSTT1^{null}*, *GSTP1^{I105V}*, *GSTA1$^{C\text{-}69T}$*.
* Significance level in the model: values in bold are P-values for each of the independent variables.

Table 5
Mean values of CA and MN according to gender and smoking habit

Subjects	Gender	CsA (%)	CtA (%)	CA tot (%)	BNMN (‰)
Smokers	All	1.1 (1.1)	3.0 (3.5)	4.1 (3.9)	8.3* (4.4)
	Female ($n=13$)	1.7$^{a\Omega}$ (1.3)	5$^{b\Phi}$ (3.9)	6.7$^{c\Psi}$ (3.9)	10.7$^{a\Omega}$ (4.1)
	Male ($n=12$)	0.5 (0.5)	0.9 (0.6)	1.3 (0.5)	5.7 (3.1)
Non-smokers	All	1.0 (1.1)	1.5 (1.2)	2.5 (1.6)	5.9 (3.8)
	Female ($n=30$)	0.9 (0.9)	1.7 (1.3)	2.5 (1.7)	7.4 (3.9)
	Male ($n=17$)	1.2 (1.3)	1.0 (0.7)	2.2 (1.5)	3.9 (2.7)

CsA: chromosome-type aberration; CtA: chromatid-type aberration; CAtot: total chromosome aberrations; BNMN: bi-nucleated cells with micronucleus. Values in parentheses are S.D. values. *Direct comparison between smokers and non-smokers, *$P<0.05$. Latin letters for direct comparison of female smokers vs. male smokers: [a]$P<0.01$; [b]$P<0.001$; [c]$P<0.0001$. Greek letters for direct comparison between female smokers versus female non-smokers. $^{\Omega}P<0.05$;$^{\Phi}P<0.01$;$^{\Psi}P<0.001$; P-value based on Mann–Whitney U-test.

Table 6
Mean values ± S.D. for BNMN frequencies stratified for a combination of *GSTM1* and *GSTP1* polymorphisms in smokers and non-smokers

Genotypes		Non-smokers		Smokers	
GSTM1	*GSTP1* 105 Ile → Val	No. of subjects	BNMN mean values ± S.D.	No. of subjects	BNMN mean values ± S.D.
Pos	Wt	11	5.3 ± 3.7	6	7.2 ± 4.5
Null	Wt	12	5.7 ± 4.3	8	**11.6 ± 3.4**
Pos	Var	12	6.2 ± 4.1	4	4.7 ± 3.9
Null	Var	12	7.2 ± 3.9	7	7.4 ± 3.8

Pos: positive; Var: variant (Ile105Val or Val105Val) genotypes; Wt: wild-type genotypes. *$P \leq 0.05$ for direct comparison among subjects with *GSTM1* null/*GSTP1* wt vs. *GSTM1*-positive/*GSTP1* variant genotypes. P-value based on Mann–Whitney U-test.

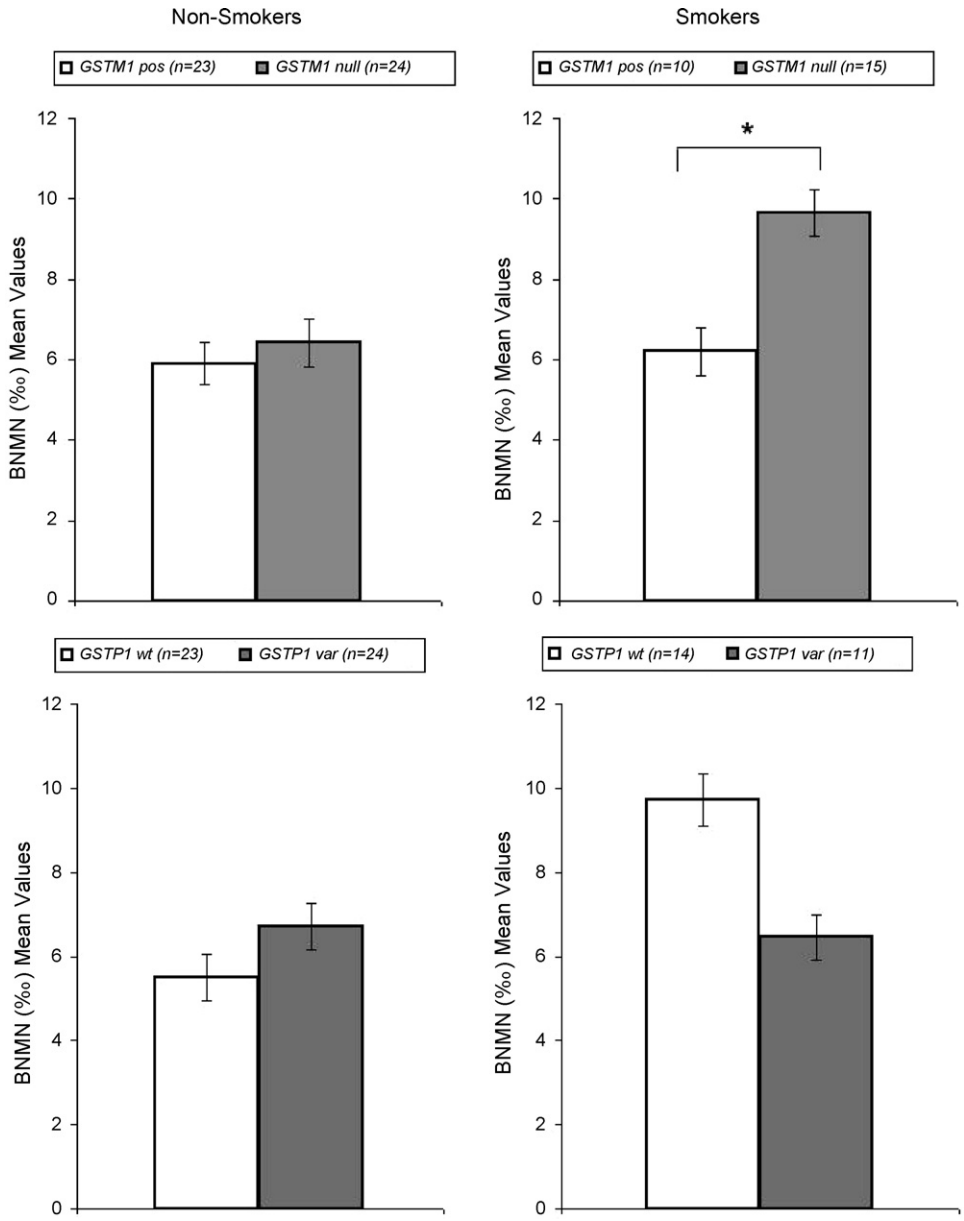

Fig. 1. Effect of *GSTM1* and *GSTP1* gene polymorphisms on BNMN values. *BNMN*: bi-nucleated cells with micronuclei; *Pos*: positive; *wt*: wild type; *var*: variant allele; $*P < 0.05$ based on Mann–Whitney *U*-test.

ers and non-smokers: smokers carrying the *GSTM1-null* genotype showed a significantly higher frequency of micronuclei compared with *GSTM1*-positive subjects ($P < 0.05$); no association was found in non-smokers. As far as *GSTP1* genotype is concerned, a slight but not significant increase was found comparing carriers of the wild-type genotype with carriers of the variant genotypes.

In Table 6 we show that a particular gene combination seems to influence BNMN frequencies in smokers, but not in non-smokers. Subjects bearing *GSTM1-null*/*GSTP1*-wild-type genotype have the highest BNMN value compared with the other three genotype combinations, showing a significant difference between *GSTM1-null*/*GSTP1*-wild-type subjects and *GSTM1*pos/*GSTP1*variant.

4. Discussion

In this study we have analysed the influence of some polymorphic genes of the glutathione *S*-transferase (GST) super-family (*GSTM1*, *GSTT1*, *GSTP1* and *GSTA1*) on the modulation of the DNA damage induced by tobacco smoke.

We found that smoke and gender are the two variables that have the strongest effect on the DNA damage, which was evaluated by means of chromosome aberration and micronucleus assays. In particular, we observed that female smokers seem to be more sensitive than male smokers, having a significantly higher frequency of chromosome aberrations, confirming previous literature data [50–52] and supporting the hypothesis for a gender difference in sensitivity to tobacco carcinogens. The underlying cause is not completely clarified, but a possible explanation could be found in the reduced capacity of early repair of the DNA damage induced by tobacco carcinogens, as was suggested by Hill et al. [52], or in an interaction between smoke and hormones (estrogens) [53]. This marked genotoxic effect induced by smoke exposure in females could be related to their assessed higher susceptibility to develop lung cancer [54,55].

We found a significant increase of DNA damage in smokers compared with non-smokers only when considering BNMN values. This increase seems to be influenced not only by age and gender as previously reported [56], but also by the genetic constitution. In particular, our results indicate that *GSTM1-null* smokers have a higher BNMN frequency than *GSTM1*-positive smokers.

An analogous effect was previously described, when an increase was reported in CA and SCE frequencies in peripheral blood lymphocytes of smokers deficient in GSTM1 [57–61], but these data were not confirmed by others [62,63]. Little is known about the role of GSTP1*A and GSTP1*B in individual response to tobacco smoke, even if GSTP1*B appears to be more efficient than GSTP1*A in conjugating PAH diol epoxides [64].

However, the correlation between BNMN, tobacco smoke and genotype is still controversial [19,30–32,40], which is probably due to the difficulty in interpreting these relationships. Moreover, the amount of DNA damage seems to be highly influenced by a particular combination of GST genotypes. In fact, we observed a significant correlation between BNMN frequency and the *GSTM1* plus *GSTP1* polymorphic genotype combination.

A lower frequency of BNMN was found in subjects carrying the *GSTP1* variant allele in combination with the *GSTM1*-positive genotype. The subjects with the highest level of DNA damage carried a combination of *GSTM1-null* and *GSTP1*-wild type, suggesting that the *GSTP1*-wild-type isoform is less active in conjugating PAH diol epoxides than the variant enzyme.

Understanding the role of different genotype combinations on the expression of DNA damage is relevant in particular when the complex network of metabolic pathways is considered. Until now, only a few studies have investigated the effects of genotype combinations on modulating the levels of genotoxicity biomarkers [65–67]. Regarding the influence of *GSTM1* and *GSTP1* genotype combinations on the DNA damage induced by tobacco-smoke exposure, Butkiewicz and co-authors found that the combined *GSTM1* and *GSTP1* genetic polymorphisms could modulate PAH-DNA adduct levels [68].

In conclusion, we found a significant correlation between the BNMN induced by tobacco-smoke exposure and the combination of two xenobiotic-metabolism genes, *GSTM1* and *GSTP1*. Although based on a small study population, our results suggest that investigations into the association between multiple gene polymorphisms and important endpoints of DNA damage could contribute to a better understanding of the role of gene–gene interactions during genotoxic processes than would result from studying single-gene effects.

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