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ORIGINAL CONTRIBUTION

Vitamin C levels in blood are influenced by polymorphisms in glutathione S-transferases

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Abstract
Purpose Glutathione S-transferases (GSTs) are intimately involved in combating oxidative stress and in detoxifying xenobiotics. Our objective was to examine possible interactions between polymorphisms in *GST* genes and plasma vitamin C, tocopherols and carotenoids in 149 reference subjects and 239 subjects occupationally exposed to mineral fibres (asbestos, rock wool, glass fibre), agents that induce oxidative stress.
Methods Deletion of *GSTM1* and *GSTT1*, and substitution 105Ile/Val in *GSTP1* genes were determined by PCR, antioxidants in plasma were measured by HPLC.
Results Tocopherols and carotenoids were affected by age, sex, smoking, occupational exposure to fibres, but not by *GST* polymorphisms. Vitamin C level was influenced by sex, smoking and occupational exposure. Subjects with deletion of *GST* had lower vitamin C levels compared with subjects carrying the functional gene variant. Vitamin C levels varied according to *GSTM1* polymorphism in the whole group ($p < 0.05$), in all reference subjects ($p < 0.05$), in the asbestos factory reference group ($p < 0.05$), and according to *GSTT1* polymorphism in reference group of the rock wool plant ($p < 0.05$). Vitamin C levels were approximately 20% lower in subjects with both functionally deficient genes in the whole group ($p < 0.01$) and in all non-exposed subjects ($p < 0.05$).
Conclusions The correspondence of lower vitamin C levels with non-functional GST isoenzymes may indicate a causal connection between two antioxidant defence pathways, also the underlying mechanism is not yet clear. It seems that supplementation by natural antioxidants is particularly important for subjects with unfavourable genetic makeup and in those exposed to oxidative stress.

Keywords GST polymorphisms · Antioxidants · Vitamin C · Oxidative stress · Exposure to mineral fibres · Individual susceptibility

Introduction

Oxidative stress occurs due to imbalance between cellular oxidants and antioxidant compounds, and it has been implicated in the pathogenesis of number of diseases. Reactive oxygen species (ROS) can damage nucleic acids, proteins and lipids and induce cellular dysfunctions. To prevent free radical-induced cellular damage, organisms have developed the complex antioxidant defence mechanism consisted of antioxidant enzymes, GSH/GSSG redox system with low-molecular antioxidants and some plasma proteins. The major exogenous antioxidants are represented by vitamin E, vitamin C and carotenoids [1]. The general defence network is completed by cooperation of immune system, repair and biotransformation machinery.

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Vitamin C belongs to the group of non-enzymatic, water-soluble antioxidants, which can scavenge radicals from a variety of sources and react with singlet oxygen [2]. It has a number of metabolically important cofactor functions in enzyme reactions, notably hydroxylation [3]. L-Ascorbate can reduce tocopheroxyl radicals directly or indirectly and thus support the antioxidant activity of vitamin E [4].

Vitamin C levels may vary in different countries. Babinska et al. [5] found suboptimal levels of vitamin C in the Slovak population: the mean level of vitamin C was 31.4 μmol/L in males (95% CI 30.1–32.7), while the value observed in females was significantly higher (43.1 μmol/L; 95% CI 41.8–44.4), but still in the range of suboptimal values (23–50 μmol/L) for vitamin C. This chronic marginal deficiency for vitamin C (and other non-enzymatic antioxidants) puts the Slovak population into the group of increased risk for oxidative stress-related diseases.

The glutathione S-transferases (GSTs) belong to large, functionally diverse family of enzymes, assigned in humans to 6 distinct classes: alpha, mu, theta, pi, zeta and omega [6–9]. Isoforms are differentially expressed in a tissue-specific manner, and the composition of enzymes in various tissues differs significantly [10]. GSTs carry out a wide range of functions in cells; removal of ROS and regeneration of S-thiolated protein (as consequences of oxidative stress), conjugation with endogenous ligands as well as reactions in metabolic pathways not associated with detoxification [11]. As phase II biotransformation enzymes, they catalyse conjugation of reduced glutathione (GSH) to electrophilic centres on a wide range of substrates [12], reactive endogenous and activated exogenous molecules, often including environmental pollutants and oxidized biomolecules, as reviewed in [13]. Quantitatively, conjugation to GSH catalysed by GSTs is the major detoxification pathway in humans. Expression of GSTs is induced by many of their substrate and non-substrate molecules, including phytochemicals and H_2O_2. Modulation of expression of detoxification enzymes is one mechanism by which diet may balance health status and modulate risk of cancer and other diseases [13, 14]. Polymorphisms in the detoxification enzymes that alter protein expression and/or function can modify risk in individuals exposed to the relevant substrates.

Several GST polymorphisms occur commonly in humans and have been associated with an increased or decreased susceptibility to certain cancers and other diseases, particularly when combined with other genetic and environmental factors such as smoking, exposure or lifestyle [15–21]. Deletion polymorphisms in the *GSTM1* and *GSTT1* genes, as important members of the GST family, result in complete lack of GSTM1 and GSTT1 proteins [22, 23]. In some observational studies of cancer, cruciferous vegetable intake confers greater protection in individuals with these polymorphisms [24]; however, in other studies, the opposite effect is observed [25]. Genetic polymorphisms in detoxification enzymes may account in part for individual variation in disease risk but have to be considered in the context of other aspects of human genetics and gene–environment interactions.

The biological potential of asbestos and other mineral fibres is determined by the length, shape, chemical composition and surface content of fibres. [26–29]. The mechanism of asbestos toxicity involves inflammation and oxidative stress, which can arise from direct generation of ROS on the fibre surface, and via activation of inflammatory cells capable to generate ROS and nitrogen (RNS) species [26, 30, 31]. There is considerable evidence that many other pneumotoxic particles act, at least in part, through mechanisms that involve production of ROS [32]. The surface of particles, with high adsorption capacity, may serve as a physical carrier of genotoxic substances and promote co-genotoxic actions of adsorbed organics [33]. Soluble compounds such as transition metals or organic molecules can also give rise to ROS, and organic molecules may serve as substrates for biotransformation enzymes and thus contribute to pathogenesis.

We were interested to know whether individual genetic variability in GSTs may influence the antioxidant status of organism, particularly levels of vitamin C, tocopherols and carotenoids, in populations exposed to asbestos, rock wool or glass fibres, i.e. agents that induce oxidative stress.

Materials and methods

Design of study

A sample of 388 volunteers, men and women, from 3 different industrial areas of western and middle part of Slovakia were recruited for this biomonitoring study. The three study groups involved 239 workers with at least 5 years of occupational exposure to asbestos (61), rock wool (98) or glass fibre (80), and 149 reference subjects from the administrative staff of each plant (21, 43 and 36, respectively). In the case of asbestos factory, 49 additional individuals from local residents were recruited. Reference volunteers were matched to exposed subjects by age, sex and smoking habit. Sampling was carried out in September–October 2000 (asbestos and rock wool factory), and in September 2001 (glass fibre factory). All participants were interviewed by trained personnel and answered detailed questionnaires relating to duration of exposure, smoking habit, alcohol consumption, lifestyle and diet. All workers underwent an additional clinical examination including a functional spirometry test, radiological and immunological

investigation. A sample of 30 mL of venous blood was collected after overnight fasting and processed immediately. Biomarkers of cellular antioxidant defences (antioxidants and antioxidant enzymes) and individual susceptibility (genetic polymorphisms of important detoxification enzymes) were measured. A urine sample (10 mL) was collected to measure cotinine, to confirm smoking status. All study participants signed an informed consent form before donating their specimens. This study was approved by the ethical committee of the Institute of Preventive and Clinical Medicine (present Slovak Medical University) and was performed according to Good Laboratory Practice. Samples were coded, and all measured parameters were performed blindly.

DNA isolation

Genomic DNA was isolated from frozen peripheral blood samples collected into potassium EDTA tubes (Sarstedt) using these standard steps: lysis of erythrocytes (0.32 M sucrose, 1% Triton X-100, 50 mM $MgCl_2$, 12 mM Tris pH = 12), destruction of lymphocytes (10% SDS, 5 M NaCl) followed by phenol–chloroform–isoamylalcohol extraction and ethanol precipitation.

Genotyping

Polymorphisms in genes for three glutathione S-transferase (GST) isoenzymes were analysed.

Deletion of *GSTM1* and *GSTT1* genes was detected by multiplex PCR, using β-globin gene as an internal positive control. PCR products were separated on 2.5% agarose gel in 1× TBE buffer (method modified from [23, 34]; for details, see Table 1). *GSTM1* gene deficiency was deduced from the absence of the specific 231-bp fragment, *GSTT1* gene deficiency from the absence of a 480-bp fragment. Presence of 268 bp β-globin fragment confirmed proper functioning of the method. In this method, heterozygous status cannot be distinguished from homozygous wild-type status.

For the *GSTP1* gene, a single nucleotide substitution A/G in coding region causing amino acid exchange Ile/Val in codon 105 was examined. Exon 5 of the gene was amplified, and substitution was identified by restriction analysis with BsmAI enzyme (NE Biolabs). Restriction products were separated on 3.5% agarose gel in 1× TBE ([35] for details, see Table 1). BsmAI recognizes variant (105Val) allele and digests 176-bp PCR product into fragments 91 and 85 bp.

Measurement of plasma antioxidants

Blood for antioxidants determination was collected into potassium EDTA tubes (Sarstedt), kept at 4 °C and

Table 1 Overview of methods for *GST* genotyping

Polymorphism	Primer sequences	PCR mixture	PCR conditions	Reference
GSTM1/GSTT1 (multiplex PCR)	*GSTM1 F:* 5′CGCCATCTTGTGCTACATTGCCCG 3′ *GSTM1 R:* 5′TTCTGGATTGTAGCAGATCA 3′ *GSTT1 F:* 5′TTCCTTACTGGTCCTCACATCTC 3′ *GSTT1 R:* 5′TCACCGGATCATGGCCAGCA 3′ *BGL F:* 5′CAACTTCATCCACGTTCACC 3′ *BGL R:* 5′GAAGAGCCAAGGACAGGTAC 3′	Reaction buffer (10 mM Tris–HCl pH = 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100), 0.033 mM dNTPs, 20 mM $NH_4(SO_4)_2$, 1.5 U DyNAzyme II DNA polymerase (Finnzymes) Primers: *GSTM1F* 0.8 µM, *GSTM1R*, *GSTT1F*, *GSTT1R* 0.67 µM each, *BGLF* and *BGLR* 0.33 µM, 100 ng of template DNA Final volume 30 µL	94 °C for 3 min, 35 cycles of (94 °C for 1 min, 61 °C for 1:30 min, 72 °C for 1 min) and 72 °C for 10 min Cycler PTC 200 (MJ RESEARCH)	[23, 34]
GSTP1 (105 Ile/Val) (RFLP)	*GSTP1 F:* 5′ACCCCAGGGCTCTATGGGAA 3′ *GSTP1 R:* 5′TGAGGGCACAAGAAGCCCCT 3′	Reaction buffer (10 mM Tris–HCl, pH = 8.8, 50 mM KCl, 0.1% Triton X-100), 1.7 mM $MgCl_2$, 0.02 mM dNTPs, 1.7 U DyNAzyme II DNA polymerase (Finnzymes), 0.63 µM each primer, 100 ng of template DNA Final volume 40 µL	94 °C for 3 min, 30 cycles of (94 °C for 30 s, 65 °C for 50 s, 72 °C for 50 s) and 72 °C for 10 min Cycler PTC 200 (MJ RESEARCH)	[35]

processed within 25 min. Blood samples were centrifuged for 10 min, 990*g* at 4 °C. Collected plasma was further processed, aliquoted and frozen in liquid nitrogen. Samples were stored up to 4 months at −80 °C until analysed.

Vitamin C

Plasma was stabilized by 10% metaphosphoric acid (HPO_3) before chilling in liquid nitrogen.

Level of L-ascorbic acid was measured by high-performance liquid chromatographic set-up consisted of a HP 1100 system (Hewlett-Packard) with diode-array detector, using LiChrospher 100 RP-18e (Merck) as analytical column, 3.7 mM KH_2PO_4 pH = 4.0 as mobile phase and detection at 245 nm. Data acquisition and analysis was achieved by HP3D ChemStation (Hewlett-Packard) [36].

Tocopherols, carotenoids

Lipophilic antioxidants were extracted from plasma using ethanol and hexane containing BHT (butylhydroxytoluene). An internal standard of retinyl palmitate was added to the mixture. Hexane fraction was dried under nitrogen and dissolved in DEA (1,4-dioxane/ethanol/acetonitrile).

α- and γ-tocopherol, β-carotene, retinol, lycopene and xanthophyll were measured by high-performance liquid chromatographic set-up consisted of a HP 1100 system (Hewlett-Packard) with visible light and fluorescence detectors connected in series, using Nucleosil C18 (Waters) as analytical column and precolumn and mixture of acetonitrile/tetrahydrofurane/methanol with BHT/ammonium acetate as mobile phase. Detection in visible light spectrum was performed at 450 nm; excitation/emission wavelengths 330/470 nm and 298/328 nm were applied for fluorescent detection [37].

Statistical analysis

Homogeneity of distribution was tested by the Kolmogorov-Smirnoff test. For normal distributed parameters, student *t* test for independent samples and the analysis of variance (ANOVA) were applied. The Mann–Whitney *U* test was applied for non-normal distributed parameters, and the chi square for categorical data. All tests were two-tailed. Statistical modelling was based on multiple regression analysis. All tests were performed at the significance level $p < 0.05$. SPSS statistical software (Ver. 13.0) was used to analyse data.

Note that small differences in study group size evidence, mentioned in Design of study and tables, occurred on account of missing values for particular analysed markers.

Results

The whole dataset included data from 388 subjects, 239 of them occupationally exposed to fibres, and 149 reference individuals. The mean age of exposed and reference subjects was 47 ± 1 (range 21–80) and 48 ± 1 (range 22–88) years, respectively. The whole study group showed a larger proportion of males (58.4%) and a remarkable prevalence of active smokers (38.2%). A wide range of biomarkers of exposure, effect and individual susceptibility was measured in the study group, and results were partially published [38–46]. Here, we report results of GST polymorphisms and levels of dietary antioxidants in plasma.

Polymorphisms in three GST isoenzymes, i.e., deletion polymorphism of *GSTM1* and *GSTT1* genes, and single nucleotide substitution 105Ile/Val in *GSTP1* gene were determined in all subjects. Relative frequencies of *GST* variants were 49.0% for *GSTM1* deletion, 16.7% for *GSTT1* deletion, 31.6% for *GSTP1*105Val* allele, and did not differ from that reported for other European populations [47–51]. No significant difference was found in the distribution of genotypes by exposure, smoking habit, age and gender (data not shown).

Set of important dietary antioxidants was measured in plasma of all volunteers.

Lipophilic antioxidants

We measured levels of non-polar antioxidants: α- and γ-tocopherol, β-carotene, retinol, lycopene and xanthophyll. The effect of age, gender, smoking habit, exposure to fibres and functional polymorphisms in *GST* genes on concentration of lipid-soluble antioxidants was explored using the univariate ANOVA.

The antioxidants in plasma of investigated subjects are influenced by age, sex, cigarette smoking and occupational exposure to fibres (Table 2). Concentrations of α-tocopherol ($p = 0.004$), γ-tocopherol ($p = 0.046$) and retinol ($p = 0.011$) were higher in older subjects compared to those younger than 40. Women had lower levels of γ-tocopherol ($p < 0.001$) and retinol ($p < 0.001$), and reversely higher levels of β-carotene ($p < 0.001$). Considerable decline of β-carotene ($p < 0.001$), lycopene ($p = 0.002$) and xanthophyll ($p = 0.037$) in smoking individuals was found. Plasmatic concentrations of β-carotene ($p = 0.001$) and lycopene ($p = 0.021$) were significantly lower in workers exposed to asbestos when compared to respective reference group.

The covariates that resulted statistically significant in the univariate ANOVA analysis were included in a multivariate model to test whether acted as confounders/effect modifiers of the action of *GST* polymorphisms with respect to antioxidants. Nevertheless, no noteworthy result was obtained from this analysis, except the *GSTP1*105Ile/Val*

Table 2 Concentration of lipophilic antioxidants in plasma (μmol/L) by major covariates and GST polymorphisms

Covariates	Size	α-Tocopherol Mean (SE)	γ-Tocopherol Mean (SE)	β-Carotene Mean (SE)	Retinol Mean (SE)	Lycopene Mean (SE)	Xanthophyll Mean (SE)
Age							
≤40	108	**18.1 (0.6)**	**2.36 (0.14)**	0.31 (0.02)	**2.03 (0.05)**	0.43 (0.02)	0.35 (0.01)
41–50	134	19.7 (0.5)	**2.81 (0.18)**•[b]	0.29 (0.02)	2.08 (0.04)	0.42 (0.01)	0.36 (0.01)
51+	146	**20.5 (0.6)**•[a]	2.67 (0.13)	0.36 (0.02)	**2.19 (0.04)**•[c]	0.42 (0.01)	0.38 (0.01)
Gender							
Male	227	19.6 (0.5)	**2.90 (0.14)**	**0.25 (0.01)**	**2.19 (0.03)**	0.41 (0.01)	0.36 (0.01)
Female	161	19.4 (0.5)	**2.25 (0.09)**•[d]	**0.43 (0.02)**•[d]	**2.00 (0.03)**•[d]	0.44 (0.01)	0.36 (0.01)
Smoking habit							
No	240	19.6 (0.4)	2.50 (0.11)	**0.37 (0.02)**	2.13 (0.03)	**0.44 (0.01)**	**0.37 (0.01)**
Yes	148	19.5 (0.5)	2.83 (0.16)	**0.25 (0.01)**•[d]	2.08 (0.04)	**0.39 (0.01)**•[e]	**0.35 (0.01)**•[f]
Asbestos factory							
Ref. group	70	20.5 (0.9)	2.53 (0.29)	**0.45 (0.03)**	2.33 (0.06)	**0.46 (0.02)**	0.38 (0.01)
Exposed	61	18.7 (0.8)	2.87 (0.24)	**0.30 (0.03)**•[g]	2.34 (0.07)	**0.40 (0.02)**•[h]	0.38 (0.02)
Rock wool factory							
Ref. group	43	18.7 (1.1)	2.40 (0.16)	0.29 (0.04)	2.07 (0.07)	0.44 (0.03)	0.34 (0.02)
Exposed	98	20.0 (0.7)	2.98 (0.19)	0.24 (0.02)	2.12 (0.05)	0.41 (0.01)	0.37 (0.01)
Glass fibre factory							
Ref. group	36	19.5 (0.9)	2.35 (0.23)	0.39 (0.06)	1.85 (0.06)	0.40 (0.03)	0.33 (0.02)
Exposed	80	19.1 (0.5)	2.36 (0.12)	0.31 (0.02)	1.88 (0.04)	0.42 (0.02)	0.36 (0.01)
GSTM1							
Present	197	19.7 (0.5)	2.59 (0.11)	0.33 (0.02)	2.11 (0.03)	0.42 (0.01)	0.37 (0.01)
Deletion	190	19.4 (0.5)	2.67 (0.14)	0.31 (0.02)	2.11 (0.03)	0.42 (0.01)	0.35 (0.01)
GSTT1							
Present	323	19.4 (0.4)	2.63 (0.10)	0.32 (0.01)	2.10 (0.03)	0.42 (0.01)	0.36 (0.01)
Deletion	64	20.3 (0.8)	2.61 (0.17)	0.33 (0.04)	2.16 (0.06)	0.43 (0.02)	0.37 (0.02)
GSTP1							
105 Ile/Ile	184	19.4 (0.5)	2.71 (0.15)	0.32 (0.02)	**2.06 (0.04)**	0.42 (0.01)	0.36 (0.01)
105 Ile/Val	160	19.8 (0.5)	2.58 (0.10)	0.33 (0.02)	**2.19 (0.04)**•[i]	0.42 (0.01)	0.37 (0.01)
105 Val/Val	42	18.6 (1.0)	2.45 (0.30)	0.30 (0.03)	2.06 (0.07)	0.43 (0.02)	0.35 (0.02)
Total	388	19.5 (0.3)	2.63 (0.09)	0.32 (0.01)	2.11 (0.02)	0.42 (0.01)	0.36 (0.01)

SE standard error of mean

• Within covariates comparison: *T* test/ANOVA *p*-value <0.05 with respect to first level

[a] $p = 0.004$

[b] $p = 0.046$

[c] $p = 0.011$

[d] $p < 0.001$

[e] $p = 0.002$

[f] $p = 0.037$

[g] $p = 0.001$

[h] $p = 0.021$

[i] $p = 0.008$

subjects showing higher level of retinol (mean difference 0.11 μmol/L, $p = 0.027$) compared to wild-type carriers. However, this trend was not found in *GSTP1*105Val/Val* subjects, neither in any other group nor in subgroup of exposed or reference subjects.

Vitamin C

The relationship between vitamin C level and major covariates was initially explored with the univariate analysis.

Table 3 Concentration of vitamin C in plasma (μmol/L) by GST polymorphism and major covariates

Covariates	Total		*GSTM1*		*GSTT1*		*GSTP1*		
	Size	Mean (SE)	Present 196 (51.0%) Mean (SE)	Deletion 188 (49.0%) Mean (SE)	Present 320 (83.3%) Mean (SE)	Deletion 64 (16.7%) Mean (SE)	105 Ile/Ile 183 (47.7%) Mean (SE)	105 Ile/Val 158 (41.3%) Mean (SE)	105 Val/Val 42 (11.0%) Mean (SE)
Age									
≤40	108	30.7 (0.8)	30.7 (1.2)	30.6 (1.1)	31.1 (0.9)	28.1 (2.1)	31.3 (1.3)	31.0 (1.1)	26.0 (3.2)
41–50	133	31.1 (0.9)	32.6 (1.4)	29.8 (1.3)	31.4 (1.1)	29.3 (2.0)	31.8 (1.5)	30.0 (1.4)	31.2 (2.1)
51+	144	28.3 (0.9)	30.0 (1.3)	26.3 (1.3)	28.6 (1.0)	27.1 (2.3)	28.1 (1.3)	27.6 (1.6)	31.9 (2.2)
Gender									
Male	225	**27.9 (0.7)**	29.0 (1.0)	27.0 (0.9)	28.5 (0.7)	25.5 (1.5)	28.0 (1.1)	27.8 (1.0)	28.3 (1.8)
Female	160	**32.7 (0.8)**•[a]	33.5 (1.1)	31.8 (1.2)	32.8 (0.9)	32.5 (1.9)	32.9 (1.2)	32.4 (1.4)	32.7 (2.2)
Smoking habit									
No	238	**31.3 (0.6)**	31.7 (0.9)	30.8 (1.0)	31.5 (0.7)	30.3 (1.5)	31.4 (1.0)	30.8 (1.0)	33.0 (1.7)
Yes	147	**27.7 (0.9)**•[b]	29.4 (1.5)	26.5 (1.1)	28.4 (1.0)	24.4 (2.1)	28.5 (1.4)	26.9 (1.4)	27.0 (2.1)
Asbestos factory									
Ref. group	68	**30.6 (1.3)**	**33.1 (1.7)**	**27.8 (1.8)***[d]	31.3 (1.4)	28.3 (3.1)	30.8 (1.6)	29.5 (2.4)	34.2 (2.8)
Exposed	60	**25.3 (1.6)**•[c]	26.9 (2.6)	23.8 (2.0)	26.1 (1.6)	19.3 (3.9)	25.3 (2.2)	24.6 (2.7)	29.0 (5.9)
Rock wool factory									
Ref. group	43	35.1 (1.5)	37.1 (2.1)	33.3 (2.0)	**36.6 (1.5)**	**27.1 (3.7)***[e]	36.2 (2.4)	33.5 (2.2)	37.1 (3.2)
Exposed	98	32.8 (0.8)	33.8 (1.2)	31.8 (1.2)	32.8 (0.9)	32.7 (2.1)	34.1 (1.4)	31.3 (1.1)	33.1 (1.7)
Glass fibre factory									
Ref. group	36	28.4 (1.7)	30.1 (2.8)	26.9 (1.9)	28.7 (1.9)	27.6 (4.1)	27.8 (2.8)	29.3 (2.7)	28.0 (2.7)
Exposed	80	27.2 (1.1)	26.6 (1.2)	27.9 (1.9)	27.1 (1.2)	27.8 (2.3)	27.9 (1.7)	27.5 (1.4)	23.8 (3.0)
Total	385	29.9 (0.5)	**31.0 (0.8)**	**28.8 (0.7)***[f]	30.3 (0.6)	28.1 (1.3)	30.3 (0.8)	29.4 (0.8)	30.3 (1.4)

SE standard error of mean

* Within polymorphisms comparison: *T* test/ANOVA *p*-value <0.05 with respect to wild-type (present gene)

• Within covariates comparison: *T* test/ANOVA *p*-value <0.05 with respect to first level

[a] $p < 0.001$

[b] $p = 0.001$

[c] $p = 0.011$

[d] $p = 0.037$

[e] $p = 0.015$

[f] $p = 0.042$

Vitamin C level measured in plasma of investigated subjects is influenced by sex, cigarette smoking and occupational exposure to fibres. L-Ascorbate was significantly higher in women ($p < 0.001$) and in non-smokers ($p = 0.001$). The concentration of vitamin C was significantly lower in workers exposed to asbestos when compared to respective reference subjects ($p = 0.011$, Table 3).

Subjects with the deletion of *GST* gene had lower level of vitamin C in plasma compared with subjects carrying functional gene. Vitamin C levels differed by *GSTM1* gene polymorphism in the whole study group (28.8 μmol/L in *GSTM1* deleted, vs. 31.0 in *GSTM1* + subjects, $p = 0.042$). After stratification by major covariates, significant differences in vitamin C level were found within the reference group of the asbestos factory (27.8 μmol/L in *GSTM1* deleted, vs. 33.1 in *GSTM1* + subjects, $p = 0.037$), and in the reference group of the rock wool plant (27.1 μmol/L in *GSTT1* deleted vs. 36.6 in *GSTT1* + subjects, $p = 0.015$). No association between *GSTP1* polymorphic variants and vitamin C in plasma was observed in this study (Table 3).

Changes in vitamin C level by *GST* polymorphisms and by major covariates were tested by multiple regression analysis of the whole study group (385 subjects) as well as reference group (146 subjects). As regards potential confounders included in the model, no significant effect of age was observed, while a highly statistically significant difference was found between men and women, with the latter reporting a vitamin C level of 4.57 μmol/L higher than men (95% CI 2.45–6.68; $p < 0.001$). Similarly, the effect of smoking was extremely evident, with smokers reporting a vitamin C level considerably lower (−2.72 μmol/L) than non-smokers (95% CI −4.79: −0.65; $p = 0.01$). The level of vitamin C in workers exposed to natural or artificial fibres, after comparison with the pool of reference subjects, showed statistically significant decreases in the asbestos and fibreglass factories, i.e., −4.29 μmol/L ($p = 0.007$) and −4.11 μmol/L ($p = 0.003$), respectively. All possible two-by-two interactions were tested in the model, but none of them explained a significant portion of variance. On the other hand, we tested the presence of gene–gene interaction between two deletion polymorphisms, which showed a stronger influence on the vitamin C level. Subjects with both functionally deficient gene variants, i.e., *GSTM1* − / *GSTT1* − had a vitamin C level 6.42 μmol/L lower that those with both isoforms functionally efficient (95% CI −10.98: −1.85; $p = 0.006$).

Given the important role of occupational exposure to fibres, and the possibility of a residual confounding, we re-run multiple regression analysis within the reference group. Interestingly, we observed a decreasing trend by age, with a significant reduction in the vitamin C level in subjects older than 50 (−4.35 μmol/L; 95% CI −8.52: −0.19; $p = 0.041$). The influence of gender ($p = 0.003$) was confirmed also in this group, despite the fact that smaller size limited statistical significance. Removing occupation from the set of predictive variables highlighted the effect of GST polymorphisms, which showed a 10% lower level of vitamin C in individuals with gene deletion, borderline significant for *GSTM1* isoform (−3.43 μmol/L, 95% CI −6.73: −0.13; $p = 0.042$). The strong gene–gene interaction observed between *GSTM1* and *GSTT1* polymorphic variants in the whole study group was further confirmed within this subset, which showed the highest reduction in vitamin C level in *GSTM1* − /*GSTT1* − subjects, i.e., −7.67 μmol/L (95% CI −14.58: −0.77; $p = 0.03$).

Discussion

Many biomonitoring studies have shown that individual susceptibility markers such as SNPs (single nucleotide polymorphisms) can modulate the body's response to endogenous and exogenous exposure. In our previous work, we have reported the associations between oxidatively damaged DNA and SNPs in various repair and metabolic genes [40, 52]. Associations between DNA damage and polymorphisms in *GST* genes have also been shown in several [53–56], but not all studies.

We have previously published results of biomonitoring of the mineral fibre-exposed subjects described here, using a wide range of biomarkers of exposure, effect and susceptibility, including genotoxic and immunomodulatory effects, nutritional factors, and interactions between genetic polymorphisms (in DNA repair genes) and oxidative damage to DNA [38–46]. In this paper, we focus on associations between plasma levels of vitamin C, lipophilic antioxidants α- and γ-tocopherol, β-carotene, retinol, lycopene and xanthophyll, and functional polymorphisms in genes coding for the detoxification and antioxidant enzyme glutathione S-transferase. The strategy that we have adopted in our molecular epidemiological studies is to trust only those results and associations that appear significant more than once, i.e. in more than one group or subgroup of analysed cohort. This approach helps us to maximize the chance of finding the true significant associations and to minimize false positive results. In our study, we found unlike association of the *GSTP1* polymorphism with retinol (higher levels of retinol in heterozygous subjects), but because it was found only in one group, we do not consider this finding as biologically reliable.

Our results show that levels of antioxidants in plasma of investigated subjects were influenced by age, sex, cigarette smoking and occupational exposure to fibres.

We observed an interaction between vitamin C levels and particular *GST* polymorphic variants. Subjects with the deletion of *GSTM1* and *GSTT1* genes had lower level of

vitamin C compared with subjects carrying functional gene variant. This association was found to be significant in the whole study group, in the group of all reference subjects and in the reference group of the asbestos factory for *GSTM1* gene, and in the reference group of rock wool plant for *GSTT1* gene. Moreover, vitamin C levels were approximately 20% lower in subjects owning both functionally deficient genes in the whole group and in all reference subjects. These findings suggest that it is unlikely that these results have arisen by chance.

In another molecular epidemiology study [52] conducted on 155 middle aged men, mostly from a rural area of Slovakia, we did not find the same association between vitamin C levels and *GST* polymorphisms. However, the mean concentration of vitamin C in this study (in unexposed men) was 42.5 μmol/L, while the values in the present study are on average 29.9 μmol/L (42% lower than in the previous study). The higher values of vitamin C in plasma of middle aged men are most likely due to their diet, rural life style and possibly genetic differences in the population living in a different part of Slovakia. Since most polymorphisms have a function which is dependent on the dose, and average concentrations of vitamin C levels in both studies differ markedly, it is not surprising that we obtained different results. We therefore suppose that the large difference in vitamin C concentrations in the two different populations—rural (previous study) and urban (present study)—is most likely the source of heterogeneity with respect to the association of vitamin C with genetic polymorphisms in metabolic genes.

Generally, concentrations of vitamin C and other antioxidants detected in Slovak population are lower compared with other European populations. We found that men have less vitamin C and β-carotene than women but more γ-tocopherol and retinol, which is in agreement with other observations [5, 57]. This may be caused by physiological differences between men and women but also by differences in nutritional habits.

Exposed workers in asbestos factory had less vitamin C, β-carotene and lycopene in plasma than non-exposed reference subjects, and smokers had less vitamin C, β-carotene, lycopene and xanthophyll than non-smokers. Lower levels of these antioxidants in exposed subjects are not surprising, especially as it is known that exposure to fibres induces oxidative stress [26, 39, 42, 58–60]. It is also known that smokers have lower levels of vitamin C and other antioxidants than non-smokers [52, 61, 62]. The α- and γ-tocopherol and retinol levels were higher in older subjects, in contrary level of vitamin C was not significantly changed. However, we observed a decreasing trend by age, with a significant reduction in the vitamin C level in reference subjects older than 50.

Vitamin C is accumulated in mammalian cells by two types of proteins: sodium-ascorbate co-transporters (SVCTs) and hexose transporters (GLUTs); in particular, SVCTs actively import ascorbate, the reduced form of this vitamin. SVCTs are surface glycoproteins encoded by two different genes, very similar in structure. SVCT1 is involved in whole-body homoeostasis of vitamin C, while SVCT2 protects metabolically active cells against oxidative stress [63]. However, there are no functional polymorphisms with frequency above 10% described in public databases for these genes, and therefore their possible contribution in metabolic fate of vitamin C was not taken into consideration.

The human GSTs possess both enzymatic and non-enzymatic functions and are involved in many important cellular processes, namely phase II metabolism, stress response, cell proliferation, apoptosis, oncogenesis, tumour progression and drug resistance. The non-enzymatic functions of GSTs involve their interactions with cellular proteins. The majority of *GST* genes harbour polymorphisms that influence their transcription and/or function of their encoded proteins [64].

It should be taken into consideration that together antioxidants and antioxidant enzymes can form synergistic complexes in cells and organisms, for example vitamin C can regenerate oxidized vitamin E [4]. The assessment of antioxidant status therefore depends on measurement of a complex battery of the compounds of the antioxidative defence system and not just one particular micronutrient. It becomes evident that the diet–plasma relationship may be influenced by the presence of various confounding factors such as body size, smoking, the use of supplements and their bioavailability, multiple sources of nutrients, food processing techniques and disease status [65]. These factors may inhibit or enhance absorption and affect nutrient concentrations in the blood [66].

Our study shows that vitamin C levels vary also according to the polymorphisms in *GST* genes, which is particularly seen in subjects with deletion in both *GSTT1* and *GSTM1* genes. We suppose that GSTs and vitamin C may partly complement each other. It is therefore not surprising that subjects missing GSTT1 and GSTM1 isoenzymes due to deletion in the genes have lower levels of vitamin C in plasma. This might imply a causal connection and suggests that GST enzymes play an important role in scavenging ROS, as well as in detoxification processes. Thus, a deficiency in GST activity might lead to greater reliance on dietary antioxidants, which might in those become depleted under conditions of oxidative stress. Higher intake of natural antioxidants seems therefore particularly important especially for subjects with unfavourable genetic makeup and in those exposed to oxidative damaging agents and other oxidative stress-related condition.

Our findings may indicate that the functions of GST and vitamin C in the antioxidant defence system are more complex than we originally thought. However, the exact mechanism underlying this interesting observation remains to be established.

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Conflict of interest The authors declare that they have no financial and commercial conflicts of interest.

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