Levels of 2-thiothiazolidine-4-carboxylic acid (TTCA) and effect modification of polymorphisms of glutathione-related genes in vulcanization workers in the southern Sweden rubber industries

Lena S Jönsson (✉), Karin Broberg, Ulf Bergendorf, Anna Axmon, Margareta Littorin, Bo AG Jönsson

Division of Occupational and Environmental Medicine,

University Hospital, SE-221 85 Lund, Sweden.

Phone no. +(46) 46 173148, Fax no. +(46) 46 143702,

E-mail: lena_s.jonsson@med.lu.se

Abstract *Objectives:* Workers in the rubber industry are exposed to a complex mixture of hazardous substances and have increased risk of developing several diseases. However, there is no up to date survey examining the exposure in the Swedish rubber industry. One of the toxic compounds in the industry is carbon disulfide (CS_2), which is biotransformed to 2-thiothiazolidine-4-carboxylic acid (TTCA). TTCA is used as a biomarker of CS_2 exposure, but there seem to exist inter- and intraindividual variability; which could partly be due to genetic variation. The aim of the study was to determine TTCA levels and the modifying effects of glutathione-related genes in a group of Swedish rubber workers. *Methods:* Urine was collected from both exposed workers and controls during the last four hours of the work shift. The level of TTCA in urine was analyzed by liquid chromatograpy tandem mass spectrometry. Genotyping of the single nucleotide polymorphisms *GCLC*-129, *GCLM*-588, *GSTA1*-52, *GSTP1*-105 and *GSTP1*-114 and deletions of *GSTM1* and *GSTT1* was performed with real-time PCR or ordinary PCR and subsequent agarose electrophoresis. *Results:* The highest levels of TTCA were found among workers curing with salt bath, hot air, microwaves or fluid-bed, and lower levels were found among workers curing with injection and compression molding. Furthermore, with respect to *GSTM1* and *GSTT1* there were statistically significant differences in TTCA-levels between genotypes among exposed workers but not among controls. The other five polymorphisms had no impact on the TTCA levels. *Conclusions:* The present study demonstrates relatively high levels of TTCA in urine from Swedish rubber workers. Polymorphisms in *GSTM1* and *GSTT1* modify the levels.

Keywords 2-Thiothiazolidine-4-carboxylic acid, Biomarkers, Polymorphisms, Rubber industry, Glutathione S-transferases

Introduction

Workers in the rubber industry are exposed to a complex mixture of hazardous substances. It has been described that the workers have increased risk of developing several diseases, e.g., coronary heart and airways diseases, as well as cancer (Gustavsson et al. 1986; Kogevinas et al. 1998; Lewis 1999). One of the toxic compounds is carbon disulfide (CS_2), which has been described to affect many different organs, although major attention has been paid to the cardiovascular and nervous systems (Stetkiewicz and Wronska-Nofer 1998). In the rubber industry, CS_2 is formed during the curing process due to decomposition of dithiocarbamates and thiurams (Craig et al. 1951), which are used as accelerators. However, there is only limited information about the levels of CS_2 in the rubber industry, which complicates the evaluation of relationships of CS_2 exposure and disease.

In the body, CS_2 is metabolized to 2-thiothiazolidine-4-carboxylic acid (TTCA, molecular weight 162.2 g/mol), which has been used as a biomarker of exposure to CS_2 (Van Doorn et al. 1981; Drexler et al. 1994). However, to the best of our knowledge, measurements of TTCA in urine from rubber workers have only been presented in a few publications (Cox et al. 1998; Vermeulen et al. 2005). CS_2 can also be formed *in vivo* when exposed to dithiocarbamates (Johnson et al. 1996). This CS_2 are subsequently metabolized to TTCA, but there are indications that this exposure may be of minor importance in the rubber industry (Vermeulen et al. 2005).

TTCA is formed metabolically via two distinct pathways (Johnson et al. 1996). On one hand, CS_2 is conjugated with reduced glutathione (GSH). This step generates trithiocarbonate, which in turn is transformed into TTCA through removal of glutamic acid and glycine in the mercapturic acid metabolic pathway. On the other hand, CS_2 could interact directly with free amine and sulfhydryl groups of amino acids or polypeptides and in that way generate dithiocarbamates and trithiocarbonates. The trithiocarbonates subsequently cyclize to form

TTCA. The concentration of GSH is generally much higher in the cell than the concentration of free amine and sulfhydryl groups and thus, the first pathway is most probably more common. TTCA is excreted in the urine; however, there seem to exist inter- and intraindividual variability of TTCA excretion (Drexler et al. 1994). Part of this variation could be due to genetic variation in the biotransformation of CS_2.

GSH is synthesized from glutamate, cysteine and glycine. The synthesis is catalyzed sequentially by two enzymes, glutamate cysteine ligase (GCL) and glutathione synthase (GS). GCL is the first and rate-limiting enzyme in *de novo* GSH synthesis and induction of GCL gene expression leads to parallel GSH production (Rahman and MacNee 1999). GCL consist of a catalytically active subunit (encoded by *GCLC*) and a modifying subunit (encoded by *GCLM*).

It is possible that, the conjugation of GSH to CS_2 is catalyzed by the family of glutathione S-transferase enzymes (GSTs). Many of them are involved in biotransformation of toxic xenobiotics and endobiotics. An increasing number of genes encoding GCL and GSTs are being recognized as polymorphic. Different alleles may give rise to different sensitivity to toxic compounds due to changed efficiency of biotransformation.

The aim of this study was to perform a survey of the TTCA levels among people currently working in the Swedish rubber industry. Eight different companies in southern Sweden were examined and the TTCA levels among employees exposed to different sulfur-curing processes at these companies were analyzed. In addition, TTCA levels in controls were analyzed.

Another aim of this study was to analyze whether part of the inter-individual variation observed in TTCA levels could be caused by genetic differences in GSH-related genes. The hypothesis is that polymorphisms causing decreased levels of GSH or reduced capacity for GSH-conjugation are associated with lower levels of TTCA in urine. For this purpose, a total

of six GSH-related genes with functional polymorphisms, were genotyped in the above-mentioned CS_2-exposed workers and in the controls.

Material and Methods

Companies

The sample of companies was made from geographical accessibility and diversity of production methods. The production profiles and methods of the eight companies participating in the study are shortly described below. Some of the companies had other divisions at the same location, which did not participate in the study. These divisions are not mentioned below if the exposure was not considered to affect the studied one. The number of study subjects from each company is presented in brackets.

Company 1 (n = 10) produced industrial rubber profiles as well as form-pressed products but also special products like seals for ship doors and lock gates. The rubber goods were produced by continuous extrusion and curing methods like microwaves, salt baths and steam-curing in an autoclave. Compression molding to join rubber profiles into rings or frames was also applied.

Company 2 manufactured components for vibration damping of heavy trucks, excavators etc. Most of the products consisted of rubber bound to metal. The workers used a number of machines for compression and injection molding, all situated in the same large production hall. However, the workers were divided into two divisions; in division A (n = 5) the vulcanization units were placed sparser and were less-frequently used compared to division B (n = 11).

Company 3 consisted of two different factories. Factory 1 produced profiles and sealing products for the industrial market. The factory was in turn divided into three divisions. Two of them were located next to each other and were treated as one unit, division A (n = 29), in our study. Division A manufactured industrial profiles; one division used continuous curing in salt bath and the other one used microwaves and fluid-bed. The third division, division B (n = 3), was well separated from the other two and was treated as a unit of its own. Here workers

produced rubber membranes for water proofing applications by steam-curing in an autoclave. Several joining operations were also performed. Factory 2 (n = 4) produced sealing strips and profiles for the consumer market using continuous curing in salt baths with non-nitrite containing salt.

Company 4 (n = 21) produced primarily industrial profiles. This was made in a large production hall with continuous curing in several salt bath lines and one line using hot air.

Company 5 (n = 15) produced different hoses and components for automotive applications and for the white goods industry. The company had a number of injection and compression molding units in two adjacent production halls. Some post-curing was performed in hot-air ovens.

Company 6 (n = 10) manufactured different rubber details like gaskets and seals for cables for the automotive industry and other industrial sectors. The company had a few types of injection and compression molding units.

Company 7 (n = 11) produced industrial rubber products for the food, offshore and automotive industries. Among the products were seals for cables and pipes and gaskets for plate heat exchangers. The company had a number of machines for compression and injection molding in three adjacent production halls. As the workers alternated between the halls, the company was considered as one division in our study.

Company 8 consisted of two different factories. Factory 1 (n = 12) produced sealing profiles, including roof and window applications, in continuous curing lines using hot air and salt bath. Furthermore, a few small compression-molding units were situated in an adjacent room where some special products were produced. Factory 2 (n = 32) primarily manufactured gaskets for plate heat exchangers in a production hall with a large number of compression molding machines. Post-curing was done in hot-air ovens. Peroxide was the most used curing agent in this factory but sulfur was also used.

Study subjects

Included in the study were 166 people currently working at the above mentioned rubber industries in the south of Sweden, and 117 controls with no occupational contact with rubber or plastic chemicals. The controls were working with assembling fittings, in the food industry, as postmen or department store workers, also in Sweden. Study subjects included were those who had given their informed consent and were present at work at the time of the medical examination. However, only rubber workers curing with sulfur, or working in the same hall as people curing with sulfur, on the same day as the urine samples were collected, were considered exposed to CS_2 and finally included in the study. The sulfur-curing workers used dithiocarbamates, thiurames, thiothiazoles or other sulfur containing accelerators. TTCA was successfully analyzed from 163 exposed workers and 114 controls. Of these, 156 – 158 (depending on gene) exposed workers and 111 controls were successfully genotyped.

The median age among exposed workers was 38 years (range 19 – 65) and among controls 42 years (20 – 63). Eighty-three exposed workers were men and 80 were women while 55 controls were men and 59 were women. There were no differences between exposed workers and controls concerning tobacco use [72 of 163 (44 %) exposed workers were using tobacco and 59 of 114 (52 %) controls] or atopy, as measured by Phadiatop [108 of 158 (68 %) exposed workers were healthy and 80 of 110 (73 %) controls]. However, there was a difference concerning ethnicity. Based on their names, the majorities in both groups were of European descent (predominating Swedish) but 21 of 163 (13 %) exposed workers were of Eastern Asian descent. With this criterion there were no individuals with Eastern Asian origin among the controls. The study subjects gave their informed written consent to take part in the study and the study was approved by the Regional Ethical Committee of Lund University.

TTCA analysis

Urine was collected from both exposed workers and controls during the last four hours of the work shift. The samples were collected on Tuesday through Thursday. The levels of TTCA were analyzed as previously described (Vermeulen et al. 2005). Briefly, the urine was acidified and the ion-strength increased, thereafter the TTCA was extracted using ethyl acetate. The organic phase was evaporated and re-dissolved in mobile phase. The analyses were performed by liquid chromatography tandem mass spectrometry (LC-MS-MS). The limit of detection (LOD) was determined to be 1 ng/ml urine. Samples <LOD were assigned a value of half the LOD. The precision, as determined from double analyses of the same sample on different days, was 11 % at 10 ng/ml and 7 % at 70 ng/ml. The analysis of TTCA was part of the Round Robin inter-comparison program (Professor Dr. Med. Hans Drexler, Institute and Out-Patient Clinic for Occupational, Social and Environmental Medicine, University of Erlangen-Nuremberg); our results were within the tolerance limits. The levels of TTCA were adjusted for creatinine content, which was analyzed enzymatically according to Mazzachi et al. (2000).

Polymorphisms analyzed

GCLC contains a polymorphism in the promoter at position –129. The T allele has shown lower promoter activity compared to the C allele in vitro in human endothelial cells (Koide et al. 2003). The 5'-flanking region of *GCLM* contains a polymorphism (-588C/T) in which the T allele has shown lower promoter activity compared to C allele when exposed to oxidants (Nakamura et al. 2002). Nakamura et al. (2002) also showed that the plasma GSH levels were significantly lower in CT and TT genotypes than in CC genotype.

Both *GSTM1* and *GSTT1* have non-functional null alleles, which are due to deletions of the genes (Hayes and Strange 2000). Thus, individuals who have a homozygous deletion have

no enzymatic activity. These genotypes are named *GSTM1**O and *GSTT1**O while genotypes with at least one functional allele are named *GSTM1**1 and *GSTT1**1.

GSTA1 contains a polymorphism in the promoter (-52G/A), which may cause differential gene activity as the variant allele misses a binding site for the transcription factor Sp1 (Morel et al. 2002).

Two functional polymorphisms in the coding region of *GSTP1* were also examined. The variant allele of the polymorphism named *GSTP1*-105 encodes valine instead of isoleucine at codon 105 due to a base pair exchange where G substitutes A. The variant allele of the polymorphism named *GSTP1*-114 encodes valine instead of alanine at codon 114 due to a base pair exchange where T substitutes C. These substitutions may affect the ability and the rate of which different toxic compounds bind to GSTP1-1 (Ali-Osman et al. 1997).

Genotyping

DNA was extracted from whole blood using QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) at the DNA/RNA Genotyping Lab, SWEGENE Resource Center for Profiling Polygenic Disease, Lund University, Malmö University Hospital, Malmö, Sweden, or by a modified salting out extraction method (Miller et al. 1988).

GCLC, GCLM, GSTA1 and *GSTP1* were genotyped on a real-time PCR intrument (ABI 7000; Applied Biosystems, Foster City, CA, USA) using probes, primers and primer concentrations as described by Custodio et al. (2004). The concentrations of probes were 0.04 µM (*GCLC, GCLM* and *GSTA1*), 0.2 µM (*GSTP1*-105) or 0.08 µM (*GSTP1*-114). Amplification of 8-16 ng of DNA was performed during 40-45 cycles in a reaction volume of 25 µl. TaqMan Universal PCR Master Mix (Applied Biosystems) was used except for the analysis of *GSTP1*-114, for which a PCR buffer (Invitrogen Corporation, Carlsbad, CA, USA) containing 2.5 mM $MgCl_2$ (Invitrogen Corporation), Rox (Invitrogen Corporation), 0.8 mM

dNTP (GE Healthcare UK Ltd, Little Chalfont, Buckinghamshire, UK) and 1 U Taq Platina (Invitrogen Corporation) was used. For genotyping of *GSTA1* the concentration of Master Mix was 0.7x instead of 1.0x.

GSTM1 and *GSTT1* were genotyped with ordinary PCR and subsequent agarose electrophoresis using *NAT*2 as an internal PCR control and primers as described by Hou et al. (1995) and Pemble et al. (1994). Amplification of 16 ng of DNA was performed during 31-32 cycles in a reaction volume of 25 µl. A PCR buffer (Invitrogen Corporation) containing 0.4 µM of each primer (Invitrogen Corporation), 1.25 mM $MgCl_2$ (Invitrogen Corporation), 0.8 mM dNTP (GE Healthcare UK Ltd) and 0.5 U Taq Platina (Invitrogen Corporation) was used. For all analyses, positive controls for the different genotypes were included in each run. Moreover, approximately 10 % of the samples were reanalyzed as a control of the method.

Statistical analysis

Genotype distribution was analyzed with χ^2, except when the expected individual frequencies were smaller than 5, then Fisher-Freeman-Halton's test was used. Regarding *GSTM1* and *GSTT1*, where heterozygous status could not be differentiated from homozygous (no deletion) status, gene frequencies found in the literature (Garte et al. 2001) was used as expected frequencies.

Differences in TTCA between exposed workers and controls, between workers curing with different methods, between men and women, and between different genotypes in each exposure group, were estimated using analysis of variance (ANOVA). Since the data were skewed, geometric means were used, and the levels of TTCA were transformed (log 10) before use in the linear model. Thus, the interpretation of the effect estimate (β) is not straightforward. However, the formula $100(10^{\beta} - 1)$ estimates the percentage increase or decrease in the average value of the TTCA levels in each genotypic subgroup compared to the

group with the reference genotype (Vittinghoff et al. 2005). The reference categories consisted of individuals with putative high-conjugation genotypes, that is *GCLC*-129 (C/C), *GCLM*-588 (C/C), *GSTA1*-52 (G/G), *GSTM1**1, *GSTP1*-105 (ile/ile), *GSTP1*-114 (ala/ala) or *GSTT1**1. The exposed workers and controls were analyzed separately since no quantitative exposure estimates were available.

Sex, age and tobacco use were considered as potential confounders. The change-in-estimate suggested by Greenland (1989) was used to evaluate which of these to be included in the final models. A change of the estimate of > 10 % was required for inclusion in the final model. However, to obtain comparable adjusted estimates, potential confounders were included in all final models for each scientific question, if they fulfilled the inclusion criteria for at least one analysis.

Results

Exposure

The levels of TTCA in urine from the exposed workers and controls are shown in Table 1. The difference between all exposed workers and all controls was statistically significant ($P < 0.001$). The geometric means of TTCA levels at different companies varied between 7.6 and 220 µmol/mol creatinine. Furthermore, the levels of TTCA differed between different subdivisions at the same company.

[Please insert Table 1 here.]

When workers curing with different methods were compared (Table 2), there were statistically significant differences in TTCA levels between workers curing with injection and compression compared to those curing with salt bath ($P < 0.001$) or compared to those curing with hot air, microwaves and fluid-bed ($P < 0.001$). However, when comparing salt bath curing with hot air, microwaves and fluid-bed curing the difference was not significant ($P = 0.098$).

[Please insert Table 2 here.]

There was no statistically significant difference ($P = 0.40$) between exposed men (geometric mean 25 µmol/mol creatinine, range 2.7 – 690) and women (20 µmol/mol creatinine, range <LOD – 950) regarding urinary TTCA levels. Neither was there any statistically significant difference ($P = 0.88$) between unexposed men and women (geometric mean for men 4.2 µmol/mol creatinine, range <LOD – 160, and for women 4.8 µmol/mol creatinine, range 0.3 – 460). The P-values were adjusted for tobacco use.

Genotype

The genotype distribution for *GCLC*-129, *GCLM*-588, *GSTA1*-52, *GSTP1*-105 and *GSTP1*-114 was consistent with the population being in Hardy-Weinberg equilibrium, both when

analyzing all study subjects together and exposed workers (with and without Eastern Asian individuals) and controls separately. Furthermore, frequencies of the deletion genotypes of *GSTM1* and *GSTT1* were in accordance with the frequencies found in the literature (Garte et al. 2001), except for the frequency of *GSTM1**O among controls, which was lower in our study than what was found in the literature.

The level of TTCA differed depending on the genotype in the analysis of *GSTM1* (P = 0.043) and *GSTT1* (P = 0.017) in the group of exposed workers (Table 3). The corresponding differences were not found in the group of controls (Table 4). There was no overrepresentation of either *GSTM1**1 or *GSTT1**O in companies 3 and 4 with the highest levels of TTCA (data not shown). Stratification on curing method (molding vs. continuous curing) for *GSTM1* and *GSTT1* did not changed the results, although statistical power was reduced due to fewer observations in the different strata (data not shown).

[Please insert Tables 3 and 4 here.]

Different combinations of *GSTM1* and *GSTT1* genotypes were further analyzed among exposed workers (Table 5). Individuals having *GSTM1**1 and *GSTT1**O were used as reference category, as the single gene analysis indicated that these individuals would have the highest levels of TTCA. Individuals with *GSTM1**O and *GSTT1**1 had the lowest levels of TTCA, which almost reached significance (P = 0.051).

[Please insert Table 5 here.]

There were no significant differences in the TTCA levels when analyzing the impact of *GCLC*-129, *GCLM*-588, *GSTA1*-52, *GSTP1*-105 and *GSTP1*-114 polymorphisms (Tables 3, 4). Neither were there any significant differences when analyzing *GSTP1*-105 and *GSTP1*-114 polymorphisms together as haplotypes (data not shown).

When exposed workers with Eastern Asian names (n = 21) were excluded from the analysis, the effect estimates were not markedly changed and we observed significant changes

in TTCA levels regarding the same genotypes as when all exposed workers were analyzed together (*GSTM1*, P = 0.040 and *GSTT1*, P = 0.045).

Discussion

This work demonstrates relatively high levels of TTCA in urine from the rubber workers in southern Sweden and indicates modifying effects of polymorphisms in GSH-related genes on the levels of TTCA.

A biological exposure index of 3500 µmol TTCA/mol creatinine (5 mg TTCA/g creatinine) is recommended by ACGIH for urine samples collected at the end of the workshift (ACGIH 2005). This corresponds to an occupational exposure level of about 8 ppm CS_2 according to Riihimäki et al. (1992). The knowledge is very limited, but the general opinion nowadays is that only levels below 4 ppm are associated with no adverse effects (Anon 1996) and ACGIH plans to lower the threshold limit value even to 1 ppm (ACGIH 2005).

In Sweden the threshold limit value is 5 ppm (Arbetsmiljöverket 2005), which corresponds to approximately 2000 µmol TTCA/mol creatinine. None of the TTCA levels in our study exceeded that value (max value 950 µmol/mol creatinine). However, using the proposed new ACGIH threshold limit value of 1 ppm, corresponding to a biological exposure index of approximately 400 µmol TTCA /mol creatinine, seven of the 163 (4.3 %) exposed workers in our survey had a TTCA level above that value.

Similar TTCA levels as in the present study were observed in a survey by Vermeulen et al. (2005) in a Dutch rubber industry study. In a survey by Cox et al. (1998) at an American rubber product facility, all 19 workers had urinary TTCA levels below the detection limits. Vermeulen et al. (2005) used the same sensitive analytical LC-MS-MS method as used in the present study while Cox et al. (1998) used a 15-times less sensitive LC method with a LOD equivalent to about 0.5 ppm CS_2 in air. This may explain the differences in results.

Urine was collected on Tuesday through Thursday. An earlier study has shown that there are little or no differences in urinary TTCA levels between different workdays (Vermeulen et al. 2005).

TTCA was found in urine from the controls and, in fact, one control had levels above 400 µmol TTCA/mol creatinine corresponding to 1 ppm. It is a well-known fact that many cruciferous vegetables contain endogenous TTCA, which is excreted unchanged in urine (Simon et al. 1994; Kivistö 2000). Although we had dietary information from the study participants, it was hard to explain why some of the controls have high levels of TTCA (data not shown). It has been suggested that tobacco use could affect the urinary levels of TTCA, but in the present study there was no such effect among the controls (median 4.7 µmol TTCA/mol creatinine for users of tobacco vs. 4.3 for non-users), and only a slight indication among the exposed workers (median 25.5 µmol TTCA/mol creatinine for users of tobacco vs. 18.7 for non-users). It is also known that the biotransformation of, for example, disulfiram, (Van Doorn 1982) and captan (Anon 1996) give rise to increased levels of TTCA in urine. A closer look into the case history showed that the above-mentioned control, who had a urinary TTCA level above 400 µmol TTCA /mol creatinine, did not take disulfiram but the use of captan cannot be excluded. The person had an untreated hypothyreosis and atopy, treated with Lomudal® eye drops, Clarityn®, cortisone lotion, Rhinocort® and Bricanyl®, but it is unclear if this can lead to elevated TTCA levels.

It was very difficult to find controls at the included rubber companies, who were not occupationally exposed to rubber chemicals, but had the same socio-economic status as the exposed workers. Instead the controls in the present study were working at different companies, but those companies were selected for including employees with similar socio-economic status as the exposed workers. Furthermore, the controls were working in the same region of Sweden as the exposed workers but differences in the local environment (e.g., proximity to highways, industries, etc) can exist.

Company 3 and especially company 4 had remarkably high levels of TTCA. A large fraction of the rubber goods were produced by continuous curing at these companies. We

observed that workers curing with salt bath and hot air, microwaves and fluid-bed curing had significantly higher TTCA levels in the urine than those working with injection and compression molding.

The use of TTCA as a biomarker in the rubber industry may have a wider application than solely as an index of CS_2 and dithiocarbamates. Since CS_2 is generated during curing with sulfur-containing accelerators, high TTCA levels may also be a biomarker of curing fumes. In fact, in the present study we found high correlations between TTCA in urine and N-nitrosamines in air (data not shown), which indicate such a relationship.

Earlier studies in the rubber industry have mainly included men. In this survey we therefore also included women.

CS_2 is biotransformed via conjugation with GSH to TTCA. We observed a significant difference in the levels of TTCA among exposed workers when genotyping *GSTM1* and *GSTT1*, but not GCL encoding genes *GCLC* and *GCLM*, suggesting that the production of TTCA might be mediated by GSTs or affected by GST activity.

Exposed workers with *GSTM1**O had lower levels of TTCA, than exposed workers with *GSTM1**1, suggesting that carrying *GSTM1**1 results in better CS_2-GSH conjugation than if carrying *GSTM1**O, and thus higher levels of the metabolites.

The opposite pattern was observed for *GSTT1*, where exposed workers with *GSTT1**O had higher levels of TTCA than exposed workers with *GSTT1**1. This pattern is not easily interpreted. A possibility would be that exposed workers with *GSTT1**O have a compensatory up-regulation of some other GSTs. It could also be that GSTT1-mediated GSH conjugation of other substances than CS_2 is interfering with the TTCA production, for example, by expending the GSH. This would results in lower levels of TTCA.

We did several analyses and cannot exclude the possibility that the significant results are a result of multiple testing. However, we believe that the significant results show a biological

pattern, but these results need to be confirmed. We observed a lower level of *GSTM1**O among controls than what was expected from the literature (Garte et al. 2001). This is probably not caused by a methodological failure as the controls were analyzed together with the exposed workers and this disequilibrium was not seen among them. In addition, there were no differences in frequencies in any group regarding *GSTT1*.

The group of exposed workers was heterogeneous regarding ethnicity. It is known for some GSH-related genes that genotype frequencies differ between different populations, e.g., *GSTT1**O is more common in Asia than in Europe, whereas *GSTM1* display similar frequencies in these two populations (Garte et al. 2001). Since there was a rather large group of study subjects with presumable Eastern Asian decent in the exposed group, we performed the analysis both with and without this group. When individuals with Eastern Asian names were excluded, the direction and significant importance of the effect of polymorphisms in *GSTM1* and *GSTT1* on urinary levels of TTCA remained. Furthermore, there was still no effect on urinary TTCA levels of the other genotypes studied. The P-value for adjusted effect estimate regarding *GSTT1* changed from 0.017 to 0.045. However, that might be explained by the few individuals having *GSTT1**O (n = 25) when Eastern Asian individuals were excluded. The used exclusion criteria might lead to that some individuals with Asian descent were not excluded, but no other information on ethnicity was available in the present study.

Next we performed analysis of the effect of the combined genotype of *GSTM1* and *GSTT1* on TTCA level. Exposed workers with *GSTM1**O and *GSTT1**1 indeed, displayed the lowest levels of TTCA compared to the reference category. However, the two other genotypes did not follow the hypothesis; exposed workers with *GSTM1**O and *GSTT1**O displayed the highest TTCA levels and exposed workers with *GSTM1**1 and *GSTT1**1 displayed the same TTCA levels as the reference category. This cannot be easily explained, but one possibility could be the low number of individuals in each group.

As described above, we also measured TTCA in the controls not exposed to CS_2. We saw no significant differences in TTCA levels when comparing groups of individuals with different genotypes. This is in accordance with the fact that some vegetables contain TTCA themselves, which are not metabolized by GSH.

Today it is unclear if a high concentration of TTCA in urine is favorable or not, but the present study indicates that it might be necessary to take into account the modifying effects of *GSTM1* and *GSTT1* on the TTCA levels in CS_2-exposed subjects to determine the actual CS_2 exposure. We found presumably modifying effects of *GSTM1* and *GSTT1* on the TTCA levels in CS_2-exposed workers even though the exposure assessment was rough. This indicates rather large differences between the genotypes. However, the results should be confirmed in a study where air levels of CS_2 exposure as well as TTCA levels in urine are determined. In the present study it was unmanageable to perform personal air sampling, but upon inclusion of that in a future study, it will be possible to find weaker associations between TTCA levels and genotypes. Moreover, phenotypic determination of GSTM1 and GSTT1 might sharpen the association between TTCA levels and glutathione conjugation capacity.

Acknowledgements

We thank Eva Assarsson, Inger Bensryd, and Kerstin Diab for collecting the samples, Margareta Warholm for genotyping a part of the study subjects and Åsa Amilon for analyzing TTCA in a skilful way. The study was supported by AFA (Swedish Labour Market Insurance Company), the Swedish Council for Work Life Research, the Swedish Research Council, Skane county council's research and development foundation, and the Medical Faculty at Lund University, Sweden.

References

Ali-Osman F, Akande O, Antoun G, Mao J-X, Buolamwini J (1997) Molecular cloning, characterization, and expression in Escherichia coli of full-length cDNAs of three human glutathione S-transferase Pi gene variants. Evidence for differential catalytic activity of the encoded proteins. J Biol Chem 272:10004-12

American Conference of Governmental Industrial Hygienists (ACGIH) (2005) TLVs and BEIs based on the documentation on the threshold limit values for chemical substances and physical agents & biological exposure indices, Cincinnati, USA

Anon (1996) Biological monitoring of chemical exposure in the workplace, vol 2. World Health Organisation, Geneva, p 203

Arbetsmiljöverket [Swedish work environment authority] (2005) Arbetsmiljöverkets författningssamling (AFS 2005:17) Hygieniska gränsvärden och åtgärder mot luftföroreningar. Stockholm (Sweden)

Cox C, Hee SS, Tolos WP (1998) Biological monitoring of workers exposed to carbon disulfide. Am J Ind Med 33:48-54

Craig D, Davidson WL, Juve AE (1951) Tetramethylthiuram disulfide vulcanization of extracted rubber. V. Low molecular products and the mechanism of zinc oxide activation. J Polym Sci 6:177-87

Custodio HM, Broberg K, Wennberg M, Jansson J-H, Vessby B, Hallmans G, et al. (2004) Polymorphisms in glutathione-related genes affect methylmercury retention. Arch Environ Health 59:588-95

Drexler H, Goen T, Angerer J, Abou-el-ela S, Lehnert G (1994) Carbon disulphide. I. External and internal exposure to carbon disulphide of workers in the viscose industry. Int Arch Occup Environ Health 65:359-65

Garte S, Gaspari L, Alexandrie A-K, Ambrosone C, Autrup H, Autrup JL, et al. (2001)

Metabolic gene polymorphism frequencies in control populations. Cancer Epidemiol Biomarkers Prev 10:1239-48

Greenland S (1989) Modeling and variable selection in epidemiologic analysis. Am J Public Health 79:340-9

Gustavsson P, Hogstedt C, Holmberg B (1986) Mortality and incidence of cancer among Swedish rubber workers, 1952-1981. Scand J Work Environ Health 12:538-44

Hayes JD, Strange RC (2000) Glutathione S-transferase polymorphisms and their biological consequences. Pharmacology 61:154-66

Hou S-M, Lambert B, Hemminki K (1995) Relationship between hprt mutant frequency, aromatic DNA adducts and genotypes for GSTM1 and NAT2 in bus maintenance workers. Carcinogenesis 16:1913-7

Johnson DJ, Graham DG, Amarnath V, Amarnath K, Valentine WM (1996) The measurement of 2-thiothiazolidine-4-carboxylic acid as an index of the in vivo release of CS2 by dithiocarbamates. Chem Res Toxicol 9:910-6

Kivistö H (2000) TTCA measurements in biomonitoring of low-level exposure to carbon disulphide. Int Arch Occup Environ Health 73:263-9

Kogevinas M, Sala M, Boffetta P, Kazerouni N, Kromhout H, Hoar-Zahm S (1998) Cancer risk in the rubber industry: a review of the recent epidemiological evidence. Occup Environ Med 55:1-12

Koide S, Kugiyama K, Sugiyama S, Nakamura S, Fukushima H, Honda O, et al. (2003) Association of polymorphism in glutamate-cysteine ligase catalytic subunit gene with coronary vasomotor dysfunction and myocardial infarction. J Am Coll Cardiol 41:539-45

Lewis R (1999) Overview of the rubber industry and tire manufacturing. Occup Med 14:707-18

Mazzachi BC, Peake MJ, Ehrhardt V (2000) Reference range and method comparison studies

for enzymatic and Jaffe creatinine assays in plasma and serum and early morning urine. Clin Lab 46:53-5

Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:1215

Morel F, Rauch C, Coles B, Le Ferrec E, Guillouzo A (2002) The human glutathione transferase alpha locus: genomic organization of the gene cluster and functional characterization of the genetic polymorphism in the hGSTA1 promoter. Pharmacogenetics. 12:277-86

Nakamura S, Kugiyama K, Sugiyama S, Miyamoto S, Koide S, Fukushima H, et al. (2002) Polymorphism in the 5'-flanking region of human glutamate-cysteine ligase modifier subunit gene is associated with myocardial infarction. Circulation 105:2968-73

Pemble S, Schroeder KR, Spencer SR, Meyer DJ, Hallier E, Bolt HM, et al. (1994) Human glutathione S-transferase theta (GSTT1): cDNA cloning and the characterization of a genetic polymorphism. Biochem J 300(Pt 1):271-6

Rahman I, MacNee W (1999) Lung glutathione and oxidative stress: implications in cigarette smoke-induced airway disease. Am J Physiol 277(6 Pt 1):L1067-88

Riihimäki V, Kivistö H, Peltonen K, Helpiö E, Aitio A (1992) Assessment of exposure to carbon disulfide in viscose production workers from urinary 2-thiothiazolidine-4-carboxylic acid determinations. Am J Ind Med 22:85-97

Simon P, Nicot T, Dieudonne M (1994) Dietary habits, a non-negligible source of 2-thiothiazolidine-4-carboxylic acid and possible overestimation of carbon disulfide exposure. Int Arch Occup Environ Health 66:85-90

Stetkiewicz J, Wronska-Nofer T (1998) Updating of hygiene standards for carbon disulfide based on health risk assessment. Int J Occup Med Environ Health 11:129-43

Van Doorn R, Delbressine LP, Leijdekkers CM, Vertin PG, Henderson PT (1981)

Identification and determination of 2-thiothiazolidine-4-carboxylic acid in urine of workers exposed to carbon disulfide. Arch Toxicol 47:51-8

Van Doorn R, Leijdekkers CM, Nossent SM, Henderson PT (1982) Excretion of TTCA in human urine after administration of disulfiram. Toxicol Lett 12:59-64

Vermeulen R, Jonsson BA, Lindh CH, Kromhout H (2005) Biological monitoring of carbon disulphide and phthalate exposure in the contemporary rubber industry. Int Arch Occup Environ Health 78:663-9

Vittinghoff E, Glidden DV, Shiboski SC, McCulloch CE (2005) Regression methods in biostatistics. Linear, logistic, survival, and repeated measures models. Springer, Berlin, p126

Table 1 Levels of 2-thiothiazolidine-4-carboxylic acid (TTCA; µmol/mol creatinine) among exposed workers and controls

Company	N	Geometric mean	Range	10^{th} and 90^{th} percentiles
Controls	114	4.4	< LOD – 460	< LOD - 27
All exposed workers	163	22	<LOD - 950	3.5 – 210
Company 1	10	11	5.5 – 25	5.7 - 24
Company 2	16	12	4.6 – 690	4.7 - 230
Division A	5	6.0	4.6 – 7.1	4.6 – 7.1
Division B	11	17	4.7 - 690	4.7 – 560
Company 3	36	55	23 – 220	27 - 110
Division A	29	64	28 – 220	33 -140
Division B	3	28	23 – 32	23 - 32
Factory 2	4	30	23 – 40	23 - 40
Company 4	21	220	72 – 950	83 - 700
Company 5	15	14	3.6 – 780	3.8 - 370
Company 6	10	17	2.8 – 45	3.2 - 44
Company 7	11	13	2.7 – 41	3.1 - 39
Company 8	44	7.6	< LOD – 410	1.4 - 42
Factory 1	12	15	5.3 – 70	5.4 - 64
Factory 2	32	5.9	< LOD – 410	< LOD – 34

Table 2 Levels of 2-thiothiazolidine-4-carboxylic acid (TTCA; µmol/mol creatinine) among workers curing with different methods

Curing method	N [a)]	Geometric mean (range)
Injection and compression molding	94	11 (< LOD -780)
Hot air, microwaves and fluid-bed	24	49 (5.3-700)
Salt bath curing	39	86 (8.0-950)

[a)] Three employees were not classified and three employees working with steam curing were not included.

Table 3 Analysis of influence of genotype on 2-thiothiazolidine-4-carboxylic acid (TTCA; µmol/mol creatinine) levels among exposed workers

Polymorphism	Genotype	N	Geometric mean (range)	Unadjusted		Adjusted [a)]	
				β [b)]	P-value	β [b)]	P-value
GCLC-129	C/C	133	24 (< LOD - 950)	Ref		Ref	
	C/T	22	21 (1.6 - 220)	-0.067	0.67	-0.064	0.69
	T/T	2	5.4 (5.3 - 5.6)	-0.64	0.19	-0.76	0.13
GCLM-588	C/C	115	24 (< LOD - 950)	Ref		Ref	
	C/T	38	21 (< LOD - 690)	-0.048	0.71	-0.060	0.65
	T/T	5	22 (1.8 - 62)	-0.033	0.92	0.065	0.84
GSTA1-52	G/G	57	25 (1.8 - 700)	Ref		Ref	
	G/A	79	21 (< LOD - 950)	-0.076	0.53	-0.093	0.44
	A/A	22	24 (< LOD - 690)	-0.025	0.88	-0.046	0.79
GSTM1	1	75	30 (1.8 - 950)	Ref		Ref	
	0	82	18 (< LOD - 780)	-0.22	**0.045**	-0.22	**0.043**
GSTP1-105	ile/ile	70	25 (< LOD - 780)	Ref		Ref	
	ile/val	78	19 (< LOD -	-0.13	0.24	-0.12	0.30

			950)				
	val/val	10	63 (8.3 - 470)	0.40	0.083	0.39	0.097
GSTP1-114	ala/ala	134	23 (< LOD - 950)	Ref		Ref	
	ala/val	23	21 (< LOD - 700)	-0.043	0.78	-0.038	0.81
	val/val	1	23 (23 - 23)	-0.044	1.0	-0.011	0.99
GSTT1	1	121	20 (< LOD - 950)	Ref		Ref	
	0	35	40 (4.2 - 780)	0.30	**0.024**	0.32	**0.017**

Ref reference category.

[a)] Effect estimates were adjusted for sex, age and tobacco use.

[b)] The interpretation of the effect estimates (β) is not straightforward. However, the formula $100(10^{\beta} - 1)$ estimates the percentage increase or decrease in the average TTCA levels in each examined group compared to the reference category (Vittinghoff et al. 2005)

Table 4 Analysis of influence of genotype on 2-thiothiazolidine-4-carboxylic acid (TTCA; µmol/mol creatinine) levels among controls

Polymorphism	Genotype	N	Geometric mean (range)	Unadjusted		Adjusted [a)]	
				β [b)]	P-value	β [b)]	P-value
GCLC	C/C	95	3.9 (< LOD - 460)	Ref		Ref	
	C/T	16	7.0 (< LOD - 47)	0.25	0.13	0.26	0.13
GCLM	C/C	82	4.1 (< LOD - 460)	Ref		Ref	
	C/T	23	4.0 (< LOD - 27)	-0.013	0.93	-0.0022	0.99
	T/T	6	8.2 (2.5 - 47)	0.30	0.25	0.27	0.31
GSTA1	G/G	35	5.4 (< LOD - 460)	Ref		Ref	
	A/G	55	4.3 (< LOD -160)	-0.096	0.47	-0.082	0.54
	A/A	21	2.7 (< LOD - 47)	-0.30	0.082	-0.28	0.10
GSTM1	1	64	3.7 (< LOD -160)	Ref		Ref	
	0	47	5.1 (< LOD - 460)	0.13	0.26	0.14	0.26
GSTP1-105	ile/ile	56	4.4 (< LOD -160)	Ref		Ref	
	ile/val	44	4.0 (< LOD - 460)	-0.042	0.74	-0.044	0.73
	val/val	11	4.8 (< LOD - 38)	0.037	0.86	0.035	0.87
GSTP1-114	ala/ala	100	4.5 (< LOD -	Ref		Ref	

			460)				
	ala/val	11	2.6 (< LOD - 24)	-0.23	0.25	-0.26	0.20
GSTT1	1	96	4.1 (< LOD - 460)	Ref		Ref	
	0	15	5.7 (0.9-47)	0.15	0.39	0.16	0.36

Ref reference category.

[a)] Effect estimates were adjusted for sex, age and tobacco use.

[b)] The interpretation of the effect estimates (β) is not straightforward. However, the formula $100(10^{\beta} - 1)$ estimates the percentage increase or decrease in the average TTCA levels in each examined group compared to the reference category (Vittinghoff et al. 2005)

Table 5 Analysis of the influence of combined *GSTM1* and *GSTT1* genotypes on the 2-thiothiazolidine-4-carboxylic acid (TTCA; µmol/mol creatinine) levels among exposed workers

GSTM1	*GSTT1*	N	Geometric mean (range)	Unadjusted		Adjusted [a)]	
				β [b)]	P-value	β [b)]	P-value
1	0	17	30 (7.3 - 680)	Ref		Ref	
0	1	63	14 (< LOD - 690)	-0.34	0.064	-0.36	0.051
0	0	18	52 (4.2 - 780)	0.24	0.29	0.24	0.29
1	1	58	30 (1.8 - 950)	0.0045	0.98	-0.014	0.94

Ref reference category.

[a)] Effect estimates were adjusted for sex, age and tobacco use.

[b)] The interpretation of the effect estimate (β) is not straightforward. However, the formula $100(10^{\beta} - 1)$ estimates the percentage increase or decrease in the average TTCA levels in each examined group compared to the reference category (Vittinghoff et al. 2005)