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Exposure, health effects, immunological markers and biomarkers of susceptibility among Swedish rubber workers

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Abstract

Workers in the rubber industry have an increased risk of several diseases, such as airways disease, cancer and probably cardiovascular disease. The exposure is very complex; carbon disulfide (CS2) and polycyclic aromatic hydrocarbons (PAHs) being two of the contaminants. The general aim of this thesis was to find exposure-response relationships usable for risk assessments in the rubber industry.

Urinary levels TTCA and 1-HP, metabolites of CS2 and one PAH, was measured in 166 workers from eight rubber industries in southern Sweden and in 117 controls. Airways symptoms were recorded by structured interviews and immunological markers in blood analysed by routine analysis. Genotypes were established using molecular biology methods.

Compared to controls, exposed workers had in average 420% higher urinary TTCA level and 200% higher urinary 1-HP level. The levels of TTCA and 1-HP differed between different rubber companies and their subdivisions, as well as between different vulcanisation methods. Exposed workers had, compared to controls, increased risk of itching, running or burning eyes, nose bleeds, throat burning and dryness, hoarseness, severe dry cough, nausea and headache. Only a few exposure-response relationships with either TTCA or 1-HP and symptoms were found, when the workers were divided into three equally sized groups according to their exposure. Increased levels of total IgG were observed among exposed workers compared to controls. No exposure-response relationship with neither TTCA nor 1-HP and immunological markers was found. Exposed workers with the GSTM1*O genotype had lower levels of TTCA compared to exposed workers with GSTM1*1 present while the opposite were found for GSTT1. The effects of GSTA1-52 (G/A+A/A) genotype on symptoms in exposed workers showed a consistent pattern of a protective effect.

The results indicate that workers in the contemporary rubber industry develop symptoms and thus, the work environment should be further improved. Moreover, better markers of exposure should be established in order to obtain better exposure-response relationships. In addition, adding information of different genotypes may sharpen the risk assessments.

Key words: TTCA, 1-hydroxypyrene, PAH, eyes, airways, symptoms, biomarkers, exposure-response relationships, headache, nausea, rubber industry, polymorphisms, vulcanisation fumes

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To my curiosity

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POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Arbetare i gummiindustrin utsätts för ett stort antal kemikalier med hög giftighet. Tidigare forskning har visat att kemikalierna innebär en fara för hälsan. Gummiarbetarna har tidigare haft en ökad risk att drabbas av olika sjukdomar, t.ex. cancer, hjärtkärlsjukdomar och luftvägsbesvär, jämfört med allmänbefolkningen. Däremot vet man inte exakt vilka ämnen som är farliga, eller i vilka koncentrationer, eftersom gummiarbetarna alltid får i sig många olika kemikalier på samma gång. Arbetsmiljön har dock förbättrats de senaste decennierna. Det är därför okänt om de halter av farliga kemikalier som förkommer idag, fortfarande ger upphov till sjukdom. Det är dessa frågor som denna avhandling syftar till att svara på.

Ett tillvägagångssätt då det gäller att kartlägga vad gummiarbetarna får i sig är att använda så kallade indexsubstanser. Dessa är enskilda ämnen som får representera en hel grupp av olika kemikalier. Genom att mäta nedbrytningsprodukter av dessa indexsubstanser i gummiarbetarnas urin går det att uppskatta halterna av de kemikalier som indexsubstansen representerar och som varje enskild gummiarbetare har fått i sig genom t.ex. inandning och hudupptag.

I denna avhandling presenteras halterna av TTCA och 1-HP som är nerbrytningsprodukter till koldisulfid och polycykliska aromatiska kolväten. Dessa är ämnen som frigörs då gummit upphettas. Vi fann att halten TTCA var i genomsnitt 420 % högre bland gummiarbetare än bland personer som inte var utsatta för gummikemikalier på arbetet (**artikel I**). Motsvarande siffra för 1-HP var 200 % (**artikel IV**). Vi fann även att gummiarbetarna hade en betydande ökad risk för ögonsymtom, näsblod, torrt och irriterat svalg, heshet, svår rethosta, illamående och huvudvärk. Vidare undersökte vi om halterna av olika immunologiska markörer (ämnen som kroppen bildar som ett försvar mot t.ex. giftiga kemikalier, bakterier och virus) förändrades (**artikel II**). Dessutom analyserade vi om det fanns ett samband mellan förhöjda halter av TTCA eller 1-HP och ökade risker för symtom eller förändrade halter av immunologiska markörer. Vi fann dock att i de flesta fall fanns inget sådant samband (**artikel II** och **IV)**.

I tidigare studier har man funnit att andelen TTCA som kommer ut i urinen hos olika individer skiljer sig åt även om de utsätts för samma halter av koldisulfid. Det skulle kunna bero på att olika individer har olika genuppsättning och därför i olika utsträckning bildar proteiner som t.ex. är med och oskadliggör farliga ämnen så att dessa kan kissas ut. Vi analyserade några av de gener som är involverade i nedbrytningen av koldisulfid och fann att två av generna, *GSTM1* och *GSTT1*, påverkade halten TTCA (**artikel I**).

Vi studerade också oxidativ stress, d.v.s. fara som orsakas av reaktiva och farliga ämnen, som antingen kan komma utifrån miljön eller från cellerna själva.

Gummiarbetarna utsätts för många ämnen som kan ge oxidativ stress, t.ex. från rök i gummiindustrin, och det kan leda till en rad olika besvär. De gener som vi undersökte ingår i nedbrytningen av farliga ämnen och därigenom också ofta i kroppens försvar mot oxidativ stress. Vi fann att personer med en särskild genuppsättning av *GSTA1* verkade ha en lägre risk för olika symtom jämfört med personer med en annan genuppsättning (**artikel III**).

Sammantaget visar artiklarna att det fortfarande finns betydande risker för olika symptom i den svenska gummiindustrin trots förbättringar i arbetsmiljön de senaste decennierna. Således krävs ytterligare förbättringar. Artiklarna visar också att personer med olika genuppsättningar har olika risk för att drabbas av besvär. I den här avhandlingen har vi dessutom tagit fram en ny strategi för att mäta farliga ämnen som gummiarbetarna utsätts för. Det hade dock varit en fördel att ha bättre indexsubstanser än de vi använde för att beskriva risken för de symtom och förändrade halter av de immunologiska markörer, som vi undersökte.

LIST OF PAPERS

This thesis is based upon the following papers, referred to in the text by their Roman numerals.

I. Jönsson LS, Broberg K, Bergendorf U, Axmon A, Littorin M, Jönsson BAG.
Levels of 2-thiothiazolidine-4-carboxylic acid (TTCA) and the effect modification of polymorphisms of glutathione-related genes in vulcanization workers in the southern Sweden rubber industries.
Int Arch Occup Environ Health, in press

II. Jönsson LS, Broberg K, Axmon A, Jönsson BAG, Littorin M.
Symptoms and immunological markers in vulcanization workers in the southern Sweden rubber industries.
Submitted

III. Jönsson LS, Jönsson BAG, Axmon A, Littorin M, Broberg K.
Influence of glutathione-related genes on symptoms and immunological biomarkers among vulcanization workers in the southern Sweden rubber industries.
Manuscript

IV. Jönsson LS, Broberg K, Axmon A, Bergendorf U, Littorin M, Jönsson BAG.
Levels of 1-hydroxypyrene, symptoms and biomarkers of response in vulcanization workers in the southern Sweden rubber industries.
Submitted

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ABBREVIATIONS

ACGIH	American Conference of Governmental Industrial Hygienists
ala	Alanine
ANOVA	Analysis of Variance
CI	Confidence interval
CRP	C reactive protein
CS_2	Carbon disulfide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
e.g.	Exempli gratia=for example
GCL	Glutamate cysteine ligase
GSH	Glutathione
GST	Glutathione S-transferase
1-HP	1-hydroxypyrene
IARC	International Agency for Research on Cancer
i.e.	Id est=that is
Ig	Immunoglobulin
ile	Isoleucine
LC	Liquid chromatography
LC-MS-MS	Liquid chromatography tandem mass spectrometry
LOD	Limit of detection
$MgCl_2$	Manganese dichloride
N or n	Number
NAT	N-acetyl transferase
OR	Odds ratio
PAH	Polycyclic aromatic hydrocarbon
PCR	Polymerase chain reaction
ppm	Part per million
RNA	Ribonucleic acid
R_S	Spearmans rank correlation coefficient
TTCA	2-thiothiazolidine-4-carboxylic acid
U	Units
val	Valine

CONTENTS

INTRODUCTION

General background

History of the rubber industry

As early as in the 6th century, natives of South America discovered natural rubber, a milky white substance that could be obtained from certain trees belonging to the family Euphorbiaceae. Later, the Amazons fashioned the rubber shoes by dipping their feet in natural rubber, drying the rubber moulds by a smoky fire and adding additional layers for strength. The discovery that rubber could be dissolved in petroleum naphtha and then applied to fabric, led to the production of waterproof garments and shoes, in the beginning of the 19th century. However, these products suffered from decomposition due to aging and temperature. In 1837, Charles Goodyear found that adding sulphur and heat to the rubber, made it more resistant to changes. He coined the term "vulcanisation", after Vulcan, the Roman god of fire, to describe the process. During World War II, the use of synthetic rubber rapidly increased due to the shortage of natural rubber and the increased demands of rubber products. A necessity for the increased exploitation was the growth of the petroleum industry and the advances in polymer technology. Still today, there is an ongoing development of the rubber by industry researchers and engineers experimenting with variations in additives and processing to achieve the best rubber characteristics for any particular application (Lewis 1999).

Occupational medicine in the rubber industry

The working environment in the rubber industry has traditionally applied a wide range of risks, including exposure to carcinogenic, allergenic, irritating and reproductive toxic compounds. Furthermore, the work has implied a risk of muscoskeletal diseases. It has been associated with a significant suffering for the person and large costs for the company and society. However, during the last decades the exposure situation has improved and the risk of symptoms in the contemporary rubber industry is not known. Also, the exposure in the rubber industry is very complex and it is unknown which agents causing the different diseases. Thus, to develop reliable methods for estimation of exposure-response relationships is important.

Exposure assessment

Establishment of reliable methods for exposure assessments are necessary for different reasons. It is essential for estimations of exposure-response relationships usable for risk assessments, as well as it facilitates the elimination of sources of exposure.

Exposure assessments have traditionally been conducted in air. However, lately so-called biological monitoring has become more common, i.e. when a compound or one of its metabolites is measured in a biological matrix, e.g. blood or urine. Biological monitoring has several advantages, e.g. it takes into consideration that the inhaled volume differs between different individuals and in different working situations, and it can be used even if personal protection devises are employed. Furthermore, it takes into account dermal absorption and it may also reflect genetic differences in biotransformation. However, it has some disadvantages, e.g. it can not be used to discover leaks. In addition, the air levels may better reflect symptoms from e.g. the eyes, than the concentrations in blood or urine. Thus, air monitoring and biological monitoring should be considered as complementary methods.

Exposure in the rubber industry

The exposure situation in the rubber industry is very complex. A large diversity of compounds is used and additional compounds are formed in the different processes. Therefore, it is not possible to conduct an exposure assessment for every single compound. One way to handle the assessment of the multitudinous exposure is to use index substances, i.e. a specific substance used as a marker of a larger group of compounds.

Vulcanisation is a basic process in the rubber industry when heat and pressure are applied to an uncured rubber. The purpose is to impart certain desirable properties such as toughness, elasticity, and resistance to corrosion, and to mould the product into its final shape. Many reactive compounds are present in vulcanisation fumes and thus, it may be interesting to study symptoms and biomarkers of response in relation to vulcanisation fumes.

Carbon disulfide (CS_2) is formed during the vulcanisation process due to decomposition of dithiocarbamates and thiurams (Craig et al. 1951), which are used as accelerators. Furthermore, sulphur is added to the rubber, which may give rise to CS_2. Large amounts of polycyclic aromatic hydrocarbons (PAHs) may be added to the rubber through extender oils, which may make up as much as 20% of the total weight of a tire or other rubber products (Talaska et al. 2002). In addition, PAHs may be present in the rubber fumes due to incomplete combustion or pyrolysis of organic material.

It is therefore relevant to investigate if biomarkers of CS_2 and PAH could be applied as index substances of vulcanisation fumes.

Biomarkers of exposure

Metabolism and monitoring of CS_2

One metabolite of CS_2 is 2-thiothiazolidine-4-carboxylic acid (TTCA), which has traditionally been used as a marker of CS_2 (Drexler et al. 1994; van Doorn et al. 1981). TTCA is a short-lived metabolite; the majority is excreted within 24 h after exposure (Riihimäki et al. 1992). CS_2 can also be formed *in vivo* when exposed to dithiocarbamates (Johnson et al. 1996). The *in vivo*-formed CS_2 are subsequently metabolised to TTCA, but there are indications that this exposure may be of minor importance in the rubber industry (Vermeulen et al. 2005). Only a few publications describe the levels of TTCA in urine from rubber workers (Cox et al. 1998; Vermeulen et al. 2005).

It is a well-known fact that many cruciferous vegetables, such as cauliflower, broccoli and radish, contain endogenous TTCA, which is excreted unchanged in urine (Kivistö 2000; Simon et al. 1994). It is also known that the biotransformation of, e.g., disulfiram (van Doorn et al. 1982) and captan (Anon 1996), give rise to increased levels of TTCA in urine. Furthermore, it has been suggested that tobacco use could affect the urinary levels of TTCA (Jian and Cao 2000). Thus, detectable urinary TTCA levels are observed in non-occupationally exposed subjects.

TTCA is formed metabolically via two distinct pathways in the human body (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, but 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. Therefore, it is interesting to study how gene variants of different metabolising genes affect the urinary levels of TTCA.

It has been observed *in vitro* that CS_2 can react with proteins (DeCaprio et al. 1992). In this way, CS_2 forms complexes large enough in size to evoke an immune response and thus, could be an inducer of airway diseases (Karol et al.

2001). Moreover, studies have indicated oxidative stress-related damage in workers exposed to CS_2 (Jian and Hu 2000; Wronska-Nofer et al. 2002).

Therefore, it is important to study urinary levels of TTCA in rubber workers for several reasons. On one hand, TTCA could be used as an index substance of vulcanization fumes, on the other as a biomarker of CS_2.

Metabolism and monitoring of PAH

PAH is a generic name for several hundred compounds. One of these is pyrene, which is metabolised into 1-hydroxypyrene (1-HP), partly conjugated with glucuronide or sulfate, and excreted in urine (Singh et al. 1995). Urinary 1-HP is suggested to be the most relevant parameter for estimating individual exposure to PAH (Dor et al. 1999). The half-life of 1-HP is approximately 18 h (Buchet et al. 1992). The exposure to PAHs in the rubber industry is not well characterized; only one earlier study describes the levels of 1-HP among workers handling cured rubber (Talaska et al. 2002).

The diet is estimated as an important source of PAH intake in the non-occupationally exposed population (IARC 1983). Food that frequently contains PAHs is, e.g. meat, meat products, cereals, potato, rice and popcorn. In meats, PAH formation is affected by the cooking method and doneness level (Kazerouni et al. 2001). Furthermore, smoking is a known source for PAH exposure (Jongeneelen 2001). Thus, detectable urinary 1-HP levels are observed in non-occupationally exposed subjects.

It has been described that metabolites of PAH react with proteins (Brunmark et al. 1997). In this way, complexes large enough in size to evoke an immune response may be formed and thus, could be an inducer of airway diseases (Karol et al. 2001). Furthermore, exposure to PAH may induce the formation of reactive oxygen species, as suggested from studies on associations between PAH exposure and a marker of oxidative damage (8-oxo-7,8-dihydro-2-deoxyguanosine) (Pilger and Rudiger 2006). Thus, it is important to study the exposure to PAH in the rubber industry for several reasons; on one hand 1-HP could be used as an index substance of vulcanisation fumes, on the other as a biomarker of PAH exposure.

Health effects in the rubber industry

Due to the complex exposure situation in the rubber industry, it has been difficult to attribute observed health effects to specific exposures. However, many of the compounds used or formed in the rubber industry are potential respiratory irritants or sensitisers with well-known acute or chronic effects on the lungs (Roth 1999).

There are several studies from the past describing diseases of the airways among rubber workers. Fine and Peters (Fine and Peters 1976) showed an

increased prevalence of chronic and acute bronchitis among vulcanisation workers compared to other rubber workers. Furthermore, Weeks et al. (Weeks et al. 1981) showed an increased risk of shortness of breath, wheeze, chest tightness, cough and sputum production in similar comparisons. In addition, Weeks et al. (Weeks et al. 1981) studied other acute symptoms such as nausea and headache. There are also indications of airway disease in more recent studies. Zuskin et al. (Zuskin et al. 1996) showed higher prevalence of chronic bronchitis, chronic cough and phlegm, dyspnoea and chest tightness among rubber workers in Croatia compared to controls. In addition, the risk of headache was investigated. However, there is no information on airways symptoms in the contemporary Swedish rubber industry. Furthermore, no information on exposure-response relationships is available. Such studies are therefore important.

Rubber workers have historically been exposed to several potential carcinogens, such as aromatic amines, nitrosamines, PAHs, solvents and asbestos and increased risks of bladder, lung and laryngeal cancer, as well as leukaemia have been observed (Roth 1999). Also, the use of solvents, such as CS_2, ethanol, phenol and butadiene, has traditionally been extensive in the rubber industry and suggested to be associated with cardiovascular disease among the exposed workers. The studies on neurological and genotoxic effects have been inconclusive (Roth 1999). Information on exposure to agents causing these diseases in the rubber industry is limited, and therefore the development of methods for exposure assessments of such agents is important.

Biomarkers of response

Biomarkers of response reflect a change in biological activity. Traditionally analysed biomarkers of response are immunological cells, antibodies and other proteins.

Circulating leukocytes consist of granulocytes (neutrophils, eosinophils and basophils), monocytes and lymphocytes. Mature blood granulocytes are normally distributed in equilibrium between the circulation and the walls of the blood vessels. A shift towards circulating granulocytes is seen in connection with physical or psychological stress. Only circulating granulocytes are analysed by routine clinical methods. Neutrophils are by far the most common blood leukocytes and in most cases, a change in the levels of circulating leukocytes reflects changed concentration of neutrophils (Ganrot et al. 1997).

The neutrophils are the “Home Guard” of the immune defence; they are recruited at short notice to the site of infection/inflammation to perform phagocytosis, i.e. killing microbes without specificity. The eosinophils make up less than 3% of the total number of circulating leukocytes. However, the number

grows, especially in the tissues where the eosinophils become activated, at infections by parasites, or at asthma and allergy.

C reactive protein (CRP) and α_1-antitrypsin are so called acute phase proteins, i.e. they belong to a group of protein whose synthesis rate increases more than 100% at an acute phase reaction. This type of reaction arises when tissue is injured and the process can be either acute (e.g. pneumonia) or chronic (e.g. rheumatoid arthritis). CRP and α_1-antitrypsin are synthesised in the liver and can be observed at increased levels in the plasma after eight hours (CRP) or 24-48 hours (α_1-antitrypsin) after the injury.

The physiological function of CRP is not completely known. Increased levels are seen e.g., during bacterial infections, diseases characterised by cell decay, and some chronic inflammations. The half-life is a couple of hours. α_1-antitrypsin is the most common protease inhibitor in plasma. It can, due to its low molecular weight, penetrate to the intercellular spaces and inactivate enzymes from granulocytes and macrophages. For example, increased levels are seen at liver damage and decreased levels at chronic obstructive lung disease. The half-life is approximately six days.

Immunoglobulins (Igs) are present in intercellular fluid, in secrete and on the cell membrane of B-lymphocytes. Their function is to inhibit invasion of pathogens and to protect against their toxic molecules. After stimulation by antigens, Igs are synthesised and usually the IgM antibodies are formed before the other Igs. The major part (75%) of the IgMs are present intravascular. The half-life is ten days. Regarding IgG, the amount localised intravascular and extravascular is approximately the same. The half-life is in most cases 23 days. IgA is known as the immunoglobulin of the mucosa, as it, apart from being present in plasma and intercellular fluid, is produced in excretory glands and in the submucosa of the airways, intestines and the urinary tract and thus, an important part of the immune defence in the mucosa. The half-life is significantly shorter than for IgG. IgE can bind to specific receptors on basophils and mast cells in the mucosa and skin. Thereby are histamine and other vasoactive substances released from these cells at antigen activation. This is a defence against parasites, but also a constituent of asthma and allergy. The half-life of IgE is 2.5 days (Hellman 2007).

In conclusion, it is of interest to study the levels of biomarkers of response in the contemporary rubber workers compared to controls and to investigate possible exposure-response relationships with TTCA and 1-HP.

Biomarkers of susceptibility

Incorporation of biomarkers of susceptibility in exposure assessments and epidemiological studies may improve the exposure and health risk estimates. An increasing number of genes involved in the biotransformation of toxic

xenobiotics and endobiotics are being recognized as polymorphic and hence to consider as biomarkers of susceptibility. A genotype associated with a decreased level of functional protein, either through lower expression of the protein or an expression of a defective protein, will cause a higher level of the parent compound and lower levels of its metabolites. Absence of the protein due to a partial or complete deletion of the gene will also be associated with a changed metabolite pattern. Thus, different alleles may give rise to different sensitivity to toxic compounds due to changed efficiency of biotransformation. Application of biomarkers of susceptibility may therefore be of importance in studies of the exposure-response relationships in the rubber industry. Several polymorphisms in different enzymes may be of interest.

GSH is synthesized from glutamate, cysteine and glycine. The synthesis is catalysed sequentially by two enzymes, glutamate cysteine ligase (GCL) and glutathione synthase. 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) (Figure 1). GCL consist of a catalytically active subunit (encoded by *GCLC*) and a modifying subunit (encoded by *GCLM*). *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 the C allele when exposed to oxidants (Nakamura et al. 2002). Nakamura et al. also showed that the plasma GSH levels were significantly lower in CT and TT genotypes than in CC genotype.

It is possible that the conjugation of GSH to CS_2 is catalysed by the family of glutathione S-transferase enzymes (GSTs; Figure 1). Many of them are involved in biotransformation of toxic xenobiotics and endobiotics. *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. GSTM1, as well as GSTT1, are expressed at high levels in the liver but also found in several other tissues including the lung (Juronen et al. 1996; Mace et al. 1998). *GSTM1**O and *GSTT1**O seem to be associated with susceptibility to asthma. Furthermore, *GSTM1* and *GSTT1* have been associated with a risk of coronary arterial disease, but studies so far have been inconclusive (Coles and Kadlubar 2005). In addition, *GSTM1**O and *GSTT1**O have shown consistent associations with an increased risk of different types of cancer (Bolt and Thier 2006). The frequency of *GSTT1**O is generally lower in Caucasians (10-25%) than in Asians (20-65%) whereas no major difference is observed between Caucasians and Asians regarding *GSTM1**O (Garte et al. 2001).

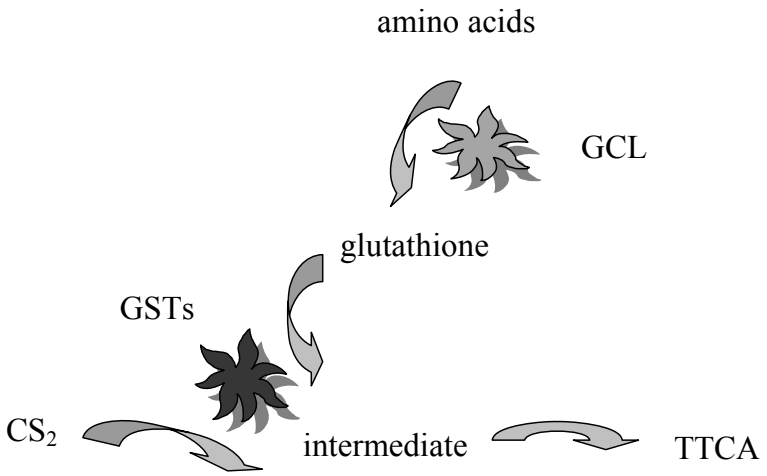

Figure 1. *An overview of glutathione-related biotransformation of CS_2. GCL (glutamate cysteine ligase) catalyses the synthesis of glutathione, which is conjugated to CS_2, possibly with help from GSTs (glutathione S-transferases).*

GSTA1-1 is present at high levels in the liver, small intestine, testis, kidney, adrenal gland, and pancreas and at low levels in a wide range of tissues (Morel et al. 2002). *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). The mean expression of GSTA1-1 in liver samples from *GSTA1*(G/G) donors was approximately fourfold higher than in samples from *GSTA1*(A/A) donors (Coles et al. 2001a). However, *GSTA1* genotype does not correlate with GSTA1-1 expression in pancreas (Coles and Kadlubar 2003). The association between *GSTA1* polymorphisms and disease has been little studied (Coles and Kadlubar 2005). The *GSTA1*(A/A) is most probably rarer in Japanese than in Caucasians (16% compared to 40-42%) (Coles et al. 2001b; Matsuno et al. 2004).

There are at least two functional polymorphisms in the coding region of *GSTP1*. The variant allele of the polymorphism named *GSTP1*-105 encodes valine (val) instead of isoleucine (ile) at codon 105 due to a base pair exchange where G substitutes A. The variant allele of the polymorphism named *GSTP1*-114 encodes val instead of alanine (ala) 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).

Thus, application of information about these polymorphisms may sharpen the understanding of exposure-response relationships in the rubber industry. An

overview of the polymorphisms and suggested mode of action is presented in Table 1.

Table 1. *An overview of several polymorphisms in glutathione-related genes.*

Gene	**Genetic location**	**Polymorphism**	**Suggested mode of action (see references in the text)**
GCLC	6p12	-129C/T	Decreased promoter activity
GCLM	1p22	-588C/T	Decreased promoter activity
GSTA1	6p12	-52G/A	Affected transcription factor binding site in the promoter
GSTM1	1p13	Deletion	No enzyme activity
GSTP1	11q13	105ile/val, 114ala/val	Affected ability and rate of binding between enzyme and toxic compounds
GSTT1	22q11	Deletion	No enzyme activity

AIMS

General aim

The overall aim of this thesis was to find exposure-response relationships usable for risk assessments in the rubber industry.

Specific aims

To describe the levels of urinary TTCA among vulcanisation workers in the rubber industry in Southern Sweden.

To describe the levels of urinary 1-HP among vulcanisation workers in the rubber industry in Southern Sweden.

To analyse the risk of symptoms and changed levels of immunological markers among vulcanisation workers in the rubber industry in Southern Sweden.

To examine the relationships between urinary TTCA levels and symptoms, as well as immunological markers.

To examine the relationships between urinary 1-HP levels and symptoms, as well as immunological markers.

To analyse how gene variants of different metabolising genes affect the urinary levels of TTCA.

To examine the association between gene variants and symptoms, as well as immunological markers.

MATERIAL AND METHODS

Companies

Companies were selected on the grounds of geographical accessibility and diversity of production methods. 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 vulcanisation methods like microwaves, salt baths and steam vulcanisation in an autoclave. Compression vulcanisation 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 vulcanisation, all situated in the same large production hall. However, the workers were divided into two divisions; in division A (n = 5) the vulcanisation units were placed sparser and were less frequently used than in 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 my study. Division A manufactured industrial profiles; one division used continuous vulcanisation 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 vulcanisation in an autoclave. Several joining operations were also performed. Factory 2 (n = 4) produced sealing strips and profiles for the consumer market, using continuous vulcanisation in salt baths with non-nitrite containing salt.

Company 4 (n = 21) primarily produced industrial profiles in a large production hall with continuous vulcanisation 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 vulcanisation units. Some post-vulcanisation was performed in hot-air ovens.

Company 6 (n = 10) manufactured different rubber details such as gaskets and seals for cables for the automotive industry and other industrial sectors. The company had both injection and compression vulcanisation 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 producing method was compression and injection vulcanisation.

Company 8 consisted of two different factories. Factory 1 (n = 12) produced sealing profiles, including roof and window applications, in continuous vulcanisation lines using hot air and salt bath. Furthermore, a few small compression vulcanisation 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 vulcanisation units. Post-vulcanisation was done in hot-air ovens. Peroxide was the most used vulcanisation agent in this factory, but sulphur was also used.

Study subjects

Included in the study were 166 exposed workers, who vulcanised with sulphur or worked in the same hall as people vulcanising with sulphur the same day as the urine samples were collected. In addition, 117 controls with no occupational contact with rubber or plastic chemicals were included. These were working as assemblers of armature at an electric light fittings company (n = 21), as meat cutters at a butchery (n = 20), as operators at a dairy plant (n = 11), as postmen (n = 27) or as department store workers (n = 38). All study subjects were working in Southern Sweden and were present at work at the time of the medical examinations. The investigation was conducted at work on Tuesday through Thursday. Urine was collected during the last four hour of an eight hour work shift. For some study subjects, data on one or more parameters was missing, but these study subjects were included in the analyses when possible. Some individual characteristics of the exposed workers and the controls are shown in Table 2. There was a difference concerning ethnicity between the two groups. Based on their names, the majority in both groups were of European descent (predominating Swedish) but 21 of 166 (13%) exposed workers, but none of the controls, were of Eastern Asian descent. 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.

Table 2. *The characteristics of the exposed workers and the controls.*

	Exposed workers n = 166	Controls n = 117
Age, median (range) years	38 (19-65)	41 (20-63)
Sex (male/female)	83 / 83	57 / 60
Smoking; n (%) [a)]	52 (31)	44 (38)
Snuffing; n (%) [a)]	22 (19)	30 (18)
Atopy; n (%)	52 (32)	31 (27)
Eastern Asian descent; n (%)	21 (13)	-

[a)] Denoted smoker or snuffer if stopped < 6 months ago

Medical examination

Medical and occupational histories were obtained by structured interviews, based on a slightly modified questionnaire adopted from Nielsen et al. (Nielsen et al. 1988) and used in Littorin et al. (Littorin et al. 2000). The structured interviews were carried out by a physician, a trained specialist in occupational medicine. The same person performed all the interviews and she was aware of the exposure status at the time of the interviews. Questions dealt with symptoms which had occurred during the past 12 months and had appeared in the eyes (itching, running, and/or burning), nose (bleeding, running, stuffiness, and/or sneezing/itching), or lower airways (severe dry cough and/or dyspnoea, wheezing, chest tightness). Furthermore, other symptoms supposed to be associated with exposure to fumes, i.e., throat burning or dryness, hoarseness, headache and nausea were recorded. At the time of the medical examinations, the results of the biomarker analyses were not known to the workers or the investigators.

Before the physician's interviews, the study subjects answered self-administered questionnaires with questions dealing with eye and nose symptoms and nose bleeds the last year. Furthermore, on the day of the medical examinations, the study subjects answered questions asked by a nurse concerning symptoms during the last three days. The medical examinations were performed on the same day as the urine samples were collected.

Blood analysis

Venous blood samples were collected. Number per litre of leukocytes, neutrophils and eosinophils was determined in blood. Plasma concentrations of α1-antitrypsin, CRP, total IgG, IgM and IgA were analysed. Concentrations in serum of total and specific IgE against ten common allergens (Phadiatop test)

were measured. All analyses were done by routine clinical methods and were performed on blinded samples. Atopy was defined as positive, when at least one of the allergens in the Phadiatop test gave a response.

Analysis of TTCA

The levels of TTCA were analysed as previously described (Vermeulen et al. 2005). Briefly, the urine was acidified and the ion-strength increased, thereafter TTCA was extracted using ethyl acetate. The organic phase was evaporated and redissolved 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 less than the 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 results were within the tolerance limits in 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). The levels of TTCA were adjusted for creatinine content.

Analysis of 1-HP

The levels of 1-HP were analysed as previously described (Jongeneelen and Anzion 1991). Briefly, the urine was acidified and the glucoronic acid and sulphate was enzymatically removed. The samples were purified using solid phase extraction and analysed by liquid chromatography (LC) and fluorescence detection. LOD was determined to be 0.01 ng/ml urine. Samples less than the LOD were assigned a value of half the LOD. The precision, as determined from double analyses of the same sample at different days, was 7% at 2.3 ng/ml. The results were within the tolerance limits in 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). The levels of 1-HP were adjusted for creatinine content.

Analysis of creatinine

Creatinine was analysed enzymatically with a routine analysis at the Department of Clinical Chemistry, Lund University Hospital, according to Mazzachi et al. (Mazzachi et al. 2000).

Genotyping

DNA was extracted from whole blood using QIAamp DNA Blood Mini Kit at the SWEGENE Resource Center for Profiling Polygenic Disease, 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 instrument using probes, primers and primer concentrations as described by Custodio et al. (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 was used except for the analysis of *GSTP1*-114, for which a PCR buffer containing 2.5 mM $MgCl_2$, Rox, 0.8 mM dNTP and 1 U Taq Platina was used. For genotyping of *GSTA1*, the concentration of Master Mix was 0.7 x instead of 1.0 x.

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. (Hou et al. 1995) and Pemble et al. (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 containing 0.4 µM of each primer, 1.25 mM $MgCl_2$, 0.8 mM dNTP and 0.5 U Taq Platina was used. For all analyses, positive controls for the different genotypes as well as a negative control (water instead of DNA) were included in each run. Moreover, approximately 10% of the samples were reanalysed as a control of the method.

Statistical analyses

Differences in metabolite levels between different exposure groups were estimated using Analysis of Variance (ANOVA). Furthermore, the effect of sex, smoking, snuffing, genotype or genotype combinations, on metabolite levels in different exposure groups were analysed using ANOVA. Effects of exposure (i.e. exposure status, TTCA subgroups and 1-HP subgroups) or genotype on symptoms was estimated by odds ratios (ORs) calculated using logistic regression, whereas effects of exposure or genotype on immunological markers was estimated using ANOVA.

Possible effect modification was estimated by analysing the effect estimates in different strata or by analysing the interaction between the exposure (e.g. exposure status or TTCA subgroups) and the independent variable (e.g. atopy or smoking) in a separate analysis.

A possible trend over exposure categories (the three TTCA or 1-HP subgroups) was evaluated using Cochran-Armitage test for trends of ORs using unadjusted data. A trend test with regards to differences in immunological

markers was performed by including TTCA or 1-HP subgroups as a continuous variable in a separate linear regression model. For all analyses two-sided significance was used.

Not normally distributed variables were $\log_{10}$-transformed (**Paper I**) or natural log-transformed (**Paper II, III, IV**). The effect estimates calculated using log-transformed data for the outcome were interpreted using the formula $100(e^{\beta} - 1)$, where β is the effect estimate. The formula describes the percentage increase or decrease in the average level of the outcome (Vittinghoff et al. 2005).

Confounders were evaluated by three different methods; the change-in-estimate approach (Greenland 1989) (**Paper I**), an analysis of the association of the potential confounder with predictor and outcome (**Paper IV**), and an inclusion of possible biological and statistical confounders (**Paper II** and **III**). For the change-in-estimate approach, a change of the estimate of more than 10% was required for inclusion of the potential confounder in the final model. In paper IV, potential confounders were evaluated for association with exposure and the dependent variables. Categorical factors were considered as confounders if the fraction between groups (e.g. men and women, smokers and non-smokers) differed more than five percent units, continuous factors if the median difference between groups exceeded ten percent. Associations between two continuous variables were determined using Spearman's coefficient of correlation (R_S), where a coefficient greater than 0.15 was considered sufficient association for inclusion. Potential confounders were included in the model only if they were found to be associated with both the exposure and the dependent variable. However, to obtain comparable adjusted estimates, potential confounders were included in all final models for each scientific question in both papers I and IV, if they fulfilled the inclusion criteria for at least one analysis.

The reason for the change in methods was that the change-in-estimates were far too time-consuming in biomedical studies of this kind with many variables and small effects, and the realisation that the results supplied by the final models were not significantly affected by the inclusion of possible confounders. Repeating the analyses without adjustments validated this.

Genotype distribution was analysed with the χ^2 test, except when the expected individual frequencies were smaller than five, 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. When the association between genotype and outcome was analysed, the following genotypes were used as reference categories: *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.

For all statistical analyses, SPSS v.13.0 (SPSS Inc., Chicago, IL, USA) was used, apart from Fisher-Freeman-Halton's test and trend test for ORs, when StatXact v. 6 (Cytel Software Corp., Cambridge, Mass, USA) was used.

An overview of the strategies used in the four papers is presented in Table 3.

Table 3. *Strategies used in the different papers.*

Paper	Studied groups	Main predictor	Adjustments	Effect modification	Outcome
I	All	Exposure status	None		TTCA
	Exposed	Vulcan. method	None		TTCA
	Exposed	Sex	Tobacco [a)]		TTCA
	Controls	Sex	Tobacco [a)]		TTCA
	Exposed	Genotype	Sex, age, tobacco [a)]		TTCA
	Controls	Genotype	Sex, age, tobacco [a)]		TTCA
	Exposed	*GSTM1+GSTT1*	Sex, age, tobacco [a)]		TTCA
II	All	Exposure status	Atopy, age, sex, smoking		Symptoms, imm. markers
	All	TTCA subgroups	Atopy, age, sex, smoking		Symptoms, imm. markers
	All	Exposure status	Atopy, age, sex, smoking	Atopy, smoking	Symptoms, imm. markers
	Exposed	TTCA subgroups	Atopy, age, sex, smoking	Atopy, smoking	Symptoms, imm. markers
	Exposed	TTCA subgroups	None; Atopy, age, sex, smoking		Symptoms, imm. markers: Trend test
III	All	Genotype	Atopy, age, sex, smoking, snuffing	Exposure status	Symptoms, imm. markers
	Exposed	Genotype	Atopy, age, sex, smoking, snuffing		Symptoms, imm. markers
	Controls	Genotype	Atopy, age, sex, smoking, snuffing		Symptoms, imm. markers
IV	All	Exposure status	Age, smoking		1-HP
	Exposed	Vulcan. method	Atopy, age, sex, smoking		1-HP
	Exposed	Sex	Atopy, age, smoking, snuffing		1-HP
	Controls	Sex	Atopy, age, smoking, snuffing		1-HP
	Exposed	Smoking	Atopy, sex		1-HP
	Controls	Smoking	Atopy, sex		1-HP
	Exposed	Snuffing	Sex, smoking		1-HP
	Controls	Snuffing	Sex, smoking		1-HP
	All	1-HP subgroups	Age, sex, smoking		Symptoms, imm. markers
	Exposed	1-HP subgroups	None; Age, sex, smoking		Symptoms, imm. markers: Trend test

[a)] Tobacco denotes smoking and/or snuffing habits.

RESULTS WITH COMMENTS

Biomarkers of exposure

Compared to controls, exposed workers were estimated to have in average 420% higher urinary TTCA level (adjusting for smoking; **Unpublished**) and in average 200% higher urinary 1-HP level (adjusting for age and smoking; **Paper IV**) (P<0.001 for both metabolites). Thus, urinary levels of TTCA and 1-HP appeared to be good markers of the exposure in vulcanisation departments. The levels of TTCA and 1-HP differed between different rubber companies and different subdivisions at the same company (Table 4).

Table 4. *Levels of 2-thiothiazolidine-4-carboxylic acid (TTCA; µmol/mol creatinine) and 1-hydroxypyrene (1-HP; µmol/mol creatinine) in controls and exposed workers at different companies and different subdivision of the same company (**Paper I** and **IV**).*

		TTCA		1-HP	
Company	N	Geometric Mean	Range	Geometric Mean	Range
Controls	106	4.3	<LOD - 460	0.042	<LOD – 0.35
All exposed workers	163	22	<LOD - 950	0.12	<LOD – 1.3
Company 1	10	11	5.5 - 25	0.15	0.085 – 0.23
Company 2	16	12	4.6 - 690	0.25	<LOD – 0.84
Division A	5	6.0	4.6 – 7.1	0.22	<LOD – 0.49
Division B	11	17	4.7 - 690	0.27	0.072 – 0.84
Company 3	36	55	23 - 220	0.12	0.003 – 1.3
Division A	29	64	28 - 220	0.13	0.003 – 1.3
Division B	3	28	23 - 32	0.095	0.050 – 0.13
Factory 2	4	30	23 - 40	0.072	0.015 – 0.41
Company 4	21	220	72 - 950	0.038	<LOD – 0.29
Company 5	15	14	3.6 - 780	0.18	<LOD – 0.61
Company 6	10	17	2.8 - 45	0.27	0.081 – 0.89
Company 7	11	13	2.7 - 41	0.074	0.015 – 0.36
Company 8	44	7.6	<LOD - 410	0.14	0.010 – 0.66
Factory 1	12	15	5.3 - 70	0.13	0.021 – 0.40
Factory 2	32	5.9	<LOD - 410	0.14	0.01 – 0.66

LOD denotes “limits of detection”

The levels of the examined metabolites differed with different vulcanisation methods and showed in general a diametrical pattern; exposed workers using injection or compression vulcanisation had low levels of TTCA but high levels

of 1-HP, whereas exposed workers using salt bath, hot air, microwaves or fluid-bed vulcanisation had high levels of TTCA and low levels of 1-HP (Table 5).

Table 5. *Levels of 2-thiothiazolidine-4-carboxylic acid (TTCA; µmol/mol creatinine) and 1-hydroxypyrene (1-HP; µmol/mol creatinine) in a group of 157 workers* [a)] *vulcanising with different methods (**Paper I** and **IV**).*

Vulcanisation method	TTCA Geometric mean (Range)	1-HP Geometric mean (Range)
Compression and injection	11 (<LOD – 780)	0.16 (< LOD – 1.3)
Hot air, microwaves, fluid-bed	49 (5.3 – 700)	0.069 (< LOD – 0.85)
Salt bath	86 (8.0 – 950)	0.087 (< LOD – 0.51)

[a)] Three employees were not possible to classify and three employees working with steam vulcanisation were not included.
LOD denotes "limit of detection"

The correlation between TTCA and 1-HP among exposed workers was rather low (R_S=-0.11, P=0.17). Thus, there seemed to be two different types of exposure in the rubber industry, and TTCA and 1-HP appeared to be good index substances of these types.

Although the overall pattern of exposure was diametrical (compressing and injection vulcanisation versus continuous vulcanisation), there was a difference, although not statistically significant, between exposed workers vulcanising with different types of continuous vulcanisation (salt bath or hot air, microwaves, fluid-bed). Exposed workers vulcanising with salt bath had higher levels of TTCA (86 µmol/mol creatinine) than exposed workers vulcanising with other types of continuous vulcanisation (49 µmol/mol creatinine; P=0.098). Salt bath vulcanisation workers also had higher levels of 1-HP (0.087 µmol/mol creatinine) than exposed workers using other types of continuous vulcanisation (0.069 µmol/mol creatinine; P=0.31). A possible explanation for the different levels of biomarkers is the use of different rubber qualities (**Paper I** and **IV**).

There were also some differences in the levels of metabolites for sex and smoking habits (Table 6). However, adjusting for age, atopy, smoking and snuffing habits, there was no statistically significant difference between exposed men and women either regarding urinary levels of TTCA (P=0.47) or 1-HP (P=0.66). The differences, calculated as suggested by Vittinghoff et al. (Vittinghoff et al. 2005), was small; women had in average 18% less TTCA and 9.2% more 1-HP than men. Nevertheless, among controls, there was a statistically significant difference (P<0.001) between sexes regarding 1-HP, women having in average 330% higher 1-HP level than men, but not regarding TTCA (P=0.87), women having only 4.9% more TTCA than men.

Table 6. *Levels of 2-thiothiazolidine-4-carboxylic acid (TTCA; µmol/mol creatinine) and 1-hydroxypyrene (1-HP; µmol/mol creatinine) among the study subjects, stratified for sex and smoking habits. Please note that the data are unadjusted (**Unpublished**).*

	TTCA		1-HP	
	Geometric mean (range)		Geometric mean (range)	
	Controls	Exposed workers	Controls	Exposed workers
Men	4.8 (<LOD – 160)	25 (2.7 – 690)	0.021 (<LOD – 0.25)	0.11 (<LOD – 0.61)
Women	4.2 (<LOD – 460)	15 (<LOD – 950)	0.080 (<LOD – 0.35)	0.13 (<LOD – 1.3)
Smokers	4.3 (<LOD – 80)	29 (2.5 – 690)	0.079 (<LOD – 0.29)	0.22 (0.021 – 1.3)
Non-smokers	4.5 (<LOD – 460)	17 (<LOD – 950)	0.029 (<LOD – 0.35)	0.096 (<LOD – 0.66)

LOD denotes “limit of detection”

Adjusting for sex and atopy, smokers had increased urinary 1-HP levels; exposed workers who smoked had in average 130% higher 1-HP level than non-smokers (P<0.001), while smoking controls had 140% higher 1-HP level than non-smokers (P=0.002). Regarding TTCA there was no difference between smokers and non-smokers; exposed workers who smoked had in average 39% higher TTCA level than non-smokers (P=0.23), while smoking controls had 2.3% lower TTCA level than non-smokers (P=0.94) (**Unpublished, Paper IV)**.

Health effects

Exposed workers had, compared to controls, increased risk of itching, running or burning eyes, nose bleeds, throat burning and dryness, hoarseness, severe dry cough, nausea and headache (**Paper II**). No increased risk of symptoms from the nose, apart from nose bleeds, was observed. The pattern was similar when comparing the ORs of symptoms recorded during 12 months and three days (Table 7).

Table 7. *Odds ratios (ORs) with 95% confidence intervals (CIs) of symptoms during the past 12 months and during the last three days, respectively, among all exposed workers compared to controls (**Unpublished**).*

Symptom	Odds ratio (95% CI) [a]	
	12 months	3 days
Itching, running or burning eyes	**3.0** (1.7 – 5.2)	**5.2** (2.3 – 12)
Running nose	1.0 (0.59 – 1.8)	1.2 (0.58 – 2.4)
Nose stuffiness	0.88 (0.51 – 1.5)	0.88 (0.49 – 1.6)
Sneezing and/or nose itching	0.89 (0.53 – 1.5)	0.93 (0.53 – 1.6)
Nose bleeds	**4.0** (1.6 – 10)	**5.2** (1.1 – 24)
Throat burning and dryness	**3.0** (1.6 – 5.5)	**2.1** (1.1 – 4.0)
Hoarseness	**2.4** (1.1 – 5.0)	-[b]
Dyspnea, wheezing and/or chest tightness	1.2 (0.66 – 2.2)	2.0 (0.83 – 4.9)
Severe dry cough	**3.8** (1.9 – 7.7)	1.9 (0.80 – 4.7)
Nausea	**4.3** (1.6 – 12)	**4.0** (1.3 – 12)
Headache	**2.5** (1.5 – 4.4)	**4.5** (2.2 – 8.9)

[a] Effect estimates are adjusted for age, sex, atopy and smoking.
[b] No control reported hoarseness.

When excluding study subjects of presumable Eastern Asian descent, the pattern was consistent with the results for the whole study population (data not shown).

No effect modification of smoking was found on the associations between exposure status and the risk of symptoms. Regarding atopy, effect modification was observed on the association between exposure status and eye symptoms (P=0.027; **Paper II**).

By using a trichotomised exposure measure (trichotomised for urinary levels of TTCA or 1-HP), possible exposure-response relationships were examined. A statistically significant trend over the three TTCA subgroups was observed for nausea (P=0.002) and headache (P=0.020; **Paper II**) and a consistent relationship were found regarding 1-HP and severe dry cough, although the trend over the three 1-HP subgroups did not reach statistical significance (**Unpublished**). No other consistent exposure-response relationships were found. Instead, a pattern with the most symptomatics belonging to the intermediate TTCA subgroup was observed (**Paper II**). For 1-HP the pattern was more divergent (**Paper IV**). This could indicate that the symptoms are caused by an exposure that does not correlate with CS_2 or PAH. Furthermore, the results could indicate a healthy worker selection.

An effect modification of smoking on the association between TTCA subgroups and running nose was observed (P=0.039). No effect modification of atopy was found on the associations between TTCA subgroups and the risk of symptoms (**Paper II**). The results of effect modification are inconsistent and may be a result of multiple testing.

Dividing the exposed workers into two equally sized groups regarding the levels of TTCA or 1-HP and thereby forming four different groups (low/low, low/high, high/low, high/high), did not improve the exposure-response relationships (**Unpublished**; Table 8). Thus, better index substances or more comprehensive exposure assessments are needed for consistent exposure-response relationships.

Different vulcanisation methods were associated with different risks of the examined symptoms compared to controls (**Paper II**). The highest risks of hoarseness, severe dry cough, nausea and headache, were observed among exposed workers using compression or injection vulcanisation. Regarding some symptoms, e.g. eye symptoms, nose bleeds and throat symptoms, the difference in risk was large between exposed workers vulcanising with different continuous vulcanisation methods (hot air, microwaves, fluid-bed vs. salt bath), whereas for other symptoms, e.g. running nose, sneezing etc and severe dry cough, it was small.

No effect modification of smoking was observed for the association between vulcanisation methods and symptoms. Regarding atopy, an effect modification was observed for running nose (P=0.045).

Table 8. *Odds ratios (ORs) with 95% confidence intervals (CIs) of symptoms during the past 12 months among exposed workers with different levels of 2-thiothiazolidine-4-carboxylic acid (TTCA; cut-off at 23 µmol/mol creatinine) and 1-hydroxypyrene (1-HP; cut-off at 0.136 µmol/mol creatinine), compared to the controls (**Unpublished**).*

Symptom	Odds ratio (95% CI) [a]			
	Low TTCA + Low 1-HP	Low TTCA + High 1-HP	High TTCA + Low 1-HP	High TTCA + High 1-HP
Itching, running or burning eyes	**3.1** (1.4 – 7.0)	**4.1** (1.8 – 9.0)	**3.0** (1.4 – 6.7)	**3.7** (1.6 – 8.4)
Running nose	0.95 (0.39 – 2.3)	0.85 (0.35 – 2.0)	1.2 (0.51 – 2.8)	1.7 (0.73 – 3.9)
Nose stuffiness	1.7 (0.75 – 3.7)	0.71 (0.30 – 1.7)	0.66 (0.27 – 1.6)	0.81 (0.33 – 1.9)
Sneezing and/or nose itching	0.69 (0.31 – 1.6)	0.84 (0.38 – 1.8)	0.97 (0.45 – 2.1)	1.3 (0.61 – 2.9)
One or more of nasal symptoms mentioned above	1.6 (0.73 – 3.7)	1.3 (0.61 – 2.9)	1.1 (0.51 – 2.3)	1.9 (0.85 – 4.4)
Nose bleeds	3.1 (0.95 – 10)	**3.8** (1.2 – 12)	**5.5** (1.8 – 16)	1.3 (0.30 – 5.6)
Throat burning and dryness	**4.3** (1.8 – 10)	**2.7** (1.1 – 6.3)	**3.5** (1.5 – 8.2)	1.5 (0.61 – 3.9)
Hoarseness	**3.4** (1.2 – 9.1)	**3.1** (1.2 – 8.3)	0.95 (0.28 – 3.2)	2.1 (0.74 – 6.2)
Dyspnea, wheezing and/or chest tightness	0.89 (0.34 – 2.3)	2.1 (0.90 – 4.8)	1.1 (0.45 – 2.8)	0.82 (0.32– 2.1)
Severe dry cough	**3.3** (1.2 – 9.1)	**9.3** (3.7 – 23)	**2.8** (1.0 – 7.6)	**4.1** (1.5 – 11)
Nausea	2.0 (0.49 – 8.5)	**7.0** (2.1 – 23)	**4.2** (1.1 – 15)	3.3 (0.93 – 12)
Headache	**2.8** (1.2 – 6.4)	**3.3** (1.5 – 7.4)	**2.9** (1.3 – 6.4)	1.6 (0.69 – 3.5)

[a] Effect estimates are adjusted for age, sex, atopy and smoking.

Immunological markers

Increased levels of total IgG were observed among exposed workers compared to controls. When excluding study subjects of presumable Eastern Asian descent, the pattern was consistent with the results for the whole study population (data not shown). No effect modification of smoking was observed on the association between exposure status and immunological markers. Regarding atopy, an effect modification was found on the association between exposure status and total IgA (P=0.003) as well as total IgG (P=0.046) (**Paper II**).

No exposure-response relationship with either TTCA or 1-HP and immunological markers was found (P>0.05). However, increased levels of leukocytes, neutrophils and eosinophils were observed in the high TTCA subgroup and an increased level of neutrophils in the high 1-HP subgroup. Regarding total IgG, the highest levels were observed in the low subgroups and the lowest levels in the high subgroups (**Paper II** and **IV**). The results indicate that total IgG may be induced even at low exposures of vulcanisation fumes. An alternative explanation is of course that the total IgGs are directed against exposures that are not related to CS_2, PAH and vulcanisation fumes.

No effect modification of smoking was observed on the association between TTCA subgroups and immunological markers. Regarding atopy, an effect modification on the association between TTCA subgroups and total IgA (P=0.045) as well as neutrophils (P=0.033) was found.

Dividing the exposed workers into two equally sized groups regarding the levels of TTCA or 1-HP and thereby forming four different groups (low/low, low/high, high/low, high/high), did not improve the exposure-response relationships (**Unpublished**; Table 9). Thus, better index substances or more comprehensive exposure assessments are needed for consistent exposure-response relationships.

Different vulcanisation methods were associated with different derangements of the levels of immunological markers compared to controls (**Paper II**). Statistically significant changes were observed regarding total IgG (increased levels among vulcanisation workers using compression, injection, hot air, microwaves or fluid-bed), total IgE (increased levels among vulcanisation workers using compression and injection) and total IgA (decreased levels among vulcanisation workers using hot air, microwaves and fluid-bed).

No effect modification of smoking on the association between vulcanisation methods and immunological markers was observed. Regarding atopy, an effect modification was observed for total IgA (P=0.024).

Table 9. *Changes (with 95% confidence intervals)[a] in the levels of immunological markers among exposed workers with different levels of 2-thiothiazolidine-4-carboxylic acid (TTCA; cut-off at 23 µmol/mol creatinine) and 1-hydroxypyrene (1-HP; cut-off at 0.136 µmol/mol creatinine), compared to the controls (**Unpublished**).*

Marker	Change (95% CI) [b]			
	Low TTCA + Low 1-HP	Low TTCA + High 1-HP	High TTCA + Low 1-HP	High TTCA + High1-HP
Leukocytes	-1.9 (-11 – 7.7)	4.9 (-4.3 – 15)	5.3 (-3.8 – 15)	7.1 (-2.4 – 18)
Neutrophils	-1.1 (-13 – 12)	8.2 (-4.1 – 22)	4.9 (-6.9 – 18)	7.6 (-4.8 – 22)
Eosinophils	-1.4 (-19 – 20)	3.6 (-15 – 26)	5.2 (-13 – 27)	**30** (7.5 – 58)
α1-antitrypsin	0.20 (-5.5 – 6.2)	4.3 (-1.5 – 10)	0.80 (-4.7 – 6.7)	1.6 (-4.1 – 7.7)
C-Reactive Protein	6.7 (-5.4 – 21)	3.4 (-8.2 – 16)	-1.9 (-13 – 10)	-2.2 (-13 – 10)
IgG	**11** (3.0 – 20)	**16** (7.7 – 25)	**16** (7.5 – 24)	2.6 (-4.8 – 11)
IgA	-1.2 (-15 – 15)	0.90 (-13 – 17)	-4.7 (-18 – 11)	-13 (-25 – 2.1)
IgM	4.4 (-11 – 22)	**19** (2.6 – 39)	12 (-3.9 – 30)	-12 (-25 – 2.8)
IgE	42 (-6.7 – 120)	**63** (8.0 – 150)	50 (-0.20 – 130)	-9.2 (-40 – 38)

[a] Percentage change in the average value of the outcome in each examined group compared to the controls (Vittinghoff et al. 2005).
[b] Adjusted for age, sex, atopy and smoking.

The results of effect modification may be a result of multiple testing. However, the pattern with an effect modification of atopy on the levels of total IgA was consistent.

Genetic effect on exposure

Polymorphisms in *GSTM1* and *GSTT1* were shown to have an effect on the urinary levels of TTCA among exposed workers (**Paper I**). Exposed workers with *GSTM1**O had lower levels of TTCA compared to exposed workers with *GSTM1**1. This suggests that carrying *GSTM1**1 results in better CS_2-GSH conjugation than if carrying *GSTM1**O, and thus higher levels of the metabolite. 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.

No effects of the polymorphisms in the other examined GST-coding or GSH synthesising genes were found.

When excluding study subjects of presumable Eastern Asian descent, the effect estimates were not markedly changed, and statistically significant changes in TTCA levels was observed regarding the same genotypes as when all exposed workers were analysed together (*GSTM1*, P=0.040 and *GSTT1*, P=0.045). The reason for the drop in significance level for *GSTT1* might be explained by the few individuals having *GSTT1**O (n = 25) when Eastern Asian individuals were excluded.

Different combinations of *GSTM1* and *GSTT1* genotypes were further analysed among exposed workers. 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 indeed the lowest levels of TTCA compared to the reference category (P=0.051). The two other genotypes did not follow the hypothesis; exposed workers with *GSTM1**O and *GSTT1**O displayed the highest TTCA levels (P=0.29) and exposed workers with *GSTM1**1 and *GSTT1**1 displayed the same TTCA levels as the reference category (P=0.94). This cannot be easily explained, but one possibility could be the low number of individuals in each group (**Paper I)**.

Influence of genotype on symptoms and immunological markers

The effects of polymorphism in *GSTA1*-52 on symptoms in exposed workers showed a consistent pattern of a protective effect of the *GSTA1*-52 (G/A+A/A) genotype, although statistical significance was not always reached. Furthermore, *GSTA1*-52 (G/A+A/A) genotype displayed a pattern of protective effect on the level of immunological markers, in particular among exposed subjects, however,

none of the associations reached statistical significance. These results are not easily interpreted, given the suggested protective role of the GSTA1-1 enzyme against oxidative stress and a lower expression level for the variant allele in liver and in rectum. The polymorphisms in the remaining genes showed no consistent pattern of effect. However, *GSTP1*-105 (ile/val+val/val) showed a statistically significant protective effect for headache. No statistically significant interactions between exposure status and genotype were found, apart from *GSTT1**O regarding headache (**Paper III**).

GCLC-129 (C/T+ T/T) genotype was associated with increased levels of leukocytes and neutrophils among exposed workers and *GCLM*-588 (C/T+T/T) genotype with increased levels of eosinophils among the controls. Exposed workers with *GSTP1*-105 (ile/val+val/val) were found to have decreased levels of total IgE. Exposed workers with *GSTT1**O genotype expressed higher levels of total IgG, and for *GSTT1**O and total IgA, the interaction between exposure status and genotype was statistically significant. Controls with *GSTP1*-114 (ala/val+val/val) genotype expressed decreased levels of total IgG (**Paper III**). It might be that the results of *GSTT1* and *GSTP1*-114, which neither showed consistency nor repeated results from earlier studies, are a result of multiple testing.

Since there were little evidence of exposure-response relationships between TTCA/1-HP levels and the risk of symptoms or changed immunological markers, no attempts were made to analyse the interaction between TTCA/1-HP levels and genotype on risk of health effects/biomarkers of response.

DISCUSSION

The exposure situation in the rubber industry is very complex and it is not possible to perform specific exposure assessments for all compounds present in the occupational environment. Different strategies have been employed to solve this problem. Thus, in earlier studies in Europe, mainly from the period from 1965 to 2003, major attention has been paid to specific *N*-nitrosamines, inhalable aerosol fraction, specific solvents (primarily toluene, heptane and benzene) and cyclohexane soluble fraction (De Vocht et al. 2005). Unfortunately, another strategy has been to not perform exposure assessments at all.

The use of index substances in an attempt to characterise the multitudinous exposure has only been applied once before by people in our research group (Vermeulen et al. 2005). In this thesis, two different index substances, TTCA and 1-HP, were investigated.

Statistically significant increases in the urinary levels of TTCA and 1-HP were found among vulcanisation workers compared to controls. This indicates that these compounds are good index substances of the exposure in the vulcanisation departments. The same was also indicated in the work by Vermeulen et al. (Vermeulen et al. 2005) who found increased levels of TTCA in vulcanisation workers during working days but not in workers in the mixing, pre-treating, finishing, shipping and service departments. The levels of TTCA found by Vermeulen et al. were similar to those in the present thesis. On the other hand, in a survey by Cox et al. (Cox et al. 1998) at an American rubber product facility, all 19 workers had urinary TTCA levels below the detection limits. Vermeulen et al. (Vermeulen et al. 2005) used the same sensitive analytical LC-MS-MS method as used in the present study while Cox et al. (Cox et al. 1998) used a 15 times less sensitive LC method. Thus, this may explain the differences in the results.

1-HP has only been measured in one earlier study in the rubber industry (Talaska et al. 2002). In that study urine samples were collected during 24 h. Geometric means of 1-HP of 0.62 and 0.48 µg/l were found among 20 workers handling vulcanised rubber. In the present thesis a geometric mean of 0.25 µg/l was found among vulcanisation workers. The 1-HP median among controls in this work was 0.071 µmol/mol creatinine, which is in accordance with an earlier study in Sweden. In that study non-smokers had 0.03 while smokers had 0.09 µmol/mol creatinine (Levin 1995).

In the non-occupationally exposed population, the diet is estimated as an important source of TTCA (Kivistö 2000) and PAH (IARC 1983). However, the information on food intake was insufficient for inclusion in the models in this study. Nevertheless, this exposure may be of minor importance, as the

differences between exposed workers and controls were rather large regarding TTCA and 1-HP.

Smoking is a known source for PAH exposure. Different reference values have therefore been described for smokers and non-smokers. Jongeneelen (Jongeneelen 2001) reported reference values for smokers of 0.76 µmol/mol creatinine and for non-smokers of 0.24 µmol/mol creatinine, using the 95th percentile. Also in this thesis an increase in 1-HP levels in smokers compared to non-smokers was found, but the difference appeared to be smaller. It has also been suggested that smoking may influence the TTCA levels (Jian and Cao 2000) but in this thesis no such effect was observed.

Earlier studies in the rubber industry have mainly included men. In this survey men as well as women was investigated. For male and female rubber workers no differences regarding urinary 1-HP were found. However, among the controls women had higher levels of 1-HP than men. There is no straightforward explanation for this observation. For TTCA there were no statistically significant differences between the sexes, neither for the rubber workers nor for the controls.

Selected for this study were rubber workers vulcanising with sulphur, or working in the same hall as these workers on the same day as the urine samples were collected. Vulcanisation workers using peroxide or not working near sulphur vulcanisation processes were excluded. The rationale for this was that TTCA was not considered to be a good index substance of vulcanisation fumes in these workers. Also, when 1-HP was used as an index substance of vulcanisation fumes, the same inclusion criterion was used for a continuity of study subjects in the thesis.

It was 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, included controls were working at companies selected for having employees with similar socio-economic status as the exposed workers but without occupational exposure to rubber chemicals. Furthermore, the controls were working in the same region in Sweden as the exposed workers.

Study subjects included encompassed those who had given their informed consent and were present at work at the time of the medical examinations. No information was available on the number of study subjects not willing to take part in the study or absent at work due to sick leave, etc., at the day of the medical examination.

There may be an information bias concerning reporting of symptoms, both on the part of the observed workers and the observer. However, at the time of the medical examinations, the results of the biomarker analyses were not known to the workers or the investigators. Furthermore, occupational and medical histories were obtained by structured interviews strictly following a questionnaire. The same specialist performed all interviews. Moreover, before

the physician's interviews, the study subjects had answered self-administered questionnaires with questions dealing with symptoms the last year. The agreement between the questionnaire and the structured interviews showed a good to moderate agreement regarding eye symptoms, nose symptoms and nose bleeds (the only symptoms asked for in both the questionnaire and the structured interviews). In the present study, the information from the interviews instead of the questionnaires was used since that was the best alternative to obtain complete history without missing data, and furthermore, the nature of the symptoms could be clarified. In conclusion, an information bias appears to be of minor importance.

An increased risk of itching, running or burning eyes was observed among the exposed workers in this thesis. This is in agreement with other studies from the rubber industry. Zuskin et al. (Zuskin et al. 1996) observed a high prevalence of irritation to eyes among rubber workers. In addition, Sparks et al. (Sparks et al. 1982) found an increased risk of burning eyes among such workers.

An increased prevalence of chronic and acute bronchitis among vulcanisation workers compared to other rubber workers was found by Fine and Peters (Fine and Peters 1976). Furthermore, Zuskin et al. (Zuskin et al. 1996) showed higher prevalence of chronic bronchitis among rubber workers in Croatia compared to controls. In this thesis, an increased risk of severe dry cough was observed.

Weeks et al. (Weeks et al. 1981) showed an increased risk of shortness of breath, wheeze, chest tightness, cough and sputum production among vulcanisation workers compared to other rubber workers. Zuskin et al. (Zuskin et al. 1996) showed higher prevalences of chronic cough and phlegm, dyspnoea and chest tightness among rubber workers in comparison with controls. However, in this thesis, no increased risk of dyspnoea and similar symptoms was observed among the vulcanisation workers compared to controls. It is possible that the exposure to the contemporary rubber workers in Sweden was lower than in the survey of 1981 and the Croatian survey.

In this thesis, an increased risk of nausea and headache was observed. Weeks et al. (Weeks et al. 1981) did not find an elevated risk of these symptoms. There is one major difference between the two studies. The controls used by Weeks et al. (Weeks et al. 1981) were rubber workers with only minor occupational contact with rubber chemicals, while in this thesis workers not employed in the rubber industry were included. Thus, it is possible that the exposure causing nausea and headache is not specific to the vulcanisation department. Zuskin et al. (Zuskin et al. 1996) reported prevalences for headache but this was not compared to the controls.

There is a possibility that exposure to PAHs itself induces airway symptoms. In this case, 1-HP may be used as a biomarker of PAH exposure (Dor et al. 1999). Statistically significant increases in eye irritation, chest tightness and chest wheezing were found by Randem et al. (Randem et al. 2004)

among asphalt workers exposed to PAHs, compared to other outdoor construction workers. In this thesis, an increase in eye symptoms was found, whereas the increase of dyspnoea, wheezing and/or chest tightness was less pronounced.

Non-atopic dockers, presumably exposed to diesel exhaust particles containing PAHs was investigated by Mastrangelo et al. (Mastrangelo et al. 2003). In that study, enhanced IgE levels but a decreased risk of rhinitis/asthma was found in the non-atopic dockers compared to controls. Unfortunately, some of the examined groups contained rather few individuals. In the present thesis, enhanced levels of total IgE levels were also found but it did not reach statistical significance.

Burstyn et al. (Burstyn et al. 2003) examined the mortality from chronic bronchitis, emphysema and asthma among asphalt workers. They found an increased risk of mortality and an association with PAH exposure. In this thesis, neither these particular symptoms nor mortality were investigated.

In the introduction of this thesis it was suggested that CS_2 exposure could induce airway symptoms by binding to endogenous proteins and thus evoke an immune response. However, no indication of such immune response from the highly CS_2-exposed rayon industry is found in the literature.

Nevertheless, increased concentrations of leukocytes, neutrophils, eosinophils and total IgG was found in the rubber workers compared to controls in the present thesis. Thus, there appeared to be an immunological response among the vulcanisation workers, but this may be due to another exposure than CS_2 or a combination of CS_2 and other agents.

Only for severe dry cough and 1-HP, but for no other symptoms or immunological markers, there was a clear exposure-response relationship, although not statistically significant. However, statistically significant trends over the three TTCA subgroups were observed for nausea, headache and leukocytes. For TTCA there was a pattern with the highest risk in the intermediate exposure group. Nevertheless, it should be stressed that exposed workers in the intermediate TTCA subgroup did not have significantly more symptoms than exposed workers with high levels of TTCA. Not even for a combination of TTCA and 1-HP levels, consistent exposure-response relationships were found.

The lack of exposure-response relationships may have several reasons. Firstly, exposures that do not correlate with CS_2 or PAH could affect the risk of symptoms and changed immunological markers. Secondly, the half-lives of the metabolites are rather short and represent the exposure only during the last couple of days (Buchet et al. 1992; Riihimäki et al. 1992), whereas the outcomes represent a longer time period. Thus, if the exposure has changed over time it may be difficult to observe an exposure-response relationship. However, when repeating the analyses using recording of symptoms from the last three days, the

pattern was similar as when using recording of symptoms from the last 12 months. Therefore, this is probably not an explanation.

Thirdly, a healthy worker selection may explain this pattern. However, eight different companies with divergent exposure levels were included in the study and a healthy worker selection between the companies is unlikely. Nevertheless, a healthy worker selection within the companies may still be possible even though representatives for the rubber industry found it unlikely (Benny Nilsson, personal communication). Furthermore, it was established in Paper II that a lower fraction of the exposed workers (5.4%) had worked less than a year in the present exposure compared to the controls (15.9%), which suggests a faster turnover of employees in the controls' occupations. It is important to emphasise that if a healthy worker selection indeed exist, this would lead to an underestimation of the risk in the rubber industry.

Another reason may be inter-individual differences in metabolism, which might lead to differences in tissue distribution and retention of toxic metabolites. It is indicated in this thesis that such a difference exists, but due to the lack of exposure assessments in air, it is hard to determine the exact extent of these individual differences.

High levels of TTCA were shown to be associated with continuous vulcanisation, which in turn was associated with an increased risk of symptoms from the eyes and throat, as well as nose bleeds and decreased levels of total IgA. On the other hand, high levels of 1-HP were associated with compression and injection vulcanisation, which was associated with hoarseness, severe dry cough, nausea, headache and increased levels of total IgE. Possibly, the difference in exposure does not depend on the vulcanisation methods itself but on the quality of the rubber vulcanised with the different methods. For example, large additions of highly aromatic oils may cause an increase in 1-HP. Unfortunately, sufficient information about the different rubber qualities was not available in the present study.

GSTM1 and *GSTT1* affected the levels of TTCA among exposed workers, but not among controls. However, the null alleles for the two genes affected the TTCA levels in different directions; missing GSTM1 was associated with lower levels of TTCA while missing GSTT1 was associated with higher levels. The hypothesis was that a lack of enzyme would result in lower conjugation and thus lower levels of TTCA, but the results of GSTT1 was opposite to what was hypothesised. A possible explanation may 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 result in lower levels of TTCA. The effect of *GSTM1* and *GSTT1* was observed only among exposed workers and not among controls. This may be explained by the probable difference in TTCA source; the TTCA among

controls may come from vegetables that contain TTCA themselves which are not metabolised by GSH.

No statistically significant changes in TTCA levels were observed for the other investigated genotypes. The reason for the negative results could be the lack of exposure assessments in air, which makes the exact modification of CS_2 levels by metabolising genes impossible to estimate.

An association with exposure to vulcanisation fumes was found for the risk of several symptoms and the changed level of several immunological markers. *GSTA1-52* (G/A+A/A) genotype displayed a pattern of protective effect on these outcomes, in particular among exposed subjects, although statistical significance was not always reached. The discrepancy between exposed workers and controls suggests that there is an association between vulcanisation fumes and *GSTA1* genotype. However, the protective effect of *GSTA1*-52 (G/A+A/A) is not easily interpreted, given the suggested protective role of the GSTA1-1 enzyme against oxidative stress and a lower expression level for the variant allele in liver and in rectum. One possibility is that GSTA1-1 is involved in the biotransformation of a compound of the vulcanization fumes that generates toxic metabolites, which in turn may have deleterious effects. Another possibility would be that subjects with *GSTA1*-52 variant genotype have a compensatory up-regulation of some other GSTs and hence a better defense against toxic compounds. It could also be that high conjugation activity of GSTA1-1 may reduce the cellular GSH pool, which in its turn causes increased oxidative stress and subsequent damage on cells and tissues.

GCLC-129 (C/T+T/T) genotype was associated with increased levels of leukocytes and neutrophils among exposed workers and *GCLM-588* (C/T+T/T) genotype with increased levels of eosinophils among the controls. Given the suggested lower expression level for the variant alleles, these results indicate that a lower glutatione activity may be associated with increased immunologic activity. The reason could be an increased burden of oxidative stress.

The decrease of total IgE among exposed workers carrying *GSTP1*-105 (ile/val-val/val) genotype is in agreement with previous findings by Fryer et al. (Fryer et al. 2000), showing a decrease in total IgE and atopy as well as the risk of asthma and bronchial hyperresponsiveness among study subjects with the *GSTP1*-105 variant genotype. However, the controls carrying *GSTP1*-105 (ile/val-val/val) genotype showed increased, although not statistically significant, levels of total IgE in the present study.

The analysis of gene-environment interaction may elucidate mechanisms of metabolism as well as mechanisms responsible of disease. Moreover, incorporation of information on polymorphisms in exposure assessments and epidemiological studies may improve the exposure and health risk estimates. However, most polymorphisms analysed until today have shown only minor effects on the effect estimates and moreover, it has been difficult to find associations between genotype and disease that is restricted to a specific

exposure. Furthermore, the interaction between different genes is complex and an individual having a polymorphism associated with increased risk may have a variant in another gene that is associated with decreased risk. Thus, it is with present knowledge not advisable to use this kind of genetic information for selection of employees. Still, it may for exposure to some agents be possible to include genetic information to improve risk management.

Multiple testing was performed in this thesis, which could lead to problems with false positive findings. These are influenced by the significance level, statistical power and prior credibility of the association tested (Wacholder et al. 2004). However, I did not formally adjust the significance level and some findings of my study could be the results of the large number of analyses performed. Nevertheless, several consistent patterns were found, thus these results are probably not chance findings.

It is known that the exposure in the rubber industry historically has induced airways symptoms. In this thesis it was established that still today there is an increased risk of symptoms in the eyes and airways, as well as for nausea and headache. Thus, the work environment needs to be further improved.

An attempt was made in the present study to use urinary TTCA and 1-HP levels as index substances of vulcanisation fumes. However, no obvious exposure-response relationships were found for the symptoms and immunological markers analysed in this thesis. Thus, a biological exposure limit based on the outcomes studied in this survey can not be proposed.

On the other hand, it is also known from earlier studies that workers in the rubber industry have an increased risk of different cancers and possibly cardiovascular disease (Boffetta et al. 1997; Stetkiewicz and Wronska-Nofer 1998). Urinary TTCA has traditionally been used as a biomarker of CS_2 exposure, a known inducer of cardiovascular disease. A biological exposure limit of 3500 µmol TTCA/mol creatinine is recommended by ACGIH for urine samples collected at the end of the work shift (ACGIH 2005). This corresponds to an occupational exposure level of about 8 ppm CS_2 according to Riihimäki et al. (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 this 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 this survey had a TTCA level above that value.

The traditional use of 1-HP is as a marker of PAHs, of which several are carcinogenic. There is no threshold limit for 1-HP in the rubber industry. However, Jongeneelen (Jongeneelen 2001) has proposed a biological exposure

index for coke works and the primary aluminium industry of 2.3 and 4.9 μmol/mol creatinine, respectively. Two indices are proposed since the proportion of pyrene, the compound that is metabolised to 1-HP, varies between the industries. The indices are not based on health effects but on what is considered to be technically manageable. A health based biological exposure limit would probably be about ten times lower (Netherlands Health Counsel 1994) and therefore, many of the subjects studied in the present thesis would be above such a low biological exposure limit.

Thus, the results for TTCA and 1-HP itself also indicated a need for improvement of the work environment in the contemporary rubber industry.

CONCLUSIONS

The levels of TTCA were increased among vulcanisation workers compared to controls. The levels of TTCA differed between different rubber companies and different subdivisions at the same company. Furthermore, the levels of TTCA differed between workers using different vulcanisation processes. Thus, salt bath, hot air, microwaves and fluid-bed vulcanisation gave rise to higher levels of TTCA compared to injection and compression vulcanisation.

The levels of 1-HP were increased among vulcanisation workers compared to controls. The levels of 1-HP differed between different rubber companies and different subdivisions at the same company. Furthermore, the levels of 1-HP differed between workers using different vulcanisation processes. Thus, compression and injection vulcanisation gave rise to higher levels of 1-HP compared to salt bath, hot air, microwaves and fluid-bed vulcanisation.

Vulcanisation workers, had compared to controls, increased risk of itching, running or burning eyes, nose bleeds, throat burning and dryness, hoarseness, severe dry cough, nausea and headache. Furthermore, they had increased levels of total IgG.

The risk of symptoms was highest among exposed workers with intermediate levels of TTCA. Statistically significant trends over the three TTCA subgroups were observed for nausea, headache and leukocytes.

1-HP showed no exposure-response relationships with symptoms or immunological markers except for severe dry cough. No statistically significant trends over the three 1-HP subgroups were observed.

GSTM1 and *GSTT1* affected the levels of TTCA among exposed workers, but not among controls. Thus, exposed workers with *GSTM1**O had decreased levels of TTCA compared to those with *GSTM1**1, whereas exposed workers with *GSTT1**O had increased levels of TTCA compared to those with *GSTT1**1.

GSTA1-52 affected the risks of symptoms and changed levels of immunological markers associated with exposure to vulcanisation fumes. The polymorphisms in the other examined genes showed no consistent pattern of effect on the risk of symptoms or immunological markers.

ISSUES FOR FUTURE RESEARCH

Levels of CS_2 and PAH in air should be measured to better evaluate associations between exposure and outcome, as well as the modifying effects of polymorphisms on these relationships. Especially a characterisation of the relationships between pyrene and more toxic PAHs is needed.

A more comprehensive exposure assessment of the exposure in the rubber industry should be conducted to facilitate the analyses of exposure-response relationships. Other groups of compounds present in the rubber industry that may be of interest to study are, e.g., nitrosamines, aldehydes, aromatic amines, phthalates, and peroxides.

Further characterisation of the urinary levels of TTCA should be conducted, as sources such as different processes and rubber qualities may be associated with high levels of TTCA and thus need to be recognised.

Further characterisation of the urinary levels of 1-HP should be conducted since sources associated with high levels of 1-HP need to be recognised. Furthermore, exposure assessments are needed at companies changing from nitrite containing salt to non-nitrite containing salt to establish whether that may be a possible source of PAHs in the rubber industry.

A survey on vulcanisation workers using peroxide needs to be performed since this type of vulcanisation is increasing in Sweden and appear to be associated with airways symptoms.

Establishments of cohorts to examine the risk of cancers in the contemporary rubber industry, using the exposure markers employed in this thesis, is desirable.

Studies on reproductive toxicity, e.g., time to pregnancy and sperm quality, among rubber workers in the contemporary rubber industry should be performed, using the exposure markers employed in this thesis.

Reliable data on symptoms for the last couple of days and information on whether or not these symptoms are associated with exposure in the rubber industry should be recorded.

Further studies to elucidate the mechanisms behind airways symptoms in the rubber industry are needed. For example, nasal lavage and induced sputum may

be conducted and analysed for immunological markers (cells, proteins and messenger RNA).

Phenotypic determination of GSTM1 and GSTT1 should be performed in order to sharpen the association between TTCA levels and GSH conjugation capacity.

Previous results of an association between *GSTA1* genotype and risk of symptoms/immunological markers should be confirmed in a new study population of rubber workers and controls. Moreover, the functional impact of the *GSTA1*-52 genotype needs to be investigated.

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