

Influence of polymorphic metabolic enzymes on biotransformation and effects of diphenylmethane diisocyanate

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Abstract

Objectives To identify effect modification produced by genetic traits found in metabolic enzymes, to investigate how these affect the levels of different biomarkers of sprayed and thermo-degraded polyurethane (PUR) based on 4,4'-diphenylmethane diisocyanate (MDI) and to determine how associated respiratory disorders are affected.

Methods Two partly overlapping groups of 141 and 158 factory employees exposed to sprayed or heated MDI-PUR glue were examined in years 0 and 2, respectively, for occurrence of polymorphisms in five genes (N-acetyltransferase *NAT2* and the glutathione S-transferases *GSTM1*, *GSTM3*, *GSTP1* [codon 105 and 114] and *GSTT1*) on the basis of the polymerase chain reaction, exposure biomarkers in plasma and urine (P- and U-MDX), by means of gas chromatography-mass spectrometry, specific serum IgG antibodies against MDI (S-IgG-MDI) by means of ELISA, total S-IgE, symptoms in the eyes, nose and lower airways as assessed by questionnaire and interview, and lung function as measured by spirometry.

Results Both the *GSTP1*¹⁰⁵ isoleucine/isoleucine and *GSTP1*¹¹⁴ alanine/alanine genotypes showed higher levels of U-MDX than the other genotypes and the *GSTP1*¹¹⁴ genotype modified the P-MDX/U-MDX relationship. *GSTP1*¹⁰⁵ isoleucine/isoleucine was found to be associated with lower levels of S-IgG-MDI and fewer eye symptoms, but with an increased risk of symptoms in the airways, as

well as with atopy. Presence of the *GSTT1* gene resulted in somewhat lower lung function levels than did the null genotype. A slow *NAT2* acetylating capacity was associated with lower P- and U-MDX and S-IgG-MDI levels, and better lung function, but a higher risk of eye and airway symptoms. Analysing the effects of combinations of the different genes provided no further information.

Conclusions Although our study has clear limitations, it reveals various effect modifications produced by the *GST* and *NAT2* genotypes. Gene-environment interactions are highly complex. Further research is needed to obtain a more comprehensive understanding of them.

Keywords Airways · Biomarkers · Genes · Isocyanates · Polymorphism

Abbreviations

Abce	Absorbance
A	Alanine
Ab	Absence, absent
Adj	Adjusted
CI	Confidence interval
FEV ₁	Forced expiratory volume in one second
FVC	Forced vital capacity
GSH	Glutathione
GST	Glutathione S-transferase
HDI	Hexamethylene diisocyanate
I	Isoleucine
Im	Intermediate acetylator, rapid/slow heterozygote
kU	Kilo units
LQ	Limit of quantification
MDA	4,4'-methylene dianiline = 4,4'-diaminodiphenylmethane
MDI	4,4'-diphenylmethane diisocyanate
MDX	Compound determined as MDA after hydrolysis

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NAT	N-acetyltransferase
OR	Odds ratio
P	Plasma
Pr	Presence, present
PUR	Polyurethane
R	Rapid acetylator, homozygote
S	Serum
Sl	Slow acetylator, homozygote
U	Urine
V	Valine

Introduction

Isocyanates are components of plastic polymers such as polyurethane (PUR). They have found increasing use both in work environments and in consumer products. They have been known for half a century to be a cause of occupationally linked respiratory disease (Baur et al. 1994; Raulf-Heimsoth and Baur 1998; Littorin et al. 2002; Liu and Wisnewski 2003).

A striking trait of isocyanate-related illness is the fact that only a fraction of those exposed to isocyanates show symptoms or become ill. This suggests genetic vulnerability to be involved. The capacity to metabolize and dispose of isocyanates may thus be of importance. Although knowledge of this is very limited, particularly regarding humans, certain basic data are available: Isocyanates, having entered the body, bind rapidly, by means of their NCO-groups, to nucleophilic extracellular glutathione (GSH), abundant in the lining fluid of the lower airways, and can be transferred to other nucleophilic sites, e.g. proteins, to form adducts which may evoke sensitisation (Lind et al. 1997; Bolognesi et al. 2001; Kelly 2003; Johannesson et al. 2004a; Wisnewski et al. 2005). They can also conjugate with intracellular GSH, either spontaneously or after catalysis by glutathione S-transferases (*GSTs*; Lange et al. 1999; Lantz et al. 2001).

The metabolic fate of the isocyanate group can likewise include non-enzymatic hydrolysis to an amino-group, which is then acetylated (by N-acetyl transferases [*NATs*]; Grant et al. 1992; Kadlubar 1994; Schutze et al. 1995; Sepai et al. 1995). The amino-group may also either be peroxidized, be oxidized and conjugated to GSH, become acetylated or be sulfonated (Sipes et al. 1991; Kadlubar 1994; Culp et al. 1997; Kautiainen et al. 1998; Lange et al. 1999; Lantz et al. 2001; Gledhill et al. 2005).

The polymorphic *GSTs* and *NATs* are expressed in human airways (Anttila et al. 1993; Windmill et al. 2005). Limited data indicate that genetic variation of these metabolic enzymes may play a role in the risk of respiratory disease induced by isocyanates, although results concerning this are contradictory (Dalene et al. 1996; Jakobsson et al. 1997; Piirilä et al. 2001; Mapp et al. 2002; Wikman et al. 2002; Berode et al. 2005).

Although isocyanate-induced respiratory disorders are accompanied by clear signs of inflammation, the underlying mechanisms are only partly understood (Brown and Wold 1971; Raulf-Heimsoth and Baur 1998; Savolainen 1999; Liu and Wisnevski 2003; cf. Johannesson et al. 2004b). Direct toxic damage to the epithelium of the airways, the neuropeptide-mediated release of histamine, and the "pharmacological" inhibition of proteases have all been postulated, although the clinical features of isocyanate-induced asthma, as well as of hypersensitivity pneumonitis, suggest these disorders to be immunologically mediated. For years, however, the possible pathogenetic role of specific IgE and IgG antibodies has been questioned (Park et al. 2002; Mapp et al. 2005). Some reports, though not others, have also indicated that certain genetic polymorphisms in the major histocompatibility complex may predispose to asthma induced by isocyanates (Rihs et al. 1997; Mapp et al. 2000; Beghe et al. 2004; Kim et al. 2006).

The overall susceptibility to bronchial hyperresponsiveness and asthma has also been suggested to be influenced by genetic disposition (Fryer et al. 2000; Kleeberger and Peden 2005).

Earlier, members of our group studied a manufacturing plant in which there were low air levels of isocyanates but employees were exposed to sprayed or heated PUR products, this being associated with increased levels of exposure biomarkers of 4,4'-diphenylmethane diisocyanate (MDI) in the plasma (P-MDX) and urine (U-MDX), together with the presence of specific serum antibodies against conjugates of MDI and human serum albumin (HSA; S-IgG-MDI), and a decrease in lung function (Skarping et al. 1996; Littorin et al. 2000). In addition, the biomarker S-IgG-MDI in particular, and the biomarkers P-MDX and U-MDX as well, showed associations with airway symptoms. In the present study, we investigate the effects genetic traits can have on the metabolite and antibody levels, symptoms and lung function and on the interrelations of these. It was hypothesized that poor metabolism of the *GST* and *NAT2* enzymes, would result in higher concentrations of the exposure biomarker P-MDX, lower concentrations of U-MDX, higher titres of specific S-IgG-MDI antibodies, more distinct symptoms and a lowering of lung function.

Materials and methods

Manufacturing plant and subjects studied

In the factory, car components containing a flexible foam of toluene diisocyanate (TDI), were joined together by means of MDI-PUR glue (Skarping et al. 1996; Littorin et al. 2000). In addition, some of the workers made use of a glue containing hexamethylene diisocyanate (HDI) as well as other, non-

PUR glues. The factory was examined twice (in years 0 and 2; Table 1). Air measurements in year 0 showed low levels of diisocyanate monomers, irrespective of whether glueing took place or not; during 40 sampling periods of 30–60 min each, concentrations of MDI were found to be <0.2–7.1, of HDI <0.1–0.7, and of TDI <0.1 $\mu\text{g}/\text{m}^3$. Use of an Isologger filter-tape instrument (MDA-Scientific, Chicago, USA) revealed peaks of isocyanate-containing compounds during heating and spraying operations, however (G. Skarping and H. Tinnerberg, personal communication). After the initial examination, measures to reduce exposure to isocyanates were taken, the glues until then having been applied by manual spraying or by a glueing machine. At the second examination, use of HDI-glue was found to have diminished substantially and most of the MDI-glueing to be performed by a robot. However, activation of the MDI-based glue by heat, particularly through use of heat guns, had increased, and a new task group that carried out heating without manual glueing had been formed. A group of workers who made armrests was still intensely exposed through use of manual spraying and heating. When the glue was heated, the materi-

als, including the flexible foam, were heated as well. At the time of both examinations, the other work tasks included cutting, sewing, assembling and repairing. Our examinations thus far (Skarping et al. 1996; Littorin et al. 2000) had indicated a possibility for all of the workers and clerks (the latter visited the production area each day and worked otherwise nearby) to become exposed. For this reason, all the employees were included in the study. In the first examination, 141 workers and clerks for whom information on genotypes was available were studied, these constituting 81% of the employees reported on in Skarping et al. (1996; Table 1). In the second examination, genetic information on 158 employees was obtained, these representing 95% of the employees examined by Littorin et al. (2000). There was an overlap between the first and second examinations, 93 subjects participating in both of them.

All the employees except one (of Asian descent) were Caucasians.

The workers gave their informed consent to participating in the study. The study was approved by the Ethical Committee at Lund University.

Table 1 Exposure categories and characteristics of 141 (year 0) and 158 (year 2) employees working in a factory in which there was exposure to sprayed and heated MDI-based polyurethane glue and genotype frequencies consistent with those of the general population according to the literature

Exposure/characteristic		Study population		General population
		Year 0	Year 2	
Exposure category				
Clerks (N)		8	13	–
Workers (N)		133	145	–
No sprayed glue, no heat		63	69	–
Either sprayed glue or heat		7	33	–
Both sprayed glue and heat		63	43	–
Female/Male (N)		117/24	122/36	–
Age (years; median, [range])		44 (19–62)	44 (20–64)	–
Atopics (%)		16	24	–
Smokers (%)		33	33	–
Genotypes (%)				
<i>GSTM1</i>	1	52	49	44 ^a
	0	48	51	56
<i>GSTM3</i>	AA	69	69	72 ^b
	AB	29.5	30	25
	BB	1.5	1	3
<i>GSTP1</i> ¹⁰⁵	I/I	38	40	43 ^b
	I/V	50	45	45
	V/V	13	15	12
<i>GSTP1</i> ¹¹⁴	A/A	83	85	84 ^b
	A/V	17	15	16
<i>GSTT1</i>	1	86	86	87 ^a
	0	14	14	13
<i>NAT2</i>	Sl	61	63	58 ^b
	Im	34	30	35
	R	5	6	7

^a Frequency among Swedes (Garte et al. 2001)

^b Frequency among Caucasians (Garte et al. 2001; Schwarzbach et al. 2007)

Methods

Exposure category

In year 0, an industrial hygienist classified the workers with regard to their exposure to MDI (by the sprayed aerosol of PUR glue, the heated glue, or both). In year 2, such information was first obtained from each subject by use of a questionnaire, followed by a supplementary interview by a physician. Then, in consultation with the hygienist, who had thorough knowledge of the company and its employees, specific information on the individual use of sprayed and heated glue was added for both years. On the basis of this classification of exposure, the employees were divided into four separate groups (clerks; workers exposed to neither sprayed nor heated glue; those exposed only to sprayed glue or only to heated glue [although in year 0, no one was exposed only to heated glue]; those exposed to both sprayed and heated glue).

Samples of venous blood and urine were collected on workdays both in years 0 and 2 and were stored at -18°C until analysis. The blood was analysed for genotypes, the plasma and urine for isocyanate metabolites, and the serum for specific antibodies.

Genotyping

Frozen whole blood was employed for DNA extraction using the Chelex 100 (BioRad) or the QIAamp 96 DNA (Qiagen) blood kit. The samples were genotyped in terms of *GSTM1*, *GSTT1* (presence = Pr, or absence = Ab, of the genes) and *NAT2* (*NAT2*5*, *NAT2*6* and *NAT2*7*), as described earlier (Pemble et al. 1994; Hou et al. 1995). The *NAT2* genotypes were characterized as being rapid (rapid homozygotes = R), intermediate (rapid/slow heterozygotes = Im) or slow (slow homozygotes = Sl) acetylators (Cascorbi et al. 1999).

Two different polymorphisms of *GSTP1* were analysed, one with the amino acid at position 105 and the other with it at position 114. The isoleucine/valine (I/V) polymorphism (amino acid 105) of *GSTP1* was determined by use of two differently labeled Taqman MGB-probes, each specifically hybridizing to one of the alleles. The probes were FAM-labeled (Isoleucine) 5'-TGCAAATACATCTCCCT-3' and VIC-labeled (Valine) 5'-TGCAAATACGTCTCCCT-3'. The primers used were 5'-CCCTGGTGGACATGGT-GAA-3' (forward) and 5'-CCTGGTGCAGATGCTCAT-3' (reverse). The MGB-probes employed for detection of the Alanine/Valine (A/V) polymorphism (amino acid 114) of *GSTP1* were FAM (Alanine) 5'-AGGAGGCGGGCAA-3' and VIC (Valine) 5'-CAG-GAGGTGGGCAA-3', whereas the primers were 5'-CCTGGTGGACATGGTGAATG-3' (forward) and 5'-GGGCAGTGCCTTCACATAGTC-3' (reverse). The 3 bp-

deletion (intron 6) of *GSTM3* was detected by the probes FAM (wild-type) 5'-AGGGAAAAGAAGAGGATA-3' and VIC (deleted allele) 5'-AGGGAAAAGAAGATACTT. The primers used were GGACAACTGAAACAAC-TGAAACAATTCTCCATGT-3' (forward) and 5'-GAG-GAG TAGTAGCCTGCAGAGATAGAG-3' (reverse).

The PCR reaction (assays *GSTP1*¹⁰⁵ and *GSTM3*) contained $1\times$ universal mix (Applied biosystems), together with $0.2\text{ }\mu\text{M}$ of each probe and $0.9\text{ }\mu\text{M}$ of each primer (for *GSTM3*, $0.3\text{ }\mu\text{M}$ of the reverse primer was employed), as well as 10–35 ng of DNA. For *GSTP1* 114, the PCR reaction mixture contained $1\times$ PCR buffer, 2.5 mM MgCl_2 , 0.8 mM dNTPs, 1 U Taq Platina (Invitrogen), $1\times$ Rox (Invitrogen), $0.04\text{ }\mu\text{M}$ of each probe, $0.9\text{ }\mu\text{M}$ of each primer and 10 ng of DNA. All the Taqman-based assays were performed in a real-time PCR machine 7000 (ABI PRISM 7000) at 50°C for 2 min, and at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. Positive and negative controls were included in all the analyses, and in each assay, 10% of the samples were reanalysed. For the *GSTP1* and *GSTM3* analyses, the genotypes for 10% of the samples were confirmed by another method as well. The concordance between the repeats was 100%.

The allelic frequencies of the *GSTM3*, *GSTP1*, and *NAT2* genotypes were compatible with the Hardy–Weinberg equilibrium. All the genotype frequencies in the study population were similar to those reported in the literature (Table 1; cf. Table 2). The frequencies of the two sexes did not differ (data not shown).

Isocyanate metabolites

Following acid hydrolysis and derivatisation, P- and U-MDX were determined in the plasma and the urine as MDA, by use of gas chromatography and chemical ionisation mass spectrometry (GC-MS; Skarping et al. 1996; Littorin et al. 2000). The limits of quantification (LQ) were $0.10\text{ }\mu\text{g/l}$ for year 0 and $0.05\text{ }\mu\text{g/l}$ for year 2. In year 0, 121 subjects, or 86% of the participants in the present study, had P-MDX, and 67 subjects (48%) U-MDX, above the LQ level. In year 2, 94 subjects (60%) had P-MDX, and only 16 subjects (10%) U-MDX, above that level.

Antibodies

S-IgG-MDI (by ELISA) antibodies were determined for both years (Skarping et al. 1996; Littorin et al. 2000). Since our findings showed S-IgG-MDI absorbance (Abce) values above 0.08 to be clearly associated with respiratory symptoms (Littorin et al. 2000), we dichotomized S-IgG-MDI at that level in the adjusted analyses. Only three of the employees at year 0 and four of them at year 2 possessed specific serum IgE (determined by RAST) antibodies

Table 2 Distribution of genotypes of various GSTs and NAT2 and levels of P- and U-MDX ($\mu\text{g/l}$; median and range) among 141 (year 0) and 158 (year 2) employees working in a factory in which there was exposure to sprayed and heated MDI-based polyurethane glue

Gene		N (%)	P-MDX ($\mu\text{g/l}$)	U-MDX ($\mu\text{g/l}$) ^a	S-IgG-MDI (abce)
Year 0					
<i>GSTP1</i> ¹⁰⁵	I/I	53 (38)	1.4 (LQ-5.5)	0.39 (LQ-4.9)	0.06 (0.00–0.58)
	I/V	70 (50)	1.0 (LQ-5.2)	LQ (LQ-2.7)	0.08 (0.01–1.3)
	V/V	18 (13)	0.9 (LQ-4.5)	LQ (LQ-1.5)	0.06 (0.02–0.52)
<i>GSTP1</i> ¹¹⁴	A/A	115 (83)	1.2 (LQ-5.5)	0.2 (LQ-4.9)	0.08 (0.00–1.3)
	A/V	24 (17)	0.86 (LQ-4.5)	LQ (LQ-1.5)	0.06 (0.01–0.12)
<i>GSTT1</i>	Pr	121 (86)	1.1 (LQ-5.5)	LQ (LQ-4.9)	0.07 (0.00–0.64)
	Ab	20 (14)	1.0 (LQ-4.1)	LQ (LQ-2.7)	0.09 (0.01–1.3)
<i>NAT2</i>	SI	86 (61)	1.0 (LQ-5.5)	LQ (LQ-4.9)	0.06 (0.00–1.3)
	Im	48 (34)	1.1 (LQ-4.5)	LQ (LQ-4.6)	0.08 (0.00–0.39)
	R	7 (5)	1.6 (0.6–4.4)	0.37 (LQ-1.5)	0.10 (0.06–0.58)
Year 2					
<i>GSTP1</i> ¹⁰⁵	I/I	64 (40)	0.35 (LQ-15)	–	0.10 (0.02–0.52)
	I/V	71 (45)	0.09 (LQ-18)	–	0.10 (0.02–1.1)
	V/V	23 (15)	0.26 (LQ-18)	–	0.11 (0.04–0.88)
<i>GSTP1</i> ¹¹⁴	A/A	132 (85)	0.16 (LQ-18)	–	0.10 (0.02–1.1)
	A/V	24 (15)	0.20 (LQ-3.0)	–	0.10 (0.02–0.17)
<i>GSTT1</i>	Pr	136 (86)	0.24 (LQ-18)	–	0.10 (0.02–0.88)
	Ab	22 (14)	0.10 (LQ-18)	–	0.11 (0.06–1.1)
<i>NAT2</i>	SI	100 (63)	0.16 (LQ-18)	–	0.10 (0.02–1.1)
	Im	48 (30)	0.19 (LQ-13)	–	0.11 (0.05–0.52)
	R	10 (6)	0.53 (LQ-18)	–	0.12 (0.06–0.41)

LQ < 0.1 $\mu\text{g/l}$ (year 0) and <0.05 $\mu\text{g/l}$ (year 2), respectively

^a In year 2, only 10% of the employees had detectable levels

against conjugates of MDI and HSA, these antibodies are not considered in the study.

Atopy was defined in year 0 in terms of medical history, whereas in year 2, the Phadiatop serological screening test (by fluoroimmunoassay) was made use of, the results being considered positive if at least one of ten common airborne allergens showed a response. Total serum IgE (S-IgE) antibodies (in kU/l = kilo units per litre) was measured as well (by fluoroimmunoassay).

Symptoms and lung function

Both years 0 and 2, the employees filled out the same self-administered questionnaires regarding their medical and occupational histories, supplemented by interviews and examinations by a physician concerning symptoms pertaining to the eyes (itching, running, or burning), nose (running, stuffiness, or sneezing/itching) and lower airways (severe dry cough or dyspnea, wheezing or tightness in the chest). In year 0, information on (total) symptoms was obtained, and in year 2, additional information on the apparent work-relatedness of these, defined in terms of the extent to which symptoms were present at work but disappeared or declined on weekends or holidays. Both these years, the frequency of symptoms (<once a month, >once a month but <once a week, >once a week, or daily) during the previous 3–6 months or longer was asked for.

Year 2, baseline spirometries were performed in accordance with the guidelines of the American Thoracic Society (1987). Forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV_1) were measured by a Vitalograph (Buckingham, UK). Performance was expressed as a percentage of what was expected for one's sex, age and height (Berglund et al. 1963).

Statistics

Subjects who had had no symptoms, or symptoms that appeared <once a month or >once a month but <once a week were grouped together as an 'asymptomatic' group, whereas those with symptoms >once a week or daily were defined as symptomatics. In analysing work-related symptoms, individuals with an uncertain relation to their work were excluded from the analyses.

Multivariate analyses of the effect of genotypes were performed using multiple linear regression for continuous responses (isocyanate metabolites, antibodies, and lung function) and multiple logistic regression for binary responses (symptoms). All continuous responses except for lung function (FEV_1 and FVC) were log transformed in the analyses. The exposure groupings were dichotomized, subjects who had worked with sprayed or heated glue constituting the first category and all other individuals the second. This binary exposure variable was included in all the multi-

variate analyses except those in which the isocyanate metabolites (or specific IgG antibodies) were used as markers of exposure. Age, classified in terms of three broad categories (<36 years, 36–50 years, and >50 years of age), gender, smoking (current or former smokers versus non-smokers), and atopy were adjusted for in modeling determinants of symptoms for the eyes, nose, and lower airways. Ratios and odds ratio (OR) estimates were obtained, showing the magnitude of the effects. Smoking and atopy were also adjusted for when determinants of lung function were modeled. The adjusted analyses did not deviate substantially from the unadjusted ones, only the adjusted analyses being presented here.

When data suggested either an increasing or a decreasing effect of the genes with three genotypes (*NAT2* and *GSTP1* 105), *P* values for trend were obtained by creating multivariate regression models in which the gene variable was coded as successive numbers (1, 2, and 3). For selected associations, the effect-modifying effect of *GSTP1* 105, *GSTP1* 114, and *GSTT1* was investigated by fitting separate multivariate models to each genotype category. Suggested effect modifications were tested statistically by including in the regression models a cross-term (genotype $\times$ exposure) together with the main effects. Effect modification for the three *NAT2* genotypes could not be analysed since there were too few rapid homozygotes; however, such analyses were performed for the rapid homozygotes and intermediate acetylators combined. When effects of separate genes were observed, the effects of combinations of genes were also analysed. For *GSTP1* and *NAT2*, the following combinations were formed: *GSTP1* I/I + *NAT2* S/I, *GSTP1* I/I + *NAT2* I/m/R, *GSTP1* I/V + *NAT2* S/I, and *GSTP1* I/V + *NAT2* I/m/R. For *GSTT1* and *NAT2*, *GSTT1*Ab + *NAT2*S/I versus all other combinations of *GSTT1* and *NAT2* genotypes was analysed.

Statistical significance refers throughout to a *P* value <0.05 (two-tailed) and to 95% confidence intervals (CIs). The term “significant” always refers to statistical significance.

Results

Influence of *GSTP1*¹⁰⁵

Metabolites

For year 0, *GSTP1*¹⁰⁵ I/I was found to be associated with higher levels of U-MDX than for the other two genotypes (Tables 2, 4). An allele-dosage effect appeared to be present for the valine (V) allele, there being a significant trend extending from I/I, which had the highest U-MDX levels, by way of I/V to V/V, which had the lowest levels. In year 2, only few subjects had detectable levels of U-MDX, the I/I carriers having slightly higher levels (not shown). No trends for P-MDX were obvious.

Antibodies

For year 0, the S-IgG-MDI levels were somewhat higher in subjects carrying the *GSTP1*¹⁰⁵ I/V or V/V genotypes than in those carrying the I/I genotype, a significant trend being found (Tables 2, 4). The same tendency was present in year 2 (not shown).

For year 2, *GSTP1*¹⁰⁵ was found to modify the association between the exposure category and S-IgG-MDI (I/I: 1.4 [1.0–1.8], I/V: 1.6 [1.2–2.2], V/V: 3.8 [2.0–7.2]; *P* = 0.02).

Atopy was more frequent in subjects carrying *GSTP1*¹⁰⁵ I/I than in the other subjects (I/I: 24/64 = 38%; V/V: 4/23 = 17%; I/V: 10/71 = 14%; not shown). *GSTP1*¹⁰⁵ was not associated significantly with total S-IgE (year 2), but the values were slightly higher in carriers of the I/I (median 24 kU/I) genotype than in carriers of the I/V (20 kU/I) and the V/V (13 kU/I) genotypes (not shown).

Symptoms and lung function

For year 0, the (total) eye symptom frequency was greater in carriers of the *GSTP1*¹⁰⁵ I/V and V/V genotypes than in carriers of the I/I genotype, a trend also being found (Tables 3, 5). In year 2, the pattern was similar, but the results were not significant (not shown). For year 0, there was no effect of the gene on the airways. For year 2, however, (when either the exposure category or S-IgG-MDI was included in the model), the nose symptoms, particularly those which were work-related, were less frequent in subjects having *GSTP1* I/V (OR = 0.23 [0.08–0.65]) or V/V (0.61 [0.18–2.1]; not shown) than in subjects having I/I. Also, subjects having *GSTP1*¹⁰⁵ I/V showed slightly (though not statistically significant), fewer work-related symptoms in the lower airways than the I/I carriers did, but no effect of *GSTP1*¹⁰⁵ on lung function was evident (Table 3).

Influence of *GSTP1* 114

Metabolites

None of the subjects was a homozygote for the rare *GSTP1*¹¹⁴ allele. In year 0, there were significantly lower U-MDX levels for *GSTP1* 114 A/V than for A/A (Tables 2, 4). In year 2, only few subjects had detectable levels of U-MDX, although the same, but not significant, tendency as in year 0 was observed (not shown). P-MDX displayed no associations with the gene.

In year 0, there was a clear effect modification of *GSTP1*¹¹⁴ concerning the relation between the exposure category and U-MDX (A/A: 2.9 [1.9–4.3], A/V: 0.98 [0.52–1.8]; *P* = 0.02)) the gene also having an effect on the U-MDX/P-MDX relation (A/A: 1.9 [1.6–2.1], A/V: 1.2 [0.87–1.5]; *P* = 0.005) that year.

Table 3 Distribution of genotypes of various GSTs and NAT2 and frequencies (%) of total or work-related symptoms in the eyes, nose and lower airways and in lung function (FEV₁ and FVC, % expected; median and range) among 141 (year 0) and 158 (year 2) employees working in a factory in which there was exposure to sprayed and heated MDI-based polyurethane glue

Gene		Symptoms (%)							
		Eyes		Nose		Lower airways		Lung function	
		Total	Work	Total	Work	Total	Work	FEV ₁	FVC
Year 0									
<i>GSTP1</i> ¹⁰⁵	I/I	8	–	23	–	9	–	–	–
	I/V	20	–	31	–	14	–	–	–
	V/V	28	–	33	–	11	–	–	–
<i>GSTP1</i> ¹¹⁴	A/A	17	–	30	–	11	–	–	–
	A/V	17	–	25	–	17	–	–	–
<i>GSTT1</i>	Pr	14	–	27	–	12	–	–	–
	Ab	30	–	35	–	15	–	–	–
<i>NAT2</i>	Sl	22	–	33	–	15	–	–	–
	Im	6	–	21	–	8	–	–	–
	R	14	–	29	–	0	–	–	–
Year 2									
<i>GSTP1</i> ¹⁰⁵	I/I	14	14	36	32	20	16	97 (71–128)	95 (70–126)
	I/V	13	9	20	11	13	4	96 (67–130)	93 (68–132)
	V/V	26	22	35	25	30	24	100 (74–119)	98 (63–130)
<i>GSTP1</i> ¹¹⁴	A/A	15	13	28	21	19	13	97 (67–130)	95 (63–132)
	A/V	17	12	29	22	17	5	97 (76–119)	94 (74–130)
<i>GSTT1</i>	Pr	15	12	29	21	16	11	97 (67–130)	94 (63–132)
	Ab	18	18	23	20	32	20	100 (81–119)	100 (82–126)
<i>NAT2</i>	Sl	19	16	36	26	22	14	97 (67–124)	95 (63–130)
	Im	8	8	17	13	8	4	98 (70–130)	95 (74–132)
	R	10	10	10	10	30	30	81 (71–117)	86 (68–110)

Table 4 Associations between genotype, on the one hand, and isocyanate metabolites (U-MDX) in urine and specific isocyanate antibodies in serum (S-IgG-MDI), on the other (point estimates [PE] of concentration ratios and 95% confidence intervals [CI])

Gene	Response	Test	Concentration ratio PE (95% CI)	Trend (P)	Exposure estimate
<i>GSTP1</i> ¹⁰⁵	U-MDX	I/V vs. I/I	0.61 (0.41–0.91)	0.007	Category
		V/V vs. I/I	0.50 (0.27–0.91)		
<i>GSTP1</i> ¹¹⁴	U-MDX	A/V vs. A/A	0.44 (0.22–0.72)		Category
<i>GSTP1</i> ¹⁰⁵	S-IgG-MDI	I/V vs. I/I	1.7 (1.1–2.6)	0.04	Category
		V/V vs. I/I	1.5 (0.82–2.8)		
		I/V vs. I/I	1.8 (1.2–2.7)		
		V/V vs. I/I	1.7 (0.95–3.0)	0.01	P-MDX
		I/V vs. I/I	2.0 (1.3–3.0)		
		V/V vs. I/I	1.8 (0.97–3.3)		

Data for year 0 for 141 employees exposed to sprayed and heated MDI-based polyurethane glue. Multivariate analysis taking exposure intensity category, P-MDX or U-MDX into account in the model, and trend tests (referent = genotype I/I = 1.0) were carried out. Associations are reported if (at least) one genotype differs significantly from the referent genotype

Antibodies

In year 2, S-IgG-MDI was influenced slightly by *GSTP1* 114 (Table 2), the point estimate of the absorbance ratio for A/V vs A/A, taking the exposure category into account, being 0.79 (0.61–1.0; not shown). The same tendency was observed in year 0.

Symptoms and lung function

No statistically significant association between either symptoms or lung function, on the one hand, and the gene in question, on the other, was observed.

The *GSTP1* haplotypes of 105 and 114 A, B and C (Fryer et al. 2000) were also examined, but this provided no

information in addition to that contained in the results presented for the separate genotypes.

Influence of *GSTT1*

Metabolites

There was only a weak association between *GSTT1* and U-MDX (point estimate of concentration ratio for Ab versus Pr: 1.7 [1.0–2.9]), the gene also not significantly affecting P-MDX (data not shown).

Antibodies

The *GSTT1* gene did not significantly affect S-IgG-MDI (not shown).

Symptoms and lung function

Subjects possessing the *GSTT1* gene displayed lower FVC (and FEV₁) than those with the null genotype did, but this had no significant effect on the symptoms (Tables 3, 6).

Influence of *GSTM1* and *GSTM3*

Metabolites, antibodies, symptoms and lung function

No associations between the *GSTM1* or *GSTM3* genotypes and metabolites, antibodies, symptoms or lung function were found (data not shown).

Influence of *NAT2*

Metabolites

The *NAT2* gene did not significantly affect either U- or P-MDX, although the extensive metabolisers showed somewhat higher levels, both years (Table 2).

Antibodies

The slow acetylators displayed lower antibody levels than the other genotypes, particularly the rapid acetylators, did (Table 2), although in the adjusted analyses the effect of *NAT2* on S-IgG-MDI was not significant. S-IgE was found to be higher in the slow than in the other *NAT2* acetylators (medians and range for the slow and the rapid acetylators: 26 [1–1,262] and 14 (1–55) kU/l, respectively; not shown).

Symptoms and lung function

In year 0, the slow acetylators showed a larger total number of eye symptoms than the other subjects, particularly the

intermediate acetylators, did (Tables 3, 5). In year 2, subjects were affected in a similar way (but not significantly so).

In year 0, the total number of nose symptoms was only slightly greater for the slow acetylators, but in year 2, this group was more strongly affected than the other groups, there also being a significant trend in this respect (Tables 3, 5). The same pattern was observed for the work-related symptoms (not shown). In year 0, in addition, a larger fraction of the slow acetylators for *NAT2* than of the other genotypes displayed symptoms in the lower airways (Table 3). In year 2, both the slow and the rapid acetylators were more strongly affected than the intermediate genotype group, which appeared to be significantly better ‘protected’ from symptoms in the lower airways than the slow ones (Table 5), the effects for the work-related symptoms being similar (not shown).

There was a certain effect of *NAT2* on FVC and a stronger effect of it on FEV₁, the rapid acetylators having a better lung function than the slow ones (Tables 3, 6).

Influence of combinations of genes

Metabolites, antibodies, symptoms and lung function

Analysis of the combined influence of *GSTP1* and *NAT2* on U-MDX and S-IgG-MDI yielded results similar to those obtained in analysing the influence of each gene separately (Tables 2, 4). This was also the case for the effect on eye symptoms in year 0 (Table 5): Some 13% of the subjects with *GSTP1* I/I and *NAT2* S/I, 27% of those with *GSTP1* I/V+V/V and *NAT2* S/I, and 12% of those with *GSTP1* I/V+V/V and *NAT2* Im+R, but none of those with *GSTP1* I/I and *NAT2* Im+R, displayed such symptoms. The adjusted OR for *GSTP1* I/V+V/V and *NAT2* S/I versus all other combinations, with use of a 95% CI, was 4.8 (1.7–14; not shown).

In line with the results of the analyses of the influence of separate genes (Tables 3, 5), the combination of *GSTP1* I/I and *NAT2* S/I displayed a higher risk level of respiratory symptoms in year 2 than any of the other combinations did (not shown). For example, the OR for work-related nose symptoms for subjects having I/V+V/V and Im+R, as compared with those having the combination of the *GSTP1* I/I and *NAT2* S/I genotypes, was 0.045 (0.005–0.40).

The combination of *GSTT1* and *NAT2* did not affect lung function differently than simply the presence of the one or the other gene (Table 6).

Discussion

The present pattern of results is complicated and there is a risk of multiple inference. There are some apparently con-

Table 5 Associations between genotype, on the one hand, and total symptoms (odds ratios [OR] and 95% confidence intervals [CI]), on the other

	Time	Gene	Symptom	Test	OR (95% CI)	Trend (P)	Exposure estimate
Data for year 0 and 2 in 141 and 158 employees, respectively, exposed to sprayed and heated MDI-based polyurethane glue. Multivariate analysis taking exposure intensity category, P-MDX, U-MDX or S-IgG-MDI into account in the model, and trend tests (referent genotype for $GSTP1=I/I = 1.0$ and for $NAT2 = SI = 1.0$) were carried out. Associations are reported if (at least) one genotype differs significantly from the referent genotype	Year 0	<i>GSTP1</i> ¹⁰⁵	Eyes	I/V vs. I/I	4.0 (1.1–14)	0.02	Category
				V/V vs. I/I	5.9 (1.2–29)		
				I/V vs. I/I	3.4 (0.97–12)	0.03	P-MDX
				V/V vs. I/I	5.0 (1.1–24)		
				I/V vs. I/I	4.5 (1.1–18)	0.02	U-MDX
				V/V vs. I/I	7.1 (1.3–39)		
				I/V vs. I/I	3.1 (0.85–11)	0.04	(S-IgG-MDI)
				V/V vs. I/I	4.8 (1.0–23)		
	Year 2	<i>NAT2</i>		Im vs. SI	0.22 (0.06–0.85)	0.05	Category
				R vs. SI	0.41 (0.04–4.3)		
		<i>NAT2</i>	Nose	Im vs. SI	0.35 (0.14–0.85)	0.009	Category
				R vs. SI	0.18 (0.02–1.5)		
				Im vs. SI	0.34 (0.14–0.84)	0.008	P-MDX
				R vs. SI	0.18 (0.02–1.5)		
				Im vs. SI	0.35 (0.14–0.87)	0.01	(S-IgG-MDI)
				R vs. SI	0.19 (0.02–1.6)		
		<i>NAT2</i>	Lower airways	Im vs. SI	0.28 (0.09–0.92)	NS ^a	Category
				R vs. SI	1.4 (0.30–6.2)		
				Im vs. SI	0.29 (0.09–0.93)	NS	P-MDX
				R vs. SI	1.2 (0.26–5.8)		
				Im vs. SI	0.27 (0.08–0.89)	NS	(S-IgG-MDI)
				R vs. SI	1.3 (0.27–5.8)		

^a NS not statistically significant

Table 6 Associations between genotype, on the one hand, and lung function (% of the expected forced expiratory volume in 1 s = FEV₁ and of the forced vital capacity = FVC; point estimate (PE) and 95% confidence interval (CI) of the difference in percentage points of the expected values), on the other, are shown

Gene	Lung function	Test	Difference PE (95% CI)	Exposure estimate
<i>NAT2</i>	FEV ₁	Im vs. SI	−0.89 (−5.5 to 3.7)	Category
		R vs. SI	−9.6 (−18 to −1.1)	
		Im vs. SI	−0.60 (−5.2 to 4.0)	P-MDX
		R vs. SI	−10 (−19 to −1.3)	
		Im vs. SI	−0.64 (−5.3 to 4.0)	(S-IgG-MDI)
		R vs. SI	−10 (−19 to −1.6)	
<i>GSTT1</i>	FVC	Ab vs. Pr	6.4 (0.46 to 12)	Category
		Ab vs. Pr	6.0 (0.01 to 12)	P-MDX
		Ab vs. Pr	6.2 (0.22 to 12)	(S-IgG-MDI)

Data for year 2 for 158 employees exposed to sprayed and heated MDI-based polyurethane glue. Multivariate analysis taking exposure intensity category, P-MDX or S-IgG-MDI into account in the model was carried out. Associations are reported if (at least) one genotype differs significantly from the referent genotype

sistent trends, however. As hypothesized, we found some *GST* genotypes (*GSTP1*¹⁰⁵ I/I and *GSTP1*¹¹⁴ A/A) to excrete higher levels of the exposure-related biomarker U-MDX than the other genotypes did. The mechanism

involved might be that of extensive metabolisers conjugating an isocyanate or its metabolite to GSH, which is excreted then into the urine, where, after hydrolysis, it was analysed as MDA, the A/A genotype thus displaying a higher U-MDX/P-MDX ratio. Data on the mercapturic acids that could have been informative concerning metabolism by the GSTs was not collected.

Interestingly, the I/I genotype showed a lower risk for the development of S-IgG-MDI antibodies than the other genotypes did, perhaps because of the sparse accumulation of MDI and metabolites in the body. These trends were also observed in the testing of formal interactions/effect modifications. *GSTP1* A/A, in contrast, appeared to be associated with higher S-IgG-MDI.

Although GSTs are known to be involved in the defense of cells affected by reactive oxygen species (Barnes 1990; Hayes and Strange 1995; Kelly 2003), and diisocyanates (i.e. TDI) have been suggested to impair the scavenging capacity of GSH-dependent enzymes in the airways through their binding to GSH (Lange et al. 1999; Wisniewski et al. 2005), our results concerning the subsequent risk of symptoms was somewhat contradictory. Thus, carriers of the *GSTP1* I/I genotype were found to have less frequent eye symptoms, but more frequent nose symptoms. No significant interactions/effect modifications were observed.

A second main hypothesis, that the slow acetylating capacity shown by *NAT2* would restrain the excretion of MDI metabolites into the urine and cause an accumulation of MDI/metabolites in the body and the development of S-IgG-MDI was not fully substantiated. However, in line with the hypothesis, a greater number of eye and nose symptoms was found to be present, whereas effects on the lower airways and on lung function were less obvious.

No evidence of gene–gene interactions for the associations obtained was detected.

Our genotyping was found to be both accurate and relevant, the analyses being checked at two different laboratories after separate DNA extractions. There is also a reasonably close agreement for the genotypes and their phenotypic expressions being obtained (Pelkonen et al. 1999; Hein et al. 2000).

There is no unexposed reference group in the study here, but we consider our exposed group, with its varying degrees of exposure, including subjects without any detectable metabolite level, to represent an acceptable study population. On the other hand, since the levels of isocyanates in the air were far below the time weighted average permissible exposure limits in Sweden, conclusions regarding the effects associated with MDI should be cautious. However, exposure to aerosols of isocyanate glues did take place, both by air and skin: Isologger measurements revealed the presence of intermittent peaks of isocyanate-containing compounds in the air and there was clear evidence of skin contamination.

A rationale employed in the present study was to establish associations in an initial examination and to then verify them in a second one (though use of largely the same population does constitute a limitation here). However, the results were not consistent. An unresolved issue is whether this is due to chance, or can be explained in terms of a change in the conditions of exposure from the one examination to the next.

There may also be a certain degree of misclassification regarding exposure, the different categories of MDI gluing not being sharply demarcated and there being exposure both to sprayed and to thermodegraded MDI glue, which varied both in extent and over time (Skarping et al. 1996; Littorin et al. 2000). These changes may have bearing on the type and frequency of the symptoms that developed.

The determination of 4,4'-MDA in the urine was probably accurate, since it agreed well with results from other laboratories (G. Skarping, personal communication). Still, the analysis of the exposure-related biomarker MDX was of limited precision (Littorin et al. 2000). In addition, some of the workers, who were presumably exposed, showed no detectable levels of exposure, whereas others, who were not engaged in jobs involving appreciable exposure displayed significant concentrations of 4,4'-MDA. This can be partly

explained in terms of the complexity of the exposure and its intermittent character. In addition, the biological half-life of P-MDX is as short as 2–3 weeks and that of U-MDX is at most days (Skarping et al. 1995; Dalene et al. 1997). Also, MDX reflects exposure associated with both sprayed and heated glue, i.e. to MDI, MDA, and possibly to aminoisocyanates as well.

The measurement of effects also involves various uncertainties. Thus, the measurements of specific IgG antibodies were performed by use of a semiquantitative method. Such imprecise information may impede the recognition of associations with antibodies that in fact are present.

Furthermore, although the use of questionnaires and supplementary interviews can provide a detailed medical history, there are uncertainties nevertheless regarding the nature of the symptoms. Also, the classification in terms of 'asymptomatic' and 'symptomatic' cases is far from self-evident. Although the symptoms used for the nose and the lower airways are probably mixed entities, separate analyses of detailed symptoms for the lower airways displayed similar patterns (unpublished data).

Since measurements of lung function were performed without reference to hours spent at work, the possibility of detecting work-related decrements of lung function, if of short duration, was limited.

Hence, there may well be biases favouring the null hypothesis as regards gene–environment interactions in the sequence of exposure, biomarkers of exposure, antibodies, symptoms and lung function. There is also a risk of a selection bias. In fact, there were indications of a selective transfer of atopic employees from high- to low-exposure tasks (Littorin et al. 2000). However, there was no indication of selection of subjects with particular genotypes to be employed. In the exposure groups, the distribution of genotypes was also similar to that of Swedes or of Caucasians in general (Garte et al. 2001; Schwarzbaum et al. 2007). This is reasonable, since there was only one subject with any other ethnicity than Caucasian. In addition, the fact that we studied effects of genetic factors on two consecutive occasions should reduce any bias due to the selection of healthy workers.

Although our study is small, it appears to be sufficiently large to detect simple and strong associations with the genes involved (Vineis et al. 1999; Gauderman 2002). For example, at a symptoms prevalence of 20% in the highest exposure group (sprayed and heated glue), there is 80% power for detecting an OR of 7.4 in subjects carrying the genotypes *GSTP1* I/V or V/V, as compared with the I/I genotype.

The information on associations between genes and isocyanate-induced risk is limited and is not consistent. Piirilä et al. (2001) found that lack of specific IgE antibodies against various isocyanates (MDI, HDI, TDI) was associ-

ated with the *GSTM1* null genotype. We did not find specific IgG to be related to that gene. Piirilä et al. (2001) and Wikman et al. (2002) found *GSTM1* null to be associated with an increased risk of isocyanate-associated asthma and with late reactions to bronchial challenge. In accordance with a study of MDI-PUR welders, we found no association between that genotype and symptoms in the lower airways (Jakobsson et al. 1997). Mapp et al. (2002) suggested the *GSTP1*¹⁰⁵ V allele to confer protection against both TDI-induced asthma and hyperresponsiveness of the airways, whereas we found the *GSTP1*¹⁰⁵ I/V genotype to protect against symptoms in the airways associated with exposure to MDI.

Wikman et al. (2002) and Berode et al. (2005) reported slow acetylators to be at risk for isocyanate-induced asthma, in particular that induced by MDI. We found eye and nose symptoms to be associated with slow acetylation, whereas effects on the lower airways appears more ambiguous.

There is also reason to consider gene associations quite apart from isocyanate exposure. Here too, the patterns are not completely clear. Fryer et al. (2000) found *GSTP1* V to be strongly associated with a protective effect on the overall risk of asthma and bronchial hyperresponsiveness, as well as of atopy and total S-IgE. In line with this, but contrary to Piirilä et al. (2001), we found the I/I genotype to be associated with higher total S-IgE levels and more frequent atopy. Others have reported *GSTP1*¹⁰⁵ V/V, *GSTT1* null and *GSTM1* null to affect lung function negatively (Gilliland et al. 2002; He et al. 2004). In our study, lung function was slightly better in the *GSTT1* null genotypes. Atopic and non-atopic asthma, asthma induced by isocyanates being an example of the latter, may well differ in their relationship to the genes (Fryer et al. 2000; Liu and Wisniewski 2003; Kleeberger and Peden 2005). There may be ethnic variations as well. In Turkey, for example, patients with *GSTT1*, *GSTM1* null and *GSTP1* V/V genotypes have been found to have a higher risk of atopic than of non-atopic asthma (Tamer et al. 2004).

We have included only some and by no means all of the genes of interest that may possibly be involved here. Also, various associations we failed to detect with the restricted approach we employed may exist, nevertheless, possibly in a more complex form than was envisioned here. The present result and earlier results of others as well show rather clearly that demonstrating gene-environment interactions in an unequivocal way is an intricate matter. It requires reasonably accurate estimates of exposure and outcomes, large populations, proper distributions of genes frequencies and strong effect modifications by the genes. Such studies are yet to be performed. Complex findings of this sort have been obtained in other gene-associated areas, in connection with tumours, for example such showing gene-modification of associations between polycyclic aromatic hydrocarbons and lung cancer (Garte 2001).

Firmer knowledge of gene–environment interactions would cast light on various pathological mechanisms and improve the understanding of exposure–response relationships. This could contribute to the development of preventive measures in this area.

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