

Respiratory symptoms, sensitization and associations with isocyanate exposure in spray painters

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This is the first paper to describe exposure-response relationships for exposure to complex mixtures of isocyanates and both work-related and non work-related respiratory symptoms and specific sensitisation.

Abstract

Rationale: Associations between oligomeric isocyanate exposure, sensitisation and respiratory disease have received little attention, despite the extensive use of isocyanate oligomers.

Objectives: To investigate exposure-response relationships of respiratory symptoms and sensitisation in a large population occupationally exposed to isocyanate oligomers during spray painting.

Methods: The prevalence of respiratory symptoms and sensitization was assessed in 581 workers in the spray-painting industry. Personal exposure was estimated by combining personal task-based inhalatory exposure measurements and time activity information. Specific IgE and IgG to hexamethylene diisocyanate (HDI) were assessed in serum by ImmunoCAP assay and enzyme immunoassays using vapor and liquid phase HDI-human serum albumin (HSA) and HSA-conjugates prepared with oligomeric HDI.

Measurements and main results: Respiratory symptoms were more prevalent in exposed than among comparison office workers. Log-linear exposure-response associations were found for asthma-like symptoms, COPD-like symptoms and work-related chest tightness (prevalence ratios for an interquartile range increase in exposure of 1.2, 1.3 and 2.0 respectively, $p \leq 0.05$). The prevalence of specific IgE sensitization was low (up to 4.2% in spray painters). Nevertheless, IgE to N100 (oligomeric HDI)-HSA was associated with exposure and work-related chest tightness. The prevalence of specific IgG was higher (2-50.4%) and strongly associated with exposure.

Conclusions: The results provide evidence of exposure-response relationships for both work-related and non work-related respiratory symptoms and specific sensitization in a population exposed to oligomers of HDI. Specific IgE was found in only a minority of symptomatic individuals. Specific IgG seems merely an indicator of exposure.

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Introduction

Isocyanates, low molecular weight compounds characterized by highly reactive NCO groups, are one of the most commonly identified causes of occupational asthma (1-3). Besides allergic asthma, isocyanate exposure may also induce irritant asthma, hypersensitivity pneumonitis and possibly accelerated lung function decline (4). Diisocyanates are used as cross-linking agents in polyurethane (PU) products such as foams, paints, lacquers, inks, insulating materials, varnishes, rubber modifiers, and bonding and vulcanizing agents (5). The PU-industry continues to increase, along with the number of workers at risk for exposure (4). Toluene diisocyanate (TDI), diphenyl-methane diisocyanate (MDI) and hexamethylene diisocyanate (HDI) are the most frequently used diisocyanate monomers.

Despite a vast amount of studies on isocyanates, aspects of the association between health effects and isocyanate exposure remain unclear. Isocyanate monomers have been studied relatively well in large TDI manufacture or foam production units. In the early years of the industry, annual occupational asthma incidence was as high as 5-6% (6). Reduction of average TDI concentrations below the 8 hr occupational exposure limit of 5 ppb ($17 \mu\text{g}/\text{m}^3$ total NCO group mass concentration) led to a decline below 1% (6). Conversely, asthma symptom prevalences up to 41% have been reported in TDI end user industries with possibly less controlled exposures (6).

Recently, diisocyanate oligomers, mainly of HDI and MDI with considerably lower vapour pressures, have been increasingly used to reduce inhalation exposure (4). In the present paper all polymeric diisocyanates, which are indicated with different terms (poly-isocyanates, oligomers, adducts) in the literature, will be referred to as oligomers. Isocyanate asthma does occur in workers exposed to oligomers and specific inhalation challenge testing of individual patients confirms that oligomers can cause asthma (7). Yet, despite the extensive use of isocyanate oligomers, exposure-response associations have hardly been investigated. Commercial products based on oligomeric isocyanates commonly contain a variable mixture of several different chemical structures. The complexity of exposure assessment of these mixtures contributes to the absence of exposure-response studies.

Spray painters, who are exposed to HDI oligomer mixtures, are among the occupational groups with the highest incidence of occupational asthma in industrialized countries (8-10). Figure 1 shows the chemical structures of HDI and two of its oligomers. This paper describes a large cross-sectional study of isocyanate exposure and health effects in spray painters. Respiratory symptoms were recorded and specific IgE and IgG antibodies to various isocyanate conjugates were measured. Over 500 task-based exposure measurements to a wide range of isocyanates were used in combination with time activity information

to estimate the exposure of each individual. We specifically aimed at establishing quantitative exposure-response relationships for a range of respiratory endpoints.

Material and methods

Population and study design

The population consists of 581 subjects working in various spray painting industries in The Netherlands. Car body repair shops, furniture paint shops and industrial paint shops specializing in ships and harbor equipment or airplanes, were contacted by mail or telephone. Companies were visited between 2003 and 2006 and a workplace survey was performed. All workers were asked to complete a self-administered questionnaire and to provide a 20 ml blood sample. All participants were actively working at the time of the study and the study was performed on a working day. The institutional review board for human studies approved of the protocols and written consent was obtained from all participants.

Questionnaire

Items included respiratory symptoms according to the Dutch version of the internationally accepted British Medical Research Council (BMRC) respiratory questionnaire (11), supplemented with questions on work-related symptoms. Symptoms were considered work-related when they were reported to occur during or shortly after work. For statistical analyses respiratory symptoms suggestive of COPD and asthma were combined: "COPD-like symptoms" included chronic cough, chronic phlegm and shortness of breath; "asthma-like symptoms" included wheezing and chest tightness.

Additional items included smoking habits, job history, present job title, personal protective equipment use and monthly task patterns. Workers reporting no tasks outside the office were classified as 'office workers'. Workers involved in spray painting were classified as 'spray painters' and all other workers involved in tasks outside the office as 'others'. The latter category consisted of mostly mechanics and metal workers. In every company, all workers were working in the same building.

Personal exposure estimates

Personal exposure estimates were obtained by combining personal task-based inhalation measurements for 23 different isocyanate compounds (4 monoisocyanates, 5 aminoisocyanates, 6 diisocyanates and 8 diisocyanate oligomers) performed in the population under study (12) and time activity information:

$$Exposure = \sum_{n=1}^n (Time)_n * (\% > LOD)_n * (Median \ NCO \ Concentration)_n$$

Exposure = Personal exposure expressed in $\mu\text{g NCO} \cdot \text{m}^{-3} \cdot \text{hr} \cdot \text{month}^{-1}$;

$n = 1, 2, \dots$ to n for the following tasks: spray painting, mixing, cleaning paint equipment, assisting a spray painter, sanding and welding;

$(Time)_n$ = Time task n was performed expressed in hours/month. On average 82 hours (standard deviation [SD]: 89) out of a 161 hour (SD: 26) working month was spent on exposed tasks;

$(\% > LOD)_n$ = Percentage of samples above the limit of detection (LOD) for task n ;

$(\text{Median NCO concentration})_n = \text{Median inhalatory isocyanate concentration}$
during task n expressed in $\mu\text{g NCO/m}^3$;

Separate task-based airborne exposure measurements were available for each combination of industry and task. The total isocyanate group (NCO) concentration and NCO from HDI and two HDI oligomers (biuret and isocyanurate) concentration were calculated.

More details are provided in the online data supplement.

Serological analysis

Blood samples were processed within 8 hours and serum aliquots were stored at -20°C until serologic assays. HDI-specific IgE and IgG antibodies were analyzed using the ImmunoCAP assay (Phadia, Uppsala, Sweden) and specific IgE to common aeroallergens using the Phadiatop as a measure of atopy. Cut-off values of 0.35 kU/l for specific IgE and 5 mg/l for specific IgG were used.

Isocyanate-specific IgE and IgG were also assessed by enzyme immunoassay (EIA) with HDI-human serum albumin (HSA) conjugates prepared in our own laboratories. HDI-HSA was prepared in liquid-phase ($\text{HDI}_L\text{-HSA}$) (13) and vapor-phase ($\text{HDI}_V\text{-HSA}$) (14) reactions essentially as described earlier. HDI oligomer-HSA conjugates were prepared with Desmodur N3300, a commercial product containing a low viscosity isocyanurate oligomer of HDI, and Desmodur N100, a trimeric biuret structure (Bayer, Pittsburgh, PA). Table 1 gives an overview of immunoassays used. Cut-off values for HSA-corrected OD values of 0.1 and 0.3 were used for IgE and IgG respectively.

Details of the EIA procedures and establishment of cut-off values are provided in the online data supplement.

Physiological testing

Bronchial hyperresponsiveness (BHR) was assessed in a subset of 229 workers. Selection of this subset is described in the online data supplement. At least 2 maximal expiratory flow-volume maneuvers were obtained to assess baseline lung function. The largest forced expiratory volume in one second (FEV1) and forced vital capacity (FVC) were recorded. Maximum mid-expiratory flow (MMEF) was obtained from the maneuver with the largest sum of FEV1+FVC as described by Miller et al. (15). BHR was assessed by methacholine challenge according to the ERJ guidelines (16). Methacholine was administered using a controlled tidal volume (Vt) breathing dosimeter technique using the Aerosol Provocation System with a Medic-Aid nebulizer (Jaeger GmbH & Co KG; Wurzburg Germany), starting with 0.019 mg methacholine following three quadrupling doses and one doubling dose up to a cumulative dose of 2.5 mg (short schedule). FEV1 was measured 30 and 90 seconds after challenge and the lowest FEV1 from a technically acceptable maneuver was used. After a fall in FEV1 of 5%, doubling doses were used (long schedule). The test was stopped when a fall of 20% in FEV1 was observed (BHR20) or the maximum cumulative dose was reached. Airway hyperresponsiveness was defined as a provocative dose of methacholine required to cause a 20% fall in FEV1 of ≤ 2.5 mg (~ 10 μ moles).

Statistical analysis

SAS v9.1 statistical software was used. Correlations between the exposure variables were assessed using Pearson correlation coefficients for log-transformed data. In cross sectional studies, the prevalence ratio (PR) is often a more easily interpretable and meaningful measure of association than the odds ratio (17). Therefore PRs and 95% confidence intervals (95%CI) were calculated by log-binomial regression (PROC GENMOD) to describe associations for binary health outcomes. Log-transformed exposure data were used and PRs per unit increase were converted to PRs per interquartile range (IQR). Associations with exposure were further explored by nonparametric regression modeling (smoothing) using generalized additive models (PROC GAM). Smoothing parameter degrees of freedom were selected by generalized crossvalidation (18) but limited to three. Unless stated otherwise all associations were adjusted for current smoking, age, gender and atopy. Possible effect modification by atopy was explored as well.

Results

Population characteristics and exposure

The 581 participating workers came from 128 companies: 88 car body repair shops; 33 furniture paint shops; and seven industrial paint shops, of which six specializing in ships and harbor equipment and one in airplanes. Of all companies contacted through surface mail and telephone 10-30% responded and the average worker participation rate per company was 67%. General characteristics of the study population are shown in Table 2.

Estimated median total NCO exposure levels were higher in the 'spray painters' category than among 'others', with a wide range in both categories. Exposure to HDI monomer represented only a very small fraction of total NCO. Of the HDI oligomers, which represented a larger fraction of total NCO, isocyanurate exposure was higher than biuret exposure.

Within the group of spray painters, those working in airplane paint shops were on average more highly exposed than those in furniture paint shops, ship and harbor equipment paint shops and car body repair shops (Median: 16600 vs 4900, 4700 and 3300 $\mu\text{g NCO}\cdot\text{m}^{-3}\cdot\text{hr}\cdot\text{month}^{-1}$ respectively). The minimum, 25th percentile, median, 75th percentile and maximum of the total exposure distribution were 0, 1.7, 165, 33821, and 66464 $\mu\text{g NCI}\cdot\text{m}^{-3}\cdot\text{hr}\cdot\text{month}^{-1}$ respectively. Pearson correlation coefficients between the exposure estimates for total NCO, HDI, biuret and isocyanurate were very high (≥ 0.95).

Prevalence of symptoms and positive serology

Exposed workers more often reported respiratory symptoms than office workers (Table 3). Asthma-like symptoms were significantly ($p \leq 0.05$) more prevalent in both spray painters and other workers (adjusted PR (95%CI): 2.8 (1.3-5.9) and 2.2 (1.0-4.8), respectively). Spray painters also reported more COPD-like symptoms (adjusted PR (95%CI): 2.9 (1.1-8.0)). No significant differences were found for any of the work-related symptoms.

Despite the high symptom prevalence, specific IgE to isocyanates were found only in a small proportion of exposed workers; 0.4-4.2% in spray painters, 0.7-3.5% in other workers and none in the office group (Table 3). The prevalence of elevated specific IgG antibody concentrations was much higher. Among spray painters prevalences up to 50% were found. Antibodies to N100-HSA and HDI_L-HSA were found most frequently, both for specific IgE and IgG.

Specific IgG to HDI_L-HSA, HDI_V-HSA and N100-HSA was significantly ($p \leq 0.05$) more prevalent among spray painters compared to office workers (adjusted PR (95% CI): 1.6 (1.0-2.6), 10.6 (1.5-75.2) and 7.8 (1.9-32.5) respectively). IgG antibodies to N100-HSA were also more often found in other workers than in office workers (adjusted PR (95% CI): 4.7 (1.1-19.4)).

Atopy was significantly ($p \leq 0.05$) less common among spray painters than office workers (adjusted PR (95%CI): 0.7 (0.5-1.0)).

Association between symptoms and serology

Table 4 shows the associations between symptoms and the presence of isocyanate-specific antibodies. A consistent pattern of significant positive associations was found for work-related rhinitis and specific IgE to each of the conjugates, with PRs between 1.8-2.8. All PRs for work-related chest tightness and specific IgE were positive but showed much more variation, and only the association with IgE to N100-HSA was significant. Overall PRs for asthma-like and COPD-like symptoms were lower and, for most conjugates, were close to 1.0.

Statistically significant associations were found for COPD-like symptoms and work-related rhinitis and conjunctivitis with IgG to N100-HSA. However, for the other conjugates PRs for the association between specific IgG and symptoms were close to 1. Exclusion of workers with a high IgG background reaction to HSA did not alter the associations (data not shown).

Associations with exposure

PRs were calculated based on log transformed exposure data and expressed for an IQR increase in exposure ($1.7\text{-}3382\ \mu\text{g NCO}\cdot\text{m}^{-3}\cdot\text{hr}\cdot\text{month}^{-1}$ or a difference in exposure of approximately a factor 2000) (Table 5). Significant positive log-linear associations with exposure were found for asthma-like symptoms, COPD-like symptoms, work-related chest tightness and work-related conjunctivitis (Table 5). Only the association between work-related conjunctivitis and exposure differed between atopic and non-atopic individuals (p interaction term ≤ 0.1). Surprisingly, the association was stronger in non-atopic than in atopic subjects (adjusted PR:

2.1 (1.2-3.9) and 1.1 (0.7-1.8) respectively). For asthma-like symptoms (Figure 2A) and COPD-like symptoms (plot not shown) the smoothed plots corroborate log-linear relations. For work-related chest tightness (Figure 2B) the smoothed plot suggests a steeper increase at high exposure levels (p spline ≤ 0.05). No statistically significant association between rhinitis and exposure was found (Figure 2C).

Interestingly, the prevalence of atopy was lower at high exposure levels. Figure 2D shows a sharp reduction for the prevalence of atopy at isocyanate exposures above $\sim 1000 \mu\text{g NCO} \cdot \text{m}^{-3} \cdot \text{hr} \cdot \text{month}^{-1}$ (p spline ≤ 0.05). Atopic subjects were significantly less exposed than non-atopic subjects (geometric mean: 24.1 and $57.9 \mu\text{g NCO} \cdot \text{m}^{-3} \cdot \text{hr} \cdot \text{month}^{-1}$ respectively, $p \leq 0.05$).

Exposure was also associated with N100-HSA-specific IgE. The smoothed plot shows a very slight increase (Figure 2E). Specific IgG antibodies to all conjugates except HDI-ImmunoCAP were positively associated with exposure. Especially strong associations were found for IgG to HDI_V-HSA and N100-HSA. For IgG measured by ImmunoCAP (p interaction term ≤ 0.1), IgG to N3300-HSA (p interaction term ≤ 0.05) and to N100-HSA (p interaction term ≤ 0.05) stronger associations were seen in atopic subjects (adjusted PR: 2.5 (0.99-6.4), 2.8 (1.6-4.8) and 3.5 (2.1-5.8) respectively) than in non-atopic subjects for whom none of the associations was significant. Exclusion of workers with a high IgG background reaction to HSA did not alter any of these associations (data not shown).

Glove use during paint related tasks, which varies among workers, did not affect exposure-response associations in this study. The use of respiratory protection during spray painting is compulsory and was always observed during the fieldwork. Therefore the effect of respiratory protection could not be investigated.

Physiological testing

Individuals with asthma-like symptoms were more likely to have bronchial hyperresponsiveness (adjusted PR (95% CI): 2.2 (1.5-3.2)). These individuals also had lower baseline FEV₁, FEV₁/FVC and MMEF between 90 and 96% compared with symptom free workers. For COPD-like symptoms the association with BHR was less strong than for asthma-like symptoms and only borderline statistically ($p=0.07$) significant (adjusted PR (95% CI): 1.6 (1.0-2.5)). In addition none of the lung function parameters was significantly associated with COPD-like symptoms. Individuals with work related symptoms were more likely to be hyperresponsive, but this was statistically significant only in those with rhinitis symptoms (PRs ≥ 1.8). No clear associations between work-related symptoms and lung function were found.

Discussion

The results of this study provide evidence for exposure-response relationships for exposure to complex mixtures of isocyanates and both work-related and non work-related respiratory symptoms and specific sensitization.

Exposure to diisocyanate monomers has been assessed in various epidemiological studies. In the majority of these studies, mean or maximum exposure levels are reported for a population in which a measure of disease frequency is investigated (19-25). However, few studies have considered the issue of quantitative exposure response in isocyanate asthma (26). Two case control studies demonstrated that higher exposure levels were more likely to be found in companies at which there were workers with a successful claim for occupational asthma (27) or in doctor diagnosed asthma cases (26) than in control companies or matched controls from the same company, respectively. Differences in study design complicate the comparison of these studies with the present study.

The use of product formulations containing complex mixtures of oligomer isocyanates is increasing (4). Currently oligomers are the major contributor to isocyanate exposure worldwide. Several studies have shown respiratory symptoms or asthma in workers exposed to oligomeric aromatic isocyanates (28-30). Oligomers of aliphatic HDI are widely used in the spray-painting industry. Decreased lung function parameters (31, 32) and high asthma symptom prevalences have been reported in this industry (33-38). Only one study has

incorporated exposure assessment. That study demonstrated a relation between peak exposure and reduced lung function in car painters who smoke (32).

However, the population size was too small ($n=36$) to be conclusive.

This is the first study performed in an end user industry in which complex exposure patterns of isocyanates were assessed. Over 500 task-based exposure measurements were taken using a state-of-the-art method (12) and used to estimate monthly cumulative personal exposure.

A working day of a spray painter consists of cycles of short tasks and even exposure during spray painting is highly variable for all workers (12). Therefore, isocyanate exposure in this study consists of a series of peaks, which is highly correlated with average exposure through the duration of the tasks.

Consequently it is not possible to differentiate between cumulative and peak exposure.

Although HDI oligomers were the major exposure factor, product formulations also contained trace amounts of monomeric HDI leading to detectable but very low monomer exposure levels. Personal task-based HDI levels up to $29 \mu\text{g NCO}/\text{m}^3$ were found which did not exceed the Dutch short-term exposure limit for HDI ($70 \mu\text{g NCO}/\text{m}^3$). In contrast, HDI oligomer levels ranged up to $3760 \mu\text{g NCO}/\text{m}^3$. Therefore, despite the high correlation between oligomer and monomer levels, it seems unlikely that these monomer levels contributed significantly to the observed associations with symptoms.

Animal studies indicate that relative potencies of different isocyanate compounds are variable (39-42). Theoretically, this kind of information might be used to calculate a weighted total NCO concentration. However, for many of the measured isocyanate compounds this information is not available, which limits the possibilities to use the information on oligomers levels for calculation of overall NCO levels weighted by toxic properties. Moreover, since exposure to HDI and its individual oligomers correlated highly, this would practically only have led to a rescaling of the exposure variable.

The company participation rate of this study was low (10-30%), while the mean worker participation rate within the companies of 67% was acceptable. Control measures are very similar among car body repair shops in the Netherlands and spray-booths and ventilation are always present. Yet, working practices may vary and it cannot be ruled out that more compliant companies were more likely to participate. The negative association between atopy and exposure may point towards another type of selection bias. Possibly, atopic workers are more likely to develop symptoms and leave the industry or atopic workers with pre-existing conditions may avoid seeking work as a spray painter. This warrants further attention in follow-up studies since it may result in a healthy worker effect.

Regardless of a possible healthy worker effect, a high prevalence of reported symptoms was noted in spray painters but also in other workers. Positive associations with exposure were found for asthma-like and COPD-like

symptoms, work-related chest tightness and work-related conjunctivitis.

Smoothed spline plots corroborated these associations and confirmed that the log-linear models describe the relation with asthma-like symptoms in a satisfactory way. For work-related chest tightness a steeper increase at high exposure levels was suggested. The surprisingly stronger association for work-related conjunctivitis in non-atopic individuals seems to be explained by the under-representation of atopic workers in the highest exposure range.

The significance of asthma-like symptoms found in this study was corroborated by the BHR results and lung function testing. Asthma-like symptoms were associated with BHR and lung function parameters indicative of obstruction. These associations were weaker or did not exist for COPD-like symptoms indicating that these symptoms may be due to other respiratory conditions.

The low prevalence of specific IgE antibodies in this population of workers that were actively working at the time of the study complicates the assessment of its association with exposure as well as with health effects. Nevertheless, an association between specific IgE to N100-HSA and work-related chest tightness as well as exposure to isocyanates was indicated. The results suggest that at most, specific IgE plays a role in a minority of individuals with symptoms. Thus, other mechanisms, like cell-mediated allergic reactions or pulmonary irritation (4, 43), are likely to be involved. The association between IgE to each of the isocyanate conjugates and work-related rhinitis in the absence of a statistically

significant association with exposure is remarkable and needs to be further explored.

IgG antibodies are usually considered an effect of exposure. The observed relationship between IgG and exposure can therefore be regarded as an external validation of the exposure assessment in this study. In addition, it shows that despite the low prevalence of specific IgE, the conjugates used are suitable reagents for the detection of isocyanate-specific immune responses. The significantly stronger association between specific IgG and exposure in atopic subjects, despite their lower exposure levels, suggests that they are immunologically more responsive to isocyanates than non-atopic individuals. A remarkable high prevalence of IgG to HDI_L-HSA was found in office workers. A recent study demonstrated specific IgG to HDI-HSA in 13% of 139 individuals without known exposure to isocyanates (44). Whether specific IgG antibodies to HDI_L-HSA in office workers represents actual exposure needs to be further explored.

Taken together, despite a possible healthy worker effect, exposure-response relationships were demonstrated for respiratory symptoms and sensitization in this population of spray painters exposed mainly to oligomers of HDI. Specific IgG antibodies seem to be primarily a marker of exposure. The association between specific IgE to N100-HSA and symptoms on one hand and exposure on the other hand is suggestive of an IgE-mediated mechanism in only a small proportion of the symptomatic individuals. A more detailed evaluation of

immunologic and physiological end points is needed to gain insight in the nature of symptoms induced by isocyanates and the role of specific antibodies in this population.

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References

1. Vandenplas O, Malo JL, Saetta M, Mapp CE, Fabbri LM. Occupational asthma and extrinsic alveolitis due to isocyanates: current status and perspectives. *Br J Ind Med* 1993;50:213-228.
2. Bernstein JA. Overview of diisocyanate occupational asthma. *Toxicology* 1996;111:181-189.
3. Wisnewski AV, Redlich CA. Recent developments in diisocyanate asthma. *Curr Opin Allergy Clin Immunol* 2001;1:169-175.
4. Wisnewski AV, Redlich C, Mapp C, Bernstein DI. Polyisocyanates and their prepolymers. In: Bernstein IL, Chan-Yeung M, Malo JL, Bernstein DI, editors. *Asthma in the workplace*. New York: Taylor & Francis Group; 2006. p. 481-504.
5. Lesage J, Goyer N, Desjardins F, Vincent JY, Perrault G. Workers' exposure to isocyanates. *Am Ind Hyg Assoc J* 1992;53:146-153.
6. Ott MG. Occupational asthma, lung function decrement, and toluene diisocyanate (TDI) exposure: a critical review of exposure-response relationships. *Appl Occup Environ Hyg* 2002;17:891-901.
7. Bello D, Woskie SR, Streicher RP, Liu Y, Stowe MH, Eisen EA, Ellenbecker MJ, Sparer J, Youngs F, Cullen MR, et al. Polyisocyanates in occupational environments: a critical review of exposure limits and metrics. *Am J Ind Med* 2004;46:480-491.
8. McDonald JC, Keynes HL, Meredith SK. Reported incidence of occupational asthma in the United Kingdom, 1989-97. *Occup Environ Med* 2000;57:823-829.
9. Karjalainen A, Kurppa K, Virtanen S, Keskinen H, Nordman H. Incidence of occupational asthma by occupation and industry in Finland. *Am J Ind Med* 2000;37:451-458.
10. Ameille J, Pauli G, Calastreng-Crinquand A, Vervloet D, Iwatsubo Y, Popin E, Bayeux-Dunglas MC, Kopferschmitt-Kubler MC. Reported incidence of occupational asthma in France, 1996-99: the ONAP programme. *Occup Environ Med* 2003;60:136-141.
11. Medical Research Council Committee on the Aetiology of Chronic Bronchitis. Instructions for the use of the questionnaire on respiratory symptoms. Dawlish, UK: Holman Ltd.; 1966.
12. Pronk A, Tielemans E, Skarping G, Bobeldijk I, van Hemmen J, Heederik D, Preller L. Inhalation exposure to isocyanates of car body repair shop workers and industrial spray painters. *Ann Occup Hyg* 2006;50:1-14.
13. Dewair MA, Baur X. Studies on antigens useful for detection of IgE antibodies in isocyanate-sensitized workers. *J Clin Chem Clin Biochem* 1982;20:337-340.
14. Wisnewski AV, Stowe MH, Cartier A, Liu Q, Liu J, Chen L, Redlich CA. Isocyanate vapor-induced antigenicity of human albumin. *J Allergy Clin Immunol* 2004;113:1178-1184.

15. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, Crapo R, Enright P, van der Grinten CP, Gustafsson P, et al. Standardisation of spirometry. *Eur Respir J* 2005;26:319-338.
16. Sterk PJ, Fabbri LM, Quanjer PH, Cockcroft DW, O'Byrne PM, Anderson SD, Juniper EF, Malo JL. Airway responsiveness. Standardized challenge testing with pharmacological, physical and sensitizing stimuli in adults. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. *Eur Respir J Suppl* 1993;16:53-83.
17. Skov T, Deddens J, Petersen MR, Endahl L. Prevalence proportion ratios: estimation and hypothesis testing. *Int J Epidemiol* 1998;27:91-95.
18. Hastie T, Tibshirani RJ. Generalized additive models. New York: Chapman & Hall; 1990.
19. Bodner KM, Burns CJ, Randolph NM, Salazar EJ. A longitudinal study of respiratory health of toluene diisocyanate production workers. *J Occup Environ Med* 2001;43:890-897.
20. White WG, Morris MJ, Sugden E, Zapata E. Isocyanate-induced asthma in a car factory. *Lancet* 1980;1:756-760.
21. Clark RL, Bugler J, McDermott M, Hill ID, Allport DC, Chamberlain JD. An epidemiology study of lung function changes of toluene diisocyanate foam workers in the United Kingdom. *Int Arch Occup Environ Health* 1998;71:169-179.
22. Jones RN, Rando RJ, Glindmeyer HW, Foster TA, Hughes JM, O'Neil CE, Weill H. Abnormal lung function in polyurethane foam producers. Weak relationship to toluene diisocyanate exposures. *Am Rev Respir Dis* 1992;146:871-877.
23. Grammer LC, Eggum P, Silverstein M, Shaughnessy MA, Liotta JL, Patterson R. Prospective immunologic and clinical study of a population exposed to hexamethylene diisocyanate. *J Allergy Clin Immunol* 1988;82:627-633.
24. Bernstein DI, Korbee L, Stauder T, Bernstein JA, Scinto J, Herd ZL, Bernstein IL. The low prevalence of occupational asthma and antibody-dependent sensitization to diphenylmethane diisocyanate in a plant engineered for minimal exposure to diisocyanates. *J Allergy Clin Immunol* 1993;92:387-396.
25. Ott MG, Klees JE, Poche SL. Respiratory health surveillance in a toluene diisocyanate production unit, 1967-97: clinical observations and lung function analyses. *Occup Environ Med* 2000;57:43-52.
26. Meredith SK, Bugler J, Clark RL. Isocyanate exposure and occupational asthma: a case-referent study. *Occup Environ Med* 2000;57:830-836.
27. Tarlo SM, Liss GM, Dias C, Banks DE. Assessment of the relationship between isocyanate exposure levels and occupational asthma. *Am J Ind Med* 1997;32:517-521.
28. Ulvestad B, Melbostad E, Fuglerud P. Asthma in tunnel workers exposed to synthetic resins. *Scand J Work Environ Health* 1999;25:335-341.
29. Simpson C, Garabrant D, Torrey S, Robins T, Franzblau A. Hypersensitivity pneumonitis-like reaction and occupational asthma associated with 1,3-

- bis(isocyanatomethyl) cyclohexane pre-polymer. *Am J Ind Med* 1996;30:48-55.
30. Petsonk EL, Wang ML, Lewis DM, Siegel PD, Husberg BJ. Asthma-like symptoms in wood product plant workers exposed to methylene diphenyl diisocyanate. *Chest* 2000;118:1183-1193.
 31. Glindmeyer HW, Lefante JJ, Jr., Rando RJ, Freyder L, Hnizdo E, Jones RN. Spray-painting and chronic airways obstruction. *Am J Ind Med* 2004;46:104-111.
 32. Tornling G, Alexandersson R, Hedenstierna G, Plato N. Decreased lung function and exposure to diisocyanates (HDI and HDI-BT) in car repair painters: observations on re-examination 6 years after initial study. *Am J Ind Med* 1990;17:299-310.
 33. Talini D, Monteverdi A, Benvenuti A, Petrozzino M, Di Pede F, Lemmi M, Carletti A, Macchioni P, Serretti N, Viegi G, et al. Asthma-like symptoms, atopy, and bronchial responsiveness in furniture workers. *Occup Environ Med* 1998;55:786-791.
 34. Mastrangelo G, Paruzzolo P, Mapp C. Asthma due to isocyanates: a mail survey in a 1% sample of furniture workers in the Veneto region, Italy. *Med Lav* 1995;86:503-510.
 35. Cullen MR, Redlich CA, Beckett WS, Weltmann B, Sparer J, Jackson G, Ruff T, Rubinstein E, Holden W. Feasibility study of respiratory questionnaire and peak flow recordings in autobody shop workers exposed to isocyanate-containing spray paint: observations and limitations. *Occup Med (Lond)* 1996;46:197-204.
 36. Eifan AO, Derman O, Kanbur N, Sekerel BE, Kutluk T. Occupational asthma in apprentice adolescent car painters. *Pediatr Allergy Immunol* 2005;16:662-668.
 37. Sari-Minodier I, Charpin D, Signouret M, Poyen D, Vervloet D. Prevalence of self-reported respiratory symptoms in workers exposed to isocyanates. *J Occup Environ Med* 1999;41:582-588.
 38. Ucgun I, Ozdemir N, Metintas M, Metintas S, Erginel S, Kolsuz M. Prevalence of occupational asthma among automobile and furniture painters in the center of Eskisehir (Turkey): the effects of atopy and smoking habits on occupational asthma. *Allergy* 1998;53:1096-1100.
 39. Pauluhn J. Acute inhalation toxicity of polymeric diphenyl-methane 4,4'-diisocyanate in rats: time course of changes in bronchoalveolar lavage. *Arch Toxicol* 2000;74:257-269.
 40. Pauluhn J. Pulmonary irritant potency of polyisocyanate aerosols in rats: comparative assessment of irritant threshold concentrations by bronchoalveolar lavage. *J Appl Toxicol* 2004;24:231-247.
 41. Pauluhn J, Eidmann P, Mohr U. Respiratory hypersensitivity in guinea pigs sensitized to 1,6-hexamethylene diisocyanate (HDI): comparison of results obtained with the monomer and homopolymers of HDI. *Toxicology* 2002;171:147-160.

42. Lee CT, Friedman M, Poovey HG, Ie SR, Rando RJ, Hoyle GW. Pulmonary toxicity of polymeric hexamethylene diisocyanate aerosols in mice. *Toxicol Appl Pharmacol* 2003;188:154-164.
43. Raulf-Heimsoth M, Baur X. Pathomechanisms and pathophysiology of isocyanate-induced diseases--summary of present knowledge. *Am J Ind Med* 1998;34:137-143.
44. Bernstein DI, Ott MG, Woolhiser M, Lummus Z, Graham C. Evaluation of antibody binding to diisocyanate protein conjugates in a general population. *Ann Allergy Asthma Immunol* 2006;97:357-364.

Figure legends:

Figure 1: Chemical structures of hexamethylene diisocyanate (HDI) and two HDI oligomers.

Figure 2: Association between log-transformed exposure to isocyanates ($\mu\text{g NCO}\cdot\text{m}^{-3}\cdot\text{hr}\cdot\text{month}^{-1}$) and selected health end points. Penalized smoothed spline plots are given with smoothed 95% confidence intervals for: A: Asthma-like symptoms, spline: $p > 0.10$, B: Work-related chest tightness, spline: $p \leq 0.05$, C: Work-related rhinitis, spline: $p > 0.10$, D: Atopy, spline: $p \leq 0.05$, E: IgE N100-HSA, spline: $p > 0.10$, F: IgG N100-HSA, spline: $p > 0.10$. Data rugs at the bottom of each graph indicate the distribution of data points.

Tables

Table 1: Overview of characteristics of assays used in specific IgE and IgG anti HDI analyses: Source where the conjugate was prepared, technical details and system in which the conjugate was used.

Conjugate	Source*	Carrier	Phase isocyanate**	Test system
HDI- ImmunoCAP	Phadia	ImmunoCAP (as solid phase)	Done by manufacturers	ImmunoCAP assay
HDI _L -HSA	IRAS	HSA	Liquid	EIA
HDI _V -HSA	Yale	HSA	Vapor	EIA
N3300-HSA	Yale	HSA	Liquid	EIA
N100-HSA	Yale	HSA	Liquid	EIA

*Source: Phadia, Sweden; Institute for Risk Assessment Sciences (IRAS), The Netherlands; Yale School of Medicine, US

** Phase of the isocyanate mixture during reaction

Table 2: General population characteristics, work history and isocyanate exposure of 581 workers in spray-painting companies.

	Office workers	Spray painters	Others
N	50	241	290
Gender (% male)	58	99	97
Age, AM (SD)*	40.1 (10.1)	36.9 (10.4)	39.0 (12.0)
Smoking status			
Smoker %	23.4	42.8	35.6
Stopped smoking within last year %	2.1	4.7	5.3
Former smoker %	40.4	19.2	23.2
Never smoked %	34.0	33.3	35.9
Total packyears, AM (SD)	7.7 (10.4)	8.2 (11.9)	8.5 (13.4)
Branch type			
Car body repair shop %	72	66	85
Furniture paint shop %	6	16	11
Boat/harbour equipment paint shop %	2	6	2
Airplane paint shop %	20	12	2
Work history			
Number of years worked, AM (SD)	15.6 (10.1)	16.3 (9.7)	19.2 (12.4)
Number of years in branch, AM (SD)	11.9 (10.7)	15.7 (9.6)	18.1 (12.4)
Number of years as spray-painter, AM (SD)	2.0 (4.7)	14.9 (9.6)	3.4 (7.5)
Isocyanate exposure ($\mu\text{g NCO}\cdot\text{m}^{-3}\cdot\text{hr}\cdot\text{month}^{-1}$):			
Total isocyanate Median (minimum-maximum)	0	3,682(4-66,464)	8 (0-13,473)
HDI Median (minimum-maximum)	0	27 (0.2-1,427)	0.3 (0-1,920)
Biuret Median (minimum-maximum)	0	269 (0.2-13,568)	2 (0-1,587)
Isocyanurate Median (minimum-maximum)	0	2,250 (6-87,623)	6 (0-30,0006)

* AM (SD): Arithmetic mean (standard deviation)

Table 3: Prevalence of respiratory and allergic symptoms and serological outcomes: atopy, and specific IgE and IgG sensitization against HDI.

	Office workers (50)	Spray painters (241)	Others (290)
Respiratory symptoms %			
Chronic cough	2.0	15.4 [^]	13.6 [^]
Chronic phlegm	4.0	13.3	10.8
Shortness of breath	4.0	8.8	8.4
Wheezing	12.0	29.1 [*]	22.6 [^]
Frequent wheezing (>1w)	4.0	12.5 ^{*#}	4.9
Shortness of breath during wheezing	4.0	16.2 [^]	10.5
Chest tightness	14.0	18.3	14.4
Chest tightness before start work	10.0	8.8	7.1
Clusters of symptoms %			
COPD-like symptoms	8.0	26.1 [*]	20.6 [^]
Asthma-like symptoms	14.0	33.6 [*]	28.0 [*]
Work related symptoms %			
Work related rhinitis	14.3	19.8	15.0
Work related chest tightness	2.0	8.3	4.0
Work related conjunctivitis	12.0	16.0	10.4
Positive serology %			
Atopy (Phadiatop)	44.0	33.6 ^{*§}	37.6
Specific IgE			
HDI- ImmunoCAP	0	2.1	1.0
HDI _L -HSA	0	2.9	3.5
HDI _V -HSA	0	0.4	0.7
N3300-HSA	0	2.1	1.0
N100-HSA	0	4.2	2.1
Specific IgG			
HDI- ImmunoCAP	4.0	9.5	7.2
HDI _L -HSA	32.0	50.4 [*]	41.5
HDI _V -HSA	2.0	20.0 ^{*‡}	9.3
N3300-HSA	10.0	23.3	15.1
N100-HSA	4.0	34.6 [*]	21.5 [*]

*p< 0.05, ^ p<0.10: Significantly different from 'office workers' category after adjustment for atopy, current smoking, age and gender

Adjusted for atopy, current smoking and gender

§ Adjusted for current smoking, age and gender

‡ Adjusted for for atopy, current smoking and age

Table 4: Association between respiratory symptoms and positive IgE and IgG sensitization: Prevalence Ratio (95% confidence interval) (PR (95%CI)) adjusted for age, gender, current smoking and atopy

	COPD-like symptoms	Asthma-like symptoms	Work related chest tightness	Work related rhinitis	Work related conjunctivitis
IgE					
HDI-ImmunoCAP	1.1 (0.3-3.6)	0.8 (0.2-2.5)	1.6 (0.2-10.3)	2.6 (1.4-4.8)*	1.6 (0.5-5.6)
HDI _L -HSA	1.2 (0.6-2.6)	0.9 (0.4-1.9)	1.8 (0.5-6.9)	2.0 (1.1-3.6)*	1.3 (0.4-3.7)
HDI _V -HSA	2.3 (0.9-5.5)	1.6 (0.7-3.7)	4.3 (0.8-23.1)	2.8 (1.1-6.7)*	- [‡]
N3300-HSA	1.0 (0.3-3.4)	0.7 (0.2-2.4)	1.5 (0.2-10.2)	2.1 (1.0-4.4)*	0.8 (0.1-5.3)
N100-HSA	1.6 (0.9-3.2)	1.1 (0.6-2.2)	3.7 (1.4-9.8)*	1.8 (1.0-3.4)*	1.2 (0.4-3.4)
IgG					
HDI-ImmunoCAP	1.4 (0.8-2.4)	1.2 (0.8-1.9)	0.8 (0.2-3.2)	1.5 (0.8-2.6)	1.0 (0.5-2.2)
HDI _L -HSA	0.9 (0.6-1.2)	1.0 (0.8-1.3)	1.4 (0.7-3.0)	1.4 (0.9-2.0)	1.3 (0.8-2.0)
HDI _V -HSA	0.8 (0.5-1.4)	1.2 (0.9-1.7)	1.2 (0.5-3.0)	1.2 (0.8-2.0)	1.3 (0.7-2.3)
N3300-HSA	1.0 (0.7-1.5)	0.9 (0.7-1.3)	1.0 (0.4-2.3)	1.3 (0.3-1.9)	1.1 (0.7-2.0)
N100-HSA	1.4 (1.0-1.9)*	1.1 (0.8-1.5)	1.7 (0.8-3.5)	1.5 (1.1-2.2)*	1.6 (1.0-2.5)*

* $p \leq 0.05$

[‡] Too few positives to calculate a PR

Table 5: Association between respiratory symptoms and specific IgE and IgG sensitization and exposure: Prevalence Ratio (95% confidence interval) (PR (95%CI)) for an interquartile range increase in exposure adjusted for age, gender, smoking and atopy.

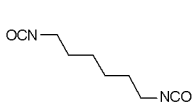
	PR (95% CI)
Symptoms	
Asthma-like symptoms	1.2 (1.0-1.5)*
COPD-like symptoms	1.3 (1.0-1.7)*
Work-related chest tightness	2.0 (1.0-3.9)*
Work-related rhinitis	1.3 (0.9-1.7)
Work-related conjunctivitis	1.5 (1.0-2.1)*
Specific IgE	
HDI- ImmunoCAP	2.2 (0.6-8.2)
HDI _L -HSA	1.4 (0.7-3.0) [#]
HDI _V -HSA	0.6 (0.1-3.9) [§]
N3300-HSA	1.8 (0.5-7.2) [§]
N100-HSA	3.0 (1.1-8.4)* [‡]
Specific IgG	
HDI- ImmunoCAP	1.2 (0.7-1.9)
HDI _L -HSA	1.2 (1.1-1.4)*
HDI _V -HSA	2.2 (1.5-3.4)*
N3300-HSA	1.6 (1.2-2.2)*
N100-HSA	2.0 (1.5-2.6)*

* $p \leq 0.05$

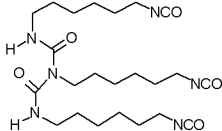
[#] Adjusted for age, smoking and atopy

[§] Adjusted for age, smoking and gender

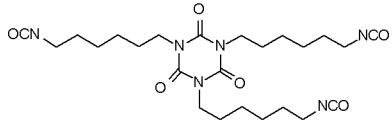
[‡] Adjusted for age, gender and atopy



Hexamethylene diisocyanate (HDI)

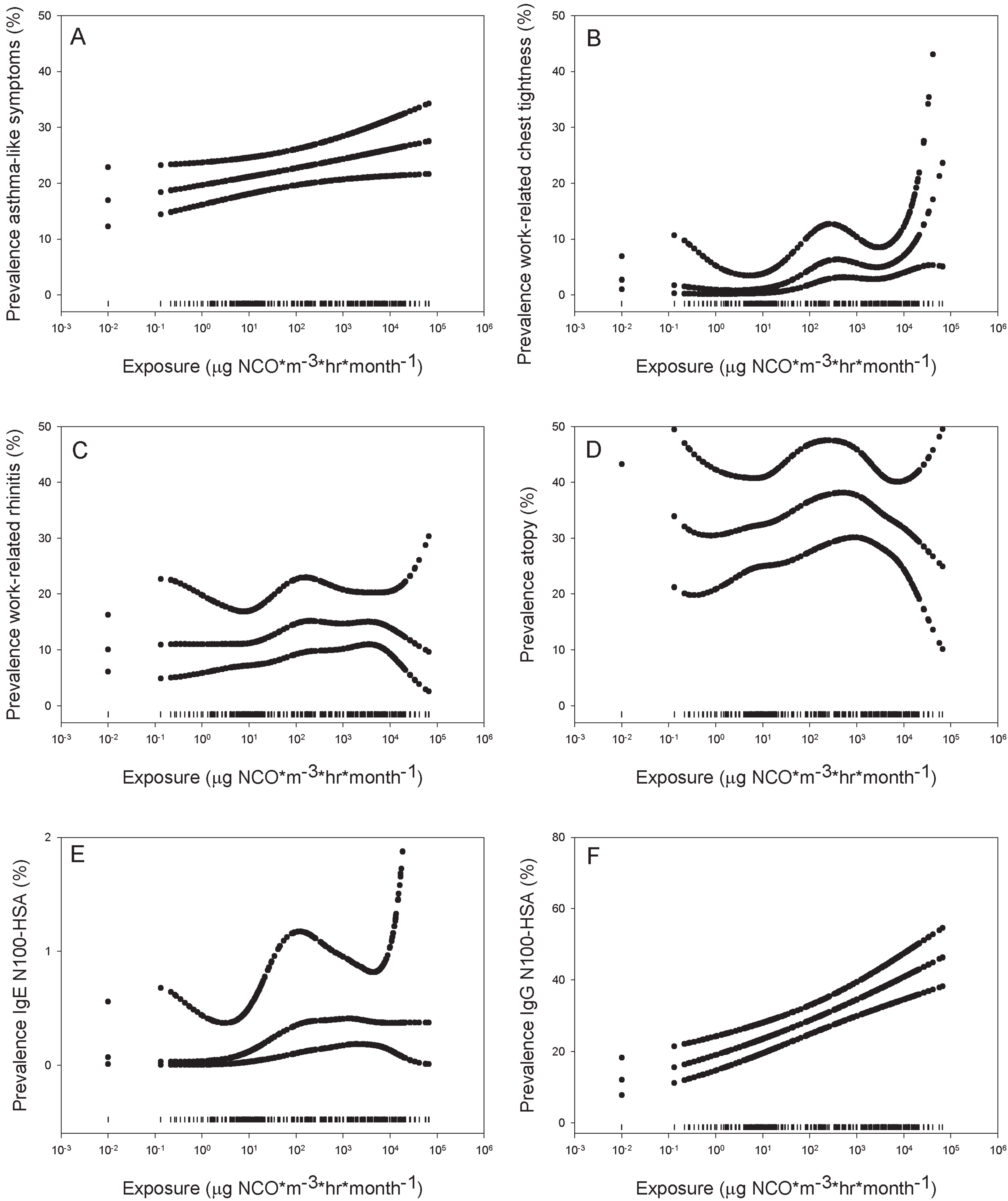


HDI Biuret



HDI Isocyanurate

Fig 2



**Respiratory symptoms, sensitization and exposure-response associations
in HDI exposed spray painters**

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Online data supplement

Material and methods

Personal exposure estimates

Detailed assessment of exposure to a range of different isocyanate compounds in the car body repair shops and industrial painting companies specialized in ships and harbor equipment has recently been published (1). Briefly, personal samples were taken using midget impingers for sampling, di-n-butylamine as a reagent and LC-MS/MS for analysis. Diisocyanates, several monoisocyanates, aminoisocyanates and oligomers of HDI and MDI were quantified. Exposure is expressed in μg reactive isocyanate group (NCO) to be able to add up exposure to different isocyanate compounds. Since a large proportion of samples was below the limit of detection (LOD) this was incorporated in the formula.

Widespread exposure to especially HDI oligomers was found with highest exposures during spray painting. Additional data from the airplane painting company indicated a similar exposure pattern to the previously reported exposures with higher exposures during especially spray painting. Separate task-based airborne exposure measurements were used for car body repair shops, industrial painting companies specializing in ships and harbor equipment and in airplanes. Estimates from car body repair shops were used for workers from companies specialized in furniture since walk-through surveys indicated that the spray-painting environment was very similar in these industries.

Cut-off values ImmunoCAP assay

For IgE antibodies a cut-off level of 0.35 kU/l was used as described by Phadia.

For IgG antibodies a cut-off level of 5 mg/l was used based on the mean + 3

*standard deviation of specific IgG levels in 20 healthy non-exposed controls.

Preparation of HDI-HSA conjugates using fluid phase reaction

HDI-HSA conjugates (HDI_L-HSA) were prepared in a liquid phase reaction essentially as described earlier (2). HDI (Sigma-Aldrich, Steinheim, Germany) was dissolved in dioxane (0.15 ml in 10 ml), and the 10 mL solution was added dropwise to 100 ml of a 1% solution of HSA (Sigma, low endotoxin, ≤0.1 ng/mg solid, St. Louis, MO, USA) in a 0.05 mol/l KCl, 0.05 mol/l Na borate buffer while stirring (3h, room temperature). Reactions were terminated by adding 2 ml of ethanolamine and after centrifugation (2100g, 1 hour), the supernatant was dialyzed against PBS (3 times, 3 hours, 4°C), dialyzed (3500 Da Spectrapor, Spectrum Laboratories Inc., Rancho Dominguez, Ca, USA) against deionized water (overnight, 4°C) and frozen. The HDI/HSA ratio of the conjugate (25-30) was based on free NH₂ groups, measured with TNBS (3).

Preparation of HDI-HSA conjugates using vapor phase reaction

Vapor-induced HDI-HSA (HDI_V-HSA) conjugates were prepared as previously described (3). HDI (Sigma; St. Louis, MO) vapor in the concentration range of 20-200 ppb was passively generated in a closed circuit system and monitored with an Autostep monitor (GMD, Pittsburgh, Pa, USA). A 0.5% solution of low

endotoxin HSA (Sigma) in tissue culture grade PBS (GibcoBRL; Grand Island, NY) was exposed overnight in open 60-mm petri dishes (Becton Dickinson, Franklin lakes, NJ, USA). The vapor exposure unit was sterilized with 70% ethanol and HSA solutions were sterile (0.2 μ m) filtered (Corning; Corning, NY) before and after exposure, aliquoted and stored at -20°C . The HDI/HSA ratio of the conjugate (9-10) was based on free NH_2 groups, measured with TNBS (3).

Preparation of oligomeric HDI-HSA conjugates

HSA-conjugates were also prepared with Desmodur N-100 and N-3300 (Bayer; Pittsburgh, PA), commercially used biuret and isocyanurate oligomers of HDI. HDI oligomers were mixed 1:1 with acetone (J.T.Baker; Phillipsburg, NJ) then with 0.5 % HSA in tissue culture-grade PBS to achieve a final concentration of 0.1% (v/v) HDI oligomer. Reactants were mixed end-over-end for 2 hr at 37°C , 0.2 μ m filtered, dialyzed against tissue-culture grade PBS 4X, using Spectra/Por cellulose membranes sterilized by irradiation (Spectrum Labs, Inc.; Los Angeles, CA), and refiltered (0.2 μ m). HDI/HSA ratios of the conjugates (8-8.5 for N100-HSA; 5.5-6 for N3300-HSA) were based on free NH_2 groups, measured with TNBS (3).

EIA

Microtiter plates (Greiner, Frickenhausen, Germany) were coated overnight at 4°C with conjugates (HDI_L -HSA, HDI_V -HSA, N100-HSA, N3300-HSA) and unmodified, non-treated HSA in PBS-azide, at 10 $\mu\text{g/ml}$ (IgE) and 20 $\mu\text{g/ml}$ (IgG).

Volumes of 0.1 ml/well were used in this step and all subsequent steps. Serum samples diluted 1/10 (IgE) or 1/100 (IgG) in PBS-Tween with 0.2% gelatin (PBTG) were added and incubated for 2 h at 37°C. In IgE analyses antibody binding was detected with mouse monoclonal anti-human IgE (CLB, Sanquin Reagents, Amsterdam, The Netherlands) 1:16000 (1h 37°C) followed by rabbit anti-mouse/biotin (DAKO, DakoCytomation Denmark, Glostrup, Denmark) 1:5000, and avidin/HrP (DAKO) 1:2000 (4). In IgG analyses antibodies were detected with peroxidase-labelled rabbit anti-human IgG/PO (DAKO) 1:2000 (1h 37°C). OPD was used as the substrate (30 min 20°C), the reaction was stopped with 2M HCl and the optical density (OD) was read at 492 nm. For each serum, the OD measured in the HSA-coated well was subtracted from the OD values measured in each of the conjugate-coated wells.

In each EIA plate a complete row of microwells was included as 'reagent blanks' or 'no serum controls'. The OD values for test sera were not further adjusted for non-specific binding of the detection reagent since these no serum control OD values showed no significant differences between wells coated with HSA alone or with any of the isocyanate-HSA conjugates.

Cut-off values EIA

All sera with a HSA-corrected OD value for an IgE reaction of > 0.05 for any of the conjugates were retested on the whole panel. A cut-off value for HSA corrected OD values of 0.1 was chosen because of optimal concordance (analyzed by kappa values [κ]) for repeated measurements ($\kappa \geq 0.8$). When using

an OD ratio ($OD(\text{conjugate})/OD(\text{HSA}) > 2$) as a cut-off level similar positive samples and associations were found (data not shown).

For IgG analyses a random sample of 145 samples was reanalyzed to determine cut-off levels. A cut-off value for HSA corrected OD values of 0.3 was chosen because of optimal concordance for repeated measurements. At this cut-off level κ was around 0.7 for HDI_L-HSA and HDI_V-HSA and around 0.9 for N3300-HSA and N100-HSA. Sera with strong reactions with HSA ($OD > 0.3$) were retested on the whole panel, and if in the second test the OD on HSA was < 0.3 , the results of the second test were used. Statistical analyses were performed with and without samples with a repeatedly strong reaction ($OD > 0.3$) in the HSA-coated well ($n=22$).

Physiological testing

At the time of selection of the subset of the population in which physiological testing was carried out results for specific IgE and IgG against diisocyanates measured by ImmunoCAP assay (Phadia, Uppsala, Sweden) were available. All workers from companies with at least one worker with detectable specific antibodies were invited to participate. The company participation rate was 90% and worker participation rate was 66%. Companies were visited between January and June 2006. All tests were carried out on a working day in a secluded room at the company or in an especially equipped van at the company premises.

References

- E1. Pronk A, Tielemans E, Skarping G, Bobeldijk I, van Hemmen J, Heederik D, Preller L. Inhalation exposure to isocyanates of car body repair shop workers and industrial spray painters. *Ann Occup Hyg* 2006;50:1-14.
- E2. Dewair MA, Baur X. Studies on antigens useful for detection of IgE antibodies in isocyanate-sensitized workers. *J Clin Chem Clin Biochem* 1982;20:337-340.
- E3. Wisnewski AV, Stowe MH, Cartier A, Liu Q, Liu J, Chen L, Redlich CA. Isocyanate vapor-induced antigenicity of human albumin. *J Allergy Clin Immunol* 2004;113:1178-1184.
- E4. Doekes G, Douwes J, Wouters I, de Wind S, Houba R, Hollander A. Enzyme immunoassays for total and allergen specific IgE in population studies. *Occup Environ Med* 1996;53:63-70.