Isocyanate exposure and respiratory health effects in the spray painting industry

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Blootstelling aan isocyanaten en respiratoire gezondheidseffecten in verfspuiters
(met een samenvatting in het Nederlands)

Proefschrift

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Contents

Chapter 1

General introduction

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General introduction

Isocyanates are a group of compounds characterised by highly reactive N=C=O groups and have been associated with a range of respiratory health effects. These include asthma (1), reactive airways dysfunction syndrome (RADS) or irritant-induced asthma (2, 3), accelerated lung function decline (4, 5), hypersensitivity pneumonitis (6-8) and rhinitis (9, 10). Of these conditions asthma is the most common syndrome linked to isocyanates (1). Occupational asthma (OA) is a 'disease characterised by variable airflow limitation and/or airway hyperresponsiveness due to causes and conditions attributable to a particular occupational environment and not to stimuli outside the workplace' (11). A detailed analysis of the combined evidence on the association between occupational exposure and asthma by a task force of the American Thoracic Society has indicated that around 15% of adult asthma cases are attributable to occupational exposures (12, 13). In industrialised countries, isocyanates are one of the most commonly identified causes of occupational asthma (up to ~30% of registered cases), along with flour dust and animal epithelia (14, 15).

Occupational isocyanate exposure and exposure assessment

Isocyanates are highly reactive because of their N=C=O groups and are used in various industrial processes. Polyurethane (PU) polymers are formed by the reaction of diisocyanates, containing two NCO groups, and polyols. PU polymers have a wide variety of applications in the manufacture of flexible and rigid foams, elastomers, adhesives and surface coatings (16). Figure 1.1 shows some common diisocyanate monomers: the aromatic toluene diisocyanate (TDI) and methylene diphenyl diisocyanate (MDI) and aliphatic hexamethylene diisocyanate (HDI) and isophorone diisocyanate (IPDI). Since the introduction of diisocyanates in the 1930's the number of industrial applications of isocyanates has increased dramatically. This has resulted in a continuous growth in their production and further growth is foreseen (1). Currently, over 3.5 million workers in the European Union are estimated by their representative trade organizations to be working with isocyanates (17, 18).
To reduce exposure levels, HDI and MDI have gradually been replaced by oligomeric isocyanates with a lower vapor pressure (19). Nowadays, so called 'technical isocyanate mixtures' are frequently used, which consist of mostly isocyanate oligomers and small fractions of the corresponding monomer. In addition to isocyanates present in these mixtures, exposure to isocyanate intermediates formed in application processes may occur. Recent attention has focused on thermal degradation products, including mono-isocyanates and amino-isocyanates that may be formed when PU products are heated (20, 21).

Since the isocyanate manufacturing process is highly controlled, exposure to isocyanates in large manufacturing facilities is generally low (22). Higher occupational exposures may occur during the application of isocyanates by end-users, e.g. the application of PU-foams as insulation material. Depending on the application process, airborne isocyanates may occur in the vapor or aerosol phase.

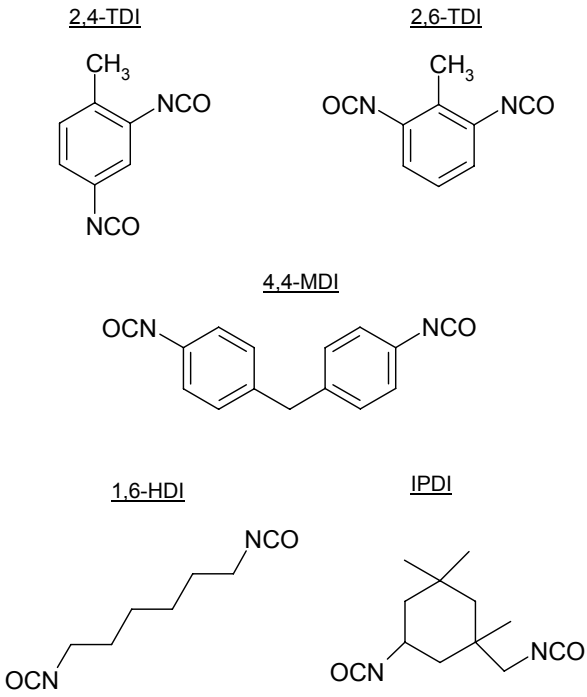

Figure 1.1: Chemical structures of some commonly used diisocyanates: 2,4- and 2,6-toluene diisocyanate (TDI), 4,4-methylene diphenyl diisocyanate (MDI), 1,6-hexamethylene diisocyanate (HDI) and isophorone diisocyanate (IPDI).

Sampling and analysis of isocyanates in the air is an active area of research. The need to immediately derivatize the reactive isocyanate compounds and to collect both aerosols and vapor efficiently greatly complicates sampling procedures (23, 24). Subsequent chemical analysis is complicated by the wide range of different isocyanate compounds present in one sample, for which analytical standards may not be available (19). Over recent years, methods for measurement of diisocyanate monomers in vapor phase only (24) have been replaced by various methods for a wider variety of isocyanates in different phases (19). In the main industrial areas in the world, including the United States, Canada, United

Kingdom and Sweden, local methods have been developed. These methods often do not include the same isocyanate compounds and results are expressed using variable units. This complicates the comparison of results between methods and surveys. The different methods have their own advantages and limitations with respect to compounds analyzed and sampling procedures employed in different exposure settings.

Although inhalation exposure is usually considered the most important exposure route, the relative contribution of dermal exposure to total internal dose has increased with the use of less volatile isocyanate oligomers. There is limited evidence that dermal contact may result in respiratory sensitization (25) as well as respiratory disease aggravation in humans (26). Animal studies have also shown that dermal exposure to isocyanates can result in respiratory sensitization (27-29). The presence of contaminated surfaces and dermal exposure to isocyanates in an industrial setting has been indicated by a qualitative study (30). However, valid methods for quantitative assessment of dermal exposure to isocyanates as well as the role of dermal exposure in disease induction remain to be established.

Isocyanate asthma and specific sensitization

Occupational asthma caused by isocyanates was first reported in 1951 (31). Mechanisms which explain the induction and development of isocyanate asthma are unclear. Many features of isocyanate asthma point towards IgE-mediated sensitization as in type I occupational allergy. First symptoms of isocyanate asthma generally occur after a latency period of months to years of exposure and in only a small proportion of the exposed population. Once asthma has developed, symptoms can arise at relatively low exposure levels (1). However, until now no valid markers of isocyanate-specific sensitization, like specific IgE antibodies or skin prick tests, have been established. Therefore, specific inhalation challenge (SIC) is regarded as the gold standard for the diagnosis and monitoring of isocyanate asthma (1). This procedure is technically and economically demanding and can be falsely negative when challenge material does not reflect the complex exposure in the work environment.
Like other low molecular weight allergens isocyanates presumably act as haptens. They are conjugated to human proteins in vivo via their highly reactive NCO-groups (32). Because of uncertainty regarding the predominant structures of isocyanate-protein conjugates causing sensitization in the exposed worker, the assessment of specific sensitization is difficult (1). Isocyanate-specific IgE antibodies are generally demonstrated in less than 20% of asthma cases confirmed by SIC (33-37). It has been suggested that specific IgG antibodies are more predictive of isocyanate asthma than specific IgE (33, 34). Other studies could not demonstrate such associations (36, 38, 39) and it is a matter of debate whether IgG antibodies only reflect exposure or also disease. Studies measuring isocyanate-specific antibodies are mostly limited to case-control

studies or small groups of exposed workers for whom no data on exposure is available. Methods used in these studies for the assessment of specific sensitization are diverse and differ with respect to the use of isocyanate-protein conjugates as test-antigens. The commercially available ImmunoCAP® method has been used (40, 41), but many research groups have employed enzyme or radio-immunoassays with 'in house' prepared isocyanate-protein conjugates (33-37). Usually, these have been prepared by liquid phase reactions of diisocyanate monomers and human serum albumin (HSA) solutions. It has been argued that conjugates prepared by incubation of an HSA solution with diisocyanate vapor may better reflect conjugation reactions in the human airways (42, 43). In addition, conjugates produced with diisocyanate oligomers may be more representative for some of the actual workplace situations (44, 45). It is a matter of debate to what extent specific antibody production may not be detected because of the use of inappropriate conjugates.
Alternatively, isocyanate asthma may be mediated by IgE independent or non-immunologic mechanisms. Evidence of cellular immune and inflammatory processes different from the typical IgE-mediated response has been reported (1, 46-48).

Exposure-response associations

Despite a vast range of studies on isocyanate related-disease, investigations that incorporate a quantitative exposure assessment component are scarce. Exposure-response relationships for isocyanate monomers have been studied relatively well in large TDI manufactures or foam production units, where exposure to monomers is predominant. The majority of these studies focused mainly on work-related lung function decline as the major health effect, which was found in some studies (4, 5) but not in others (49-52). In the majority of population studies where asthma and monomer exposure were both investigated, only mean or maximum exposure levels were reported (39, 49-54). Often exposure data in these studies consisted of a relatively small measurement series or existing monitoring data that was previously collected. Thus far, few studies have considered the issue of quantitative exposure-response relationships in isocyanate asthma (55). Two studies demonstrated that higher isocyanate levels occurred in companies at which there were workers with a successful claim for occupational asthma compared to control companies (56) or in doctor diagnosed asthma cases compared to matched controls from the same company (55). Despite the widespread use of isocyanate oligomers, only one small study has incorporated isocyanate oligomer exposure assessment. This study demonstrated a relation between peak exposure and reduced lung function but only in smoking car painters (57). The lack of epidemiological studies incorporating accurate quantitative oligomer exposure assessment is most likely explained by the complexity of the exposure assessment. Additionally, isocyanate oligomers are frequently used in end-user activities, which are difficult to study (58).

The spray painting industry

The spray-painting industry is an example of an end-user activity in which exposure to a range of isocyanates may occur. Oligomers of HDI present in hardeners of PU lacquers are the main source of exposure. In addition, TDI and MDI may be present in kits, glues, pastes and insulating materials used in this industry. Moreover, a variety of thermal degradation products may be formed during heating of PU, e.g. when using a heater for curing paint or during welding. Several studies have measured isocyanate exposure in spray painters (57, 59-64), focussing mostly on HDI, HDI oligomers or total NCO.
Spray painters are estimated to be the largest working population with high isocyanate exposure in The Netherlands (22). However, due to the absence of accurate disease registries in The Netherlands no reliable information on the magnitude of health risks caused by isocyanates is available. High mean annual incidence rates of (compensable) occupational asthma among spray painters, between 300 and >2000 per million workers, have been reported in other European countries (15, 65, 66). In addition, decreased lung function parameters (57, 67) and high asthma symptom prevalence have been found in surveys (10, 68-72). Yet, with the exception of one small study (57) associations between isocyanate exposure levels and respiratory effects have not been investigated in this industry.

Aims and outline of this thesis

Many elements of the association between isocyanate exposure and respiratory health effects, including relevant host factors, biologically-relevant exposure proxies, disease mechanisms and respiratory health effects are unclear (Figure 1.2).

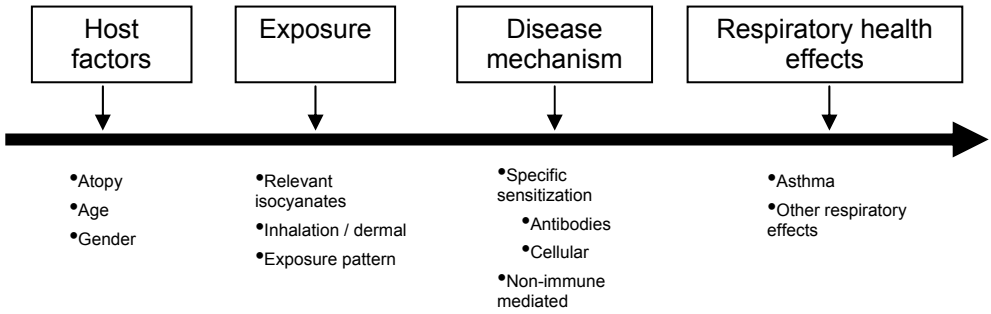

Figure 1.2: Schematic representation of various elements relevant to the association between isocyanate exposure and respiratory health effects.

The primary objective of this thesis was to establish exposure-response relationships between isocyanate exposure and respiratory health end-points and specific sensitization in spray painters. Specific aims were: 1) To identify relevant isocyanate compounds, exposure sources, exposure routes and possible determinants of exposure for the identification of control measures and ultimately to establish personal exposure estimates for exposure-response modeling, 2) To study the prevalence of respiratory health effects and their association with exposure, and 3) To study the prevalence of specific sensitization and its association with exposure and health effects.
Chapter 2 comprises isocyanate exposure assessment. Chapter 2.1 describes an extensive inhalation exposure study in car body repair shops and industrial paint shops in The Netherlands. Task-based inhalation exposure to 23 isocyanate compounds was analyzed using a state-of-the-art method. Chapter 2.2 explores dermal exposure and biomonitoring. A method for dermal exposure assessment was developed and used to investigate dermal exposure. In addition the use of biomonitoring for exposure assessment was explored. Exposure-response studies are investigated in chapter 3. Chapter 3.1 describes respiratory symptoms and specific IgE and IgG serology in a population of spray painters. Personal exposure estimates obtained by combining task-based exposure estimates and time activity information were used to investigate exposure-response relationships. In a subset of this population more objective respiratory parameters, like spirometry and bronchial hyper-responsiveness and their association with exposure and respiratory symptoms were investigated, and these are reported in Chapter 3.2. Chapter 4 focuses on immunological tests for specific sensitization. Specific IgE and IgG reactivity was assessed by immunoassays using different HDI-HSA conjugates and by a commercially available immunoassay; ImmunoCAP. In Chapter 4.1 these assays were compared and cross-reactivity of different conjugates is investigated. In addition, the use of specific antibody reactivity as measured by the various assays is evaluated as a marker of exposure or respiratory health end-points. In chapter 4.2 the use of a new cellular test based on the production of monocyte chemotactic protein-1 by peripheral blood mononuclear cells was explored. Chapter 5 evaluates the main findings presented in this thesis and discusses limitations and implications.

References

1. 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.
2. Perfetti L, Brame B, Ferrari M, Moscato G. Occupational asthma (OA) with sensitization to diphenylmethane diisocyanate (MDI) presenting at the onset like a reactive airways dysfunction syndrome (RADS). Am J Ind Med 2003;44(3):325-8.
3. Leroyer C, Perfetti L, Cartier A, Malo JL. Can reactive airways dysfunction syndrome (RADS) transform into occupational asthma due to "sensitisation" to isocyanates? Thorax 1998;53(2):152-3.

4. Diem JE, Jones RN, Hendrick DJ, Glindmeyer HW, Dharmarajan V, Butcher BT, et al. Five-year longitudinal study of workers employed in a new toluene diisocyanate manufacturing plant. Am Rev Respir Dis 1982;126(3):420-8.
5. Wegman DH, Musk AW, Main DM, Pagnotto LD. Accelerated loss of FEV- in polyurethane production workers: a four-year prospective study. Am J Ind Med 1982;3(2):209-15.
6. Vandenplas O, Malo JL, Dugas M, Cartier A, Desjardins A, Levesque J, et al. Hypersensitivity pneumonitis-like reaction among workers exposed to diphenylmethane [correction to piphenylmethane] diisocyanate (MDI). Am Rev Respir Dis 1993;147(2):338-46.
7. Nakashima K, Takeshita T, Morimoto K. Occupational hypersensitivity pneumonitis due to isocyanates: mechanisms of action and case reports in Japan. Ind Health 2001;39(3):269-79.
8. Baur X. Hypersensitivity pneumonitis (extrinsic allergic alveolitis) induced by isocyanates. J Allergy Clin Immunol 1995;95(5 Pt 1):1004-10.
9. Littorin M, Welinder H, Skarping G, Dalene M, Skerfving S. Exposure and nasal inflammation in workers heating polyurethane. Int Arch Occup Environ Health 2002;75(7):468-74.
10. 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(7):582-8.
11. Bernstein IL, Bernstein DI, Chan-Yeung M, Malo JL. Definition and classification of asthma in the workplace. In: Bernstein IL, Chan-Yeung M, Malo JL, Bernstein DI, editors. Asthma in the workplace. New York: Taylor & Francis Group; 2006.
12. Balmes J, Becklake M, Blanc P, Henneberger P, Kreiss K, Mapp C, et al. American Thoracic Society Statement: Occupational contribution to the burden of airway disease. Am J Respir Crit Care Med 2003;167(5):787-97.
13. Blanc PD, Toren K. How much adult asthma can be attributed to occupational factors? Am J Med 1999;107(6):580-7.
14. Latza U, Baur X. Occupational obstructive airway diseases in Germany: Frequency and causes in an international comparison. Am J Ind Med 2005;48(2):144-52.
15. 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(5):451-8.
16. Allport DC, Gilbert DS, Outterside SM. MDI & TDI Safety, Health and the Environment. Chichester: John Wiley & Sons; 2003.
17. www.alipa.org
18. www.isopa.org
19. Streicher RP, Reh CM, Key-Schwartz RJ, Schlecht PC, Cassinelli ME, O'Connor PF. Determination of airborne isocyanate exposure: considerations in method selection. Am Ind Hyg Assoc J 2000;61(4):544-56.
20. Karlsson D, Dahlin J, Skarping G, Dalene M. Determination of isocyanates, aminoisocyanates and amines in air formed during the thermal degradation of polyurethane. J Environ Monit 2002;4(2):216-22.
21. Henriks-Eckerman ML, Valimaa J, Rosenberg C, Peltonen K, Engstrom K. Exposure to airborne isocyanates and other thermal degradation products at polyurethane-processing workplaces. J Environ Monit 2002;4(5):717-21.
22. Snippe RJ, Gijsbers JHJ, Drooge HL, Preller EA. Chemische allergenen in Nederland. Een onderzoek naar de blootstelling aan diisocyanaten en zuuranhydriden in Nederland. 's-Gravenhage: Ministerie van Sociale Zaken en Werkgelegenheid; february 2001.
23. Molander P, Levin JO, Ostin A, Rosenberg C, Henriks-Eckerman ML, Brodsgaard S, et al. Harmonized Nordic strategies for isocyanate monitoring in workroom atmospheres. Journal of Environmental Monitoring 2002;4(5):685-687.
24. Streicher RP, Kennedy ER, Lorberau CD. Strategies for the simultaneous collection of vapours and aerosols with emphasis on isocyanate sampling. Analyst 1994;119(1):89-97.
25. Kimber I, Dearman RJ. Chemical respiratory allergy: role of IgE antibody and relevance of route of exposure. Toxicology 2002;181-182:311-5.
26. 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(4):1183-93.
27. Scheerens H, Buckley TL, Muis TL, Garssen J, Dormans J, Nijkamp FP, et al. Long-term topical exposure to toluene diisocyanate in mice leads to antibody production and in vivo airway hyperresponsiveness three hours after intranasal challenge. Am J Respir Crit Care Med 1999;159(4 Pt 1):1074-80.

28. Rattray NJ, Botham PA, Hext PM, Woodcock DR, Fielding I, Dearman RJ, et al. Induction of respiratory hypersensitivity to diphenylmethane-4,4'-diisocyanate (MDI) in guinea pigs. Influence of route of exposure. Toxicology 1994;88(1-3):15-30.
29. Karol MH, Hauth BA, Riley EJ, Magreni CM. Dermal contact with toluene diisocyanate (TDI) produces respiratory tract hypersensitivity in guinea pigs. Toxicol Appl Pharmacol 1981;58(2):221-30.
30. Liu Y, Sparer J, Woskie SR, Cullen MR, Chung JS, Holm CT, et al. Qualitative assessment of isocyanate skin exposure in auto body shops: a pilot study. Am J Ind Med 2000;37(3):265-74.
31. Fuchs S, Valade P. Clinical and experimental study of some cases of poisoning by desmodur T (1-2-4 and 1-2-6 diisocyanates of toluene). Arch Mal Prof 1951;12(2):191-196.
32. Wisnewski AV, Redlich CA. Recent developments in diisocyanate asthma. Curr Opin Allergy Clin Immunol 2001;1(2):169-75.
33. Park HS, Kim HY, Nahm DH, Son JW, Kim YY. Specific IgG, but not specific IgE, antibodies to toluene diisocyanate-human serum albumin conjugate are associated with toluene diisocyanate bronchoprovocation test results. J Allergy Clin Immunol 1999;104(4 Pt 1):847-51.
34. Cartier A, Grammer L, Malo JL, Lagier F, Ghezzo H, Harris K, et al. Specific serum antibodies against isocyanates: association with occupational asthma. J Allergy Clin Immunol 1989;84(4 Pt 1):507-14.
35. Baur X, Dewair M, Fruhmann G. Detection of immunologically sensitized isocyanate workers by RAST and intracutaneous skin tests. J Allergy Clin Immunol 1984;73(5 Pt 1):610-8.
36. Kim H, Kim YD, Choi J. Seroimmunological characteristics of Korean workers exposed to toluene diisocyanate. Environ Res 1997;75(1):1-6.
37. Butcher BT, O'Neil CE, Reed MA, Salvaggio JE. Radioallergosorbent testing of toluene diisocyanate-reactive individuals using p-tolyl isocyanate antigen. J Allergy Clin Immunol 1980;66(3):213-6.
38. Paggiaro PL, Filieri M, Loi AM, Roselli MG, Cantalupi R, Parlanti A, et al. Absence of IgG antibodies to TDI-HSA in a radioimmunological study. Clin Allergy 1983;13(1):75-9.
39. 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(4):627-33.
40. Pezzini A, Riviera A, Paggiaro P, Spiazzi A, Gerosa F, Filieri M, et al. Specific IgE antibodies in twenty-eight workers with diisocyanate-induced bronchial asthma. Clin Allergy 1984;14(5):453-61.
41. Keskinen H, Tupasela O, Tiikkainen U, Nordman H. Experiences of specific IgE in asthma due to diisocyanates. Clin Allergy 1988;18(6):597-604.
42. Ye YM, Kim CW, Kim HR, Kim HM, Suh CH, Nahm DH, et al. Biophysical determinants of toluene diisocyanate antigenicity associated with exposure and asthma. J Allergy Clin Immunol 2006;118(4):885-91.
43. Wisnewski AV, Stowe MH, Cartier A, Liu Q, Liu J, Chen L, et al. Isocyanate vapor-induced antigenicity of human albumin. J Allergy Clin Immunol 2004;113(6):1178-84.
44. Welinder H, Nielsen J, Bensryd I, Skerfving S. IgG antibodies against polyisocyanates in car painters. Clin Allergy 1988;18(1):85-93.
45. Aul DJ, Bhaumik A, Kennedy AL, Brown WE, Lesage J, Malo JL. Specific IgG response to monomeric and polymeric diphenylmethane diisocyanate conjugates in subjects with respiratory reactions to isocyanates. J Allergy Clin Immunol 1999;103(5 Pt 1):749-55.
46. Jones MG, Floyd A, Nouri-Aria KT, Jacobson MR, Durham SR, Taylor AN, et al. Is occupational asthma to diisocyanates a non-IgE-mediated disease? J Allergy Clin Immunol 2006;117(3):663-9.
47. Lummus ZL, Alam R, Bernstein JA, Bernstein DI. Diisocyanate antigen-enhanced production of monocyte chemoattractant protein-1, IL-8, and tumor necrosis factor-alpha by peripheral mononuclear cells of workers with occupational asthma. J Allergy Clin Immunol 1998;102(2):265-74.
48. Raulf-Heimsoth M, Baur X. Pathomechanisms and pathophysiology of isocyanate-induced diseases--summary of present knowledge. Am J Ind Med 1998;34(2):137-43.
49. 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(10):890-7.
50. Ott MG, Klees JE, Poche SL. Respiratory health surveillance in a toluene di-isocyanate production unit, 1967-97: clinical observations and lung function analyses. Occup Environ Med 2000;57(1):43-52.

51. 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(3):169-79.
52. Jones RN, Rando RJ, Glindmeyer HW, Foster TA, Hughes JM, O'Neil CE, et al. Abnormal lung function in polyurethane foam producers. Weak relationship to toluene diisocyanate exposures. Am Rev Respir Dis 1992;146(4):871-7.
53. White WG, Morris MJ, Sugden E, Zapata E. Isocyanate-induced asthma in a car factory. Lancet 1980;1(8171):756-60.
54. Bernstein DI, Korbee L, Stauder T, Bernstein JA, Scinto J, Herd ZL, et al. 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(3):387-96.
55. Meredith SK, Bugler J, Clark RL. Isocyanate exposure and occupational asthma: a case-referent study. Occup Environ Med 2000;57(12):830-6.
56. 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(5):517-21.
57. 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(3):299-310.
58. Bello D, Woskie SR, Streicher RP, Liu Y, Stowe MH, Eisen EA, et al. Polyisocyanates in occupational environments: a critical review of exposure limits and metrics. Am J Ind Med 2004;46(5):480-91.
59. Rosenberg C, Tuomi T. Airborne isocyanates in polyurethane spray painting: determination and respirator efficiency. Am Ind Hyg Assoc J 1984;45(2):117-21.
60. Woskie SR, Sparer J, Gore RJ, Stowe M, Bello D, Liu Y, et al. Determinants of isocyanate exposures in auto body repair and refinishing shops. Ann Occup Hyg 2004;48(5):393-403.
61. Carlton GN, England EC. Exposures to 1,6-hexamethylene diisocyanate during polyurethane spray painting in the U.S. Air Force. Appl Occup Environ Hyg 2000;15(9):705-12.
62. Maitre A, Leplay A, Perdrix A, Ohl G, Boinay P, Romazini S, et al. Comparison between solid sampler and impinger for evaluation of occupational exposure to 1,6-hexamethylene diisocyanate polyisocyanates during spray painting. Am Ind Hyg Assoc J 1996;57(2):153-160.
63. Pisaniello DL, Muriale L. The use of isocyanate paints in auto refinishing--a survey of isocyanate exposures and related work practices in South Australia. Ann Occup Hyg 1989;33(4):563-72.
64. Myer HE, O'Block ST, Dharmarajan V. A survey of airborne HDI, HDI-based polyisocyanate and solvent concentrations in the manufacture and application of polyurethane coatings. Am Ind Hyg Assoc J 1993;54(11):663-70.
65. McDonald JC, Keynes HL, Meredith SK. Reported incidence of occupational asthma in the United Kingdom, 1989-97. Occup Environ Med 2000;57(12):823-9.
66. Ameille J, Pauli G, Calastreng-Crinquand A, Vervloet D, Iwatsubo Y, Popin E, et al. Reported incidence of occupational asthma in France, 1996-99: the ONAP programme. Occup Environ Med 2003;60(2):136-41.
67. 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(2):104-11.
68. Talini D, Monteverdi A, Benvenuti A, Petrozzino M, Di Pede F, Lemmi M, et al. Asthma-like symptoms, atopy, and bronchial responsiveness in furniture workers. Occup Environ Med 1998;55(11):786-91.
69. 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(6):503-10.
70. Cullen MR, Redlich CA, Beckett WS, Weltmann B, Sparer J, Jackson G, et al. 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(3):197-204.
71. Eifan AO, Derman O, Kanbur N, Sekerel BE, Kutluk T. Occupational asthma in apprentice adolescent car painters. Pediatr Allergy Immunol 2005;16(8):662-8.
72. 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(11):1096-100.

Chapter 2

Isocyanate exposure

Chapter 2.1

Inhalation exposure to isocyanates of car body repair shop workers and industrial spray painters

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Abstract

As part of a large-scale epidemiological study, occupational isocyanate exposure was assessed in spray-painting environments. The aim was to assess which compounds contribute to isocyanate exposure in car body repair shops and industrial painting companies and to identify tasks with high risk of isocyanate exposure. Mainly personal task-based samples (n=566) were collected from 24 car body repair shops and 5 industrial painting companies using impingers with DBA in toluene. Samples were analyzed by LC-MS for isocyanate monomers, oligomers and products of thermal degradation.
From the 23 analyzed compounds 20 were detected. Exploratory factor analysis resulted in a HDI, TDI and MDI factor with the thermal degradation products divided over the TDI and MDI factors. The HDI factor mainly consisted of HDI oligomers and was dominant in frequency and exposure levels in both industries. Spray painting of PU lacquers resulted in the highest exposures for the HDI factor (<LOD - 2643 $\mu g/m^3$ NCO), with no significant difference between the industries. Exposure variability during PU spray painting was large with a variability over time of ${}_{ww}S^2=9.1$ compared to between worker variability of ${}_{bw}S^2=1.6$. Lower level exposure to the HDI factor was found during other painting related tasks and even tasks without direct exposure to paint. Exposure to the TDI factor was found more regularly in car body repair shops than in industrial painting companies. Exposure levels were low (<LOD – 5 $\mu g/m^3$ NCO) compared to the HDI factor and no clear contrast in levels between the tasks was observed. Exposure to the MDI factor was found incidentally during spraying and welding in car body repair shops (<LOD-0.5 $\mu g/m^3$ NCO).
The results indicate that paint is the most important source and major contributor of isocyanate exposure in both industries with highest exposures during PU spraying. However, since respiratory protection is less extensively used during other tasks, lower level exposure during these other tasks may significantly contribute to the internal dose.

Introduction

Isocyanates are industrial chemicals containing highly unsaturated N=C=O groups which are used in polyurethane (PU) products such as foams, paints, lacquers, inks, insulating materials, varnishes, rubber modifiers, and bonding and vulcanizing agents (1). In Western countries these low molecular weight allergens are one of the most commonly identified causes of occupational asthma (2-4) with the highest risk among spray painters (5). Despite their widespread use and serious consequences a lack of insight in the relationship between exposure and disease still exists due to uncertainties on health endpoints, underlying mechanisms and biologically relevant exposure (4, 6). Exposure assessment is complicated by the variety of isocyanate compounds present in different occupational settings (7). Moreover peak exposure (8) and dermal exposure (9, 10) may play an important role in the development or aggravation of disease.

In The Netherlands, data on the magnitude of health risks are lacking due to the absence of occupational disease registries. A large-scale epidemiological study is being set up to evaluate isocyanate exposure, health effects and exposure-response relationships. This study mainly focuses on end users of PU lacquers in car body repair shops and industrial painting companies. Within these industries exposure to a range of isocyanates with variable physical and chemical properties may occur. Hardeners of PU lacquers used generally contain hexamethylene diisocyanate (HDI) based compounds and isophorone diisocyanate (IPDI). To reduce the vapor hazards associated with monomeric HDI, the HDI monomer has mostly been replaced by its oligomers. Other isocyanate containing intermediates can be formed during application (7). In addition toluene diisocyanate (TDI) and methylene bisphenyl diisocyanate (MDI) can be present in kits, glues, pastes and insulating materials used in these industries. Moreover, a variety of thermal degradation products (mono isocyanates and amino isocyanates) can be formed when cured PU products are heated (11-13), e.g. by welding and possibly by grinding and sanding of spray painted parts.

So far, several studies have measured isocyanate exposure in spray painters (14-20). However, information on the occurrence of a wide range of isocyanate compounds is not available since most studies focus on exposure to either HDI, oligomers of HDI or total NCO. Additionally, in most studies only exposure during spray painting is sampled. In the present paper we differentiate between task-based exposures to a range of isocyanate compounds in car body repair shops and industrial painting companies. The aim of this study is to assess which compounds, including monomers, oligomers and thermal degradation products, contribute to isocyanate exposure in these industries, to identify tasks with high risk of isocyanate exposure, and to describe control measures currently in use in The Netherlands.

Methods

Population and sampling strategy
Exposure assessment was carried out as part of an epidemiological study on isocyanate exposure and health effects in car body repair shop workers and industrial spray painters in The Netherlands. The study currently involves 520 workers from some 100 companies. Car body repair shops were enrolled using databases from the branch organization (written information on study design and a request to participate in exposure as well as health assessment part of the study was sent to 763 companies; positive response rate: 11%) and the Chamber of Commerce (written information and request to 93 companies; positive response rate: 9%). Industrial painting companies were contacted through shipyards resulting in companies specialized in painting of mainly ships and harbour equipment (telephone contact with 16 companies, positive response rate: 69%). Air measurements were performed in a random selection of these companies.
Mainly task-based personal air samples were collected. Prior to the selection of tasks, a thorough walk through survey was carried out to identify tasks involving isocyanate exposure. Some tasks with no obvious direct exposure were also included to assess possible bystander exposure. Coinciding with the personal exposure measurements, data on work circumstances, e.g. ventilation and personal protection equipment (PPE) use, and product samples were collected.
In twenty four car body repair shops a total of 475 task-based samples were taken during the following tasks: preparatory/finishing work, sanding/grinding of painted parts, mixing lacquers, spraying PU lacquer, spraying water-based color lacquer, cleaning spray gun, welding, assembly work and use of isocyanate containing glues, kits and pastes.
In five industrial painting companies a total of 36 task-based samples were taken during the following tasks: mixing lacquers, PU spray painting, applying PU lacquer with a roller or brush, assisting a spray painter and other activities near the spray painter (bystander).
Besides task-based samples, a background office sample was taken on most measurement days (28 in car body repair shops and 2 in industrial painting companies) and stationary samples were taken when high-risk tasks were performed on the general work floor at a distance of 0.5-5 meter (22 in car body repair shops and 3 in industrial painting companies).
Sampling periods were September-December 2003 and June-November 2004. Samples were taken on one measurement day per company except for eight car body repair shops where samples were taken on two measurement days within the same sampling period.

Air sampling and analysis
Task-based personal air samples were collected at 1 l/min using midget impingers, containing 10 g (=11.5 ml) 0.01 M di-n-butylamine (DBA) in toluene, attached to the lapel (11)**.** During 31% of the measurements filter back up samples (13 mm glass fiber filters) were taken that were submerged in 10 ml

DBA in toluene after the measurement day. Gillian personal sampling pumps were calibrated before and after sampling with a rotameter and average flows were used for calculations. Task-based measurement times varied between 1-64 minutes depending on task duration. When spray painting involved several layers, sampling was stopped during intermitting times. The use of impingers in combination with the volatile toluene results in limited sampling times and therefore eliminates the possibility of taking 8-hour samples.
Immediately upon sampling, isocyanate groups derivatize with DBA in the impinger. After sampling, samples were stored at 4°C. Derivatization of amine groups was performed with a chloroformate reagent in a two-phase system (toluene/water) within 3 weeks after sampling, resulting in the determination of amine groups (amino isocyanates) as carbamate esters (11).
Since on material safety data sheets isocyanate compounds are only roughly defined (e.g. poly isocyanates, polymeric HDI) 23 product samples have been randomly collected coinciding with air sampling. These samples were qualitatively analyzed for isocyanate compounds to investigate if compounds in the exposure samples are present in the products.
Compounds were separated by reversed phase high performance liquid chromatography (RP-HPLC) ionized with electrospray in the positive ionization mode and detected with tandem mass spectrometric detection (MS-MS). 23 compounds were quantified in a single analytical run. Analyzed compounds were: HDI, IPDI, MDI, TDI, oligomers of HDI (uretidone, isocyanurate, biuret, diisocyanurate, unknown oligomer of HDI) and MDI (3-ring, 4-ring and 5-ring MDI) and thermal degradation products. Thermal degradation products included mono isocyanates: methyl isocyanate (MIC), ethyl isocyanate (EIC), propyl isocyanate (PIC), phenyl isocyanate (PhI) and amino isocyanates of HDI, TDI and MDI: hexamethylene amino isocyanate (HAI), toluene amino isocyanate (TAI), methylene bisphenyl amino isocyanate (MAI). Only thermal degradation products containing N=C=O groups were analyzed. In the present paper all polymeric isocyanates, which are indicated with different terms (polyisocyanates, oligomers, adducts) in the literature, will be indicated as oligomers. The analysis of all compounds in a single analytical run resulted in a high and unstable background signal and limit of quantification for isocyanic acid (ICA). Therefore ICA was excluded from this study.

Quantification
Deuterated internal standards (derivatized with DBA) and derivatized external standards were used for calibration of monomers, monisocyanates and aminoisocyanates (11). D9-DBA derivatized external standards were available for the quantification of biuret, isocyanurate and diisocyanurate. For uretidone and an unknown oligomer of HDI no standards were available. Based on the structure of the molecule it was decided to use the biuret calibration for uretidone and consequently express uretidone in biuret equivalents. The unknown oligomer of HDI was expressed in diisocyanurate equivalents. Bobeldijk et al. gives a more detailed description of derivatization, calibration and analysis of the isocyanate compounds within the present study (21).

To be able to interpret the contribution of individual compounds, all concentrations are expressed in $\mu g/m^3$ NCO in air calculated as the concentration of the compound divided by its molecular weight times the number of NCO groups times the molecular weight (MW) of NCO (MW=42):

$(C_{compound} / MW_{compound})*N_{NCO}*MW_{NCO}$.

The limit of detection (LOD) depends on compound and measurement time. The maximum LOD (calculated with the minimum measurement time of 1 min, standard volume of 11.5 ml and standard flow of 1 litre/min) in this study was roughly 0.1-2.4 $\mu g/m^3$ NCO for diisocyanates, 0.03-0.2 $\mu g/m^3$ for aminoisocyanates, 0.1-2.9 $\mu g/m^3$ for monoisocyanates and 1.4-37.7 $\mu g/m^3$ for oligomers of HDI. These LODs decrease linearly when measurement time increases.

Statistical analysis
SAS statistical software (SAS System for Windows, version 8.02; SAS Institute, Cary, NC) was used for data analysis. Due to the large proportion of samples with non-detectable concentrations, exposure distributions were (severely) truncated to the left for the majority of the individual compounds. Therefore exposure was described by the frequency of detects and the minimum, median and maximum concentration for samples above the LOD.
Because it is impossible to study exposure determinants or exposure-response relationships for all compounds separately, methods for aggregation of compounds were explored. Next to aggregation based on chemical properties exploratory factor analysis (PROC FACTOR) was used on the presence of 19 compounds (above / below LOD) to identify clusters of compounds that occurred regularly in combination with each other during personal exposure. Only compounds that were found multiple times were included. Factors were retained if eigenvalues were greater than 1. Factors were identified using the factor pattern matrix after orthogonal varimax rotation. Compounds with a factor loading score greater than 0.25 were included in a factor.
Sum measures were calculated for grouped components. These were calculated by adding up detectable levels only, resulting in a non-detectable sum measure of zero when the concentrations of all compounds were below the limit of detection.
Since the compounds in the 'HDI factor' were the most dominant in both frequency and concentration this factor was selected for more detailed analyses. Within worker ($_{ww}S^2$) and between worker ($_{bw}S^2$) variance components for exposure to the HDI factor during PU spray painting were obtained by PROC MIXED with worker as a random component and no fixed components. Mixed effect models were also used to assess the following fixed effects: company type, lacquer type and water-based clear coat lacquer. In all mixed effect models worker was considered a random effect and a compound symmetric covariance structure was assumed. The between worker variance components were used to estimate the range within which 95% of the individual mean

exposures fall: $_{bw}R_{0.95}$ = exp[3.92 * ($_{bw}S$)]. Analogous a $_{ww}R_{0.95}$ was calculated within which 95% of the estimates for an individual fall (22). For the mixed model analyses exposure levels were (natural) log transformed and non-detectable values were substituted by the lowest LOD/2 in the factor (LOD of HDI).

Results

Task description and control measures
Table 2.1.1 gives an overview of the location in which tasks were performed and control measures used during the measurements.

Table 2.1.1: Workplace characteristics and control measures: overview of task-based use of respiratory protection and local exhaust ventilation during the measurements in car body repair shops and industrial painting companies based on data collected during the measurements.

	Location	Inhalatory protection* (yes/no)	Local exhaust ventilation (yes/no)
Car body repair shops			
Use of PU kit, glue, paste	General work floor	none	none
Preparatory/ finishing work	Spray department	11% Filtering mask	none
Sanding, grinding	General work floor/ Spray department	35% Dust mask	42%
Mixing	Mixing room	17% Filtering mask	82%
Spray painting PU based lacquer	Base coat: 44% spray booth, 56% spray bay Clear/2-component coat: 100 % spray booth	97% Filtering mask	100%
Spray painting water based lacquer	Spray booth	100% Filtering mask	100%
Gun cleaning	Mixing room	46% Filtering mask	70%
Welding	General work floor	11% Welding mask	17%
Assembly work	General work floor	none	none
Industrial painting companies			
Spray painting PU lacquer	77 % hall, 23% outside	86% Filtering mask	Hall: 30% Outside: none
Applying PU lacquer with brush or roller	8 % hall, 92% outside	20% Filtering mask	none
Mixing	67% hall, 33%outside	67% Filtering mask	none
Spray assistent	Hall	100% Filtering mask	none
Near spray painter	Hall	none	none

* No supplied air masks were observed in this study

Car body repair shops: In the spray departments 1-8 spray cycles occurred that each consisted of sanding /grinding of the car (parts), masking, spraying base coat (PU), color coat (water-based) and clear coat (PU) and de-masking. Occasionally a PU color top coat (2-component coat) was used. One company had just started to use newly developed water-based clear coats.

For each coat the lacquer was mixed, sprayed on in multiple layers and spray guns were cleaned by the same worker. The use of high volume low pressure (HVLP) spray guns is widespread in Dutch car body repair shops (>90%). Spray booths were equipped with downdraft ventilation except for one company (4%), which had cross draft ventilation. In 75% of the companies base coat was sometimes sprayed outside the spray booth in a spray bay with local exhaust ventilation. To accelerate curing, inside the spray booth cars were heated with warm air for 30-60 minutes. Outside the booth small IR or UV heaters were used in 75% of the companies. Automated gun cleaning machines were encountered in 75% of the companies but cleaning always also involved manual handling. Usually spray painters alternate daily or weekly between spraying inside the booth and activities outside the booth (sanding, masking, de-masking, incidentally spraying base coat). In this study preparatory and finishing work includes (de-) masking, spraying of non-PU coats and polishing. Questionnaires among 186 workers in spray departments indicate that the amount of time a worker spends on actual spraying is very limited and varies greatly. A mean spraying time inside the booth of 37 hours per month (standard deviation (sd)=37) and spraying time outside the booth of 9 hours per month (sd=18) were reported. This results in a mean spraying time per day of 111 minutes inside the booth and 27 minutes outside the booth in case of 20 working days per month. Preparatory work like masking and de-masking and also waiting between different layers and coats makes up most of the working day.
The general work floor was always connected to the spray department. On the general work floor metal sheet workers and mechanics incidentally performed tasks with possible isocyanate exposure: i.e. welding and using isocyanate containing glues, kits and pastes. All other activities with no obvious source of isocyanate exposure were classified as assembly work. Sanding was done in both the spray department and on the general work floor.
Industrial painting companies: Most industrial painting companies had sandblasting departments that were not included in the study since they comprised a separate group of workers in an isolated area. Painted objects varied from ships to small parts of a crane. Consequently a paint job lasted longer (7 min-several hours) and mixing and gun cleaning was performed less frequently (1-2 per day) than in car body repair shops. All PU lacquers encountered were color top coats (2-component). In addition to (airless) spray guns, rollers and brushes were used to apply lacquers. Painters worked alone or with an assistant who conducted supportive tasks. Objects were painted in a hall or outdoors. Lacquers were mixed in the same area in which often also other workers (bystanders) were present. Sampling of long paint jobs was truncated at 30-45 minutes since the use of impingers limits sampling time.

Isocyanate compounds
A total of 566 air samples were collected from 29 companies. In the samples 23 isocyanate compounds were analyzed of which 20 could be detected. Table 2.1.2 gives an overview of the distribution of personal and stationary samples over the branches, tasks, workers and companies.

Table 2.1.2: Overview of personal and stationary samples in car body repair shops and industrial painting companies.

Task description	Type P/S*	Sampling time (min) Median (range)	N samples	N workers	N companies
Car body repair shop workers			525	94	24
Use of PU kit, glue, paste	P	4 (2-18)	11	9	7
Preparatory/ finishing work	P	6 (2-64)	18	14	12
Sanding, grinding	P	11 (2-30)	48	36	19
Mixing	P	3 (1-23)	101	48	24
Spray painting PU lacquer	P	7 (1-40)	148	51	24
Spray painting water based lacquer	P	11 (3-34)	27	17	14
Gun cleaning	P	3 (1-13)	77	41	24
Welding	P	12 (1-46)	29	21	17
Assembly work	P	23 (10-43)	16	15	13
Office	S	39 (20-113)	28	-	21
Near drying with portable IR/UV lamp (1-5 m)	S	28 (15-61)	16	-	13
Near spraying (1-5 m)	S	24 (12-97)	4	-	4
Near mixing (1-5 m)	S	2 -	1	-	1
Near welding (1-5 m)	S	10 -	1	-	1
Industrial spray painters			41	15	5
Spray painting PU lacquer	P	25 (7-33)	13	6	4
Applying PU lacquer with brush or roller	P	25 (7-41)	12	5	3
Mixing	P	8 (4-10)	3	3	3
Spray assistant	P	30 (27-40)	4	2	1
Near spray painter (1-100 m)	P	30 (16-31)	4	3	2
Office	S	- (24-26)	2	-	2
Near spraying	S	13 (13-26)	3	-	2

* P= personal sample, S= stationary sample

In many samples most of the compounds were below the LOD. In both car body repair shops and industrial painting companies the most dominant compounds, in frequency and concentration, were HDI oligomers and to a lesser degree HDI monomers (Table 2.1.3). IPDI and 2,4-TDI were found regularly in car body repair shops but levels were low compared to HDI and its oligomers. Measurements indicate that exposure to MDI related compounds occurred only to a limited extent. Most mono isocyanates and amino isocyanates were found infrequently and in low concentrations in both industries. Yet in car body repair shops these products of thermal degradation were encountered relatively more frequently and in a larger variety than in spray-painting companies. The most dominant thermal degradation products were MIC, 2,4-TAI and 1,6-HAI.
On 180 filter samples that were collected, oligomers of HDI were the most dominant compounds. Frequencies of detectable oligomers were lower (5-18% of filters) and levels were a fraction of levels found in the impinger samples. Thermal degradation products were also detectable, with frequencies and levels more comparable to levels in impingers. Since detectable frequencies and levels on filters were low, filter samples have not been collected in later sampling periods and the results are not presented in the present paper.

Table 2.1.3: Descriptive statistics of personal samples in car body repair shops and industrial painting companies; number of detectable samples and minimum, median and maximum concentration (μg/m^3 NCO) for the samples above the LOD for all analyzed compounds.

	Car body repair shop workers N=475			**Industrial spray painters N=36**		
Compound	n >LOD	median	(range)	n>LOD	median	(range)
MIC[1]	51	0.05	(0.01-3.1)	6	0.05	(0.01-0.65)
EIC[1]	1	0.54	-	0	-	
PIC[1]	6	0.43	(0.04-0.54)	0	-	
PhI[1]	8	0.04	(0.01-0.48)	0	-	
2,4-TAI[2]	14	0.04	(0.001-0.59)	7	0.01	(0.004-0.04)
4,2-TAI[2]	5	0.02	(0.003-0.54)	0	-	
2,6-TAI[2]	8	0.05	(0.001-0.74)	0	-	
1,6-HAI[2]	43	0.21	(0.02-1.82)	14	0.19	(0.02-3.95)
4,4-MAI[2]	7	0.03	(0.02-0.10)	0	-	
2,4-TDI[3]	63	0.07	(0.005-1.16)	0	-	
2,6-TDI[3]	3	0.67	(0.27-2.88)	0	-	
1,6-HDI[3]	183	0.44	(0.002-15.5)	34	0.11	(0.01-28.8)
4,4-MDI[3]	3	0.02	(0.02-0.06)	0	-	
IPDI[3]	44	0.08	(0.004-1.72)	0	-	
IPDI isomer[3]	26	0.23	(0.01-1.10)	0	-	
Uritidone[4]	77	1.29	(0.12-47.5)	19	3.2	(0.07-61.9)
Isocyanurate[4]	213	13.29	(0.02-892)	21	5.31	(0.06-1931)
Biuret[4]	142	8.11	(0.06-306)	28	2.78	(0.11-552)
Diisocyanurate[4]	90	24.27	(0.84-149)	11	4.21	(0.65-577)
Unknown poly HDI[4]	92	10.58	(0.26-79.9)	4	1.06	(0.42-5.89)
Three ring MDI[5]	0	-		0	-	
Four ring MDI[5]	0	-		0	-	
Five ring MDI[5]	0	-		0	-	

[1]mono isocyanate, [2]amino isocyanate, [3]diisocyanate, [4]oligo HDI, [5]oligo MDI

In 23 hardener product samples collected coinciding with paint related tasks the following compounds were detected: HDI (26% of samples), IPDI (13%), IPDI-isomer (13%), uretidone (22%), isocyanurate (74%), biuret (34%), diisocyanurate (61%) and unknown oligomer of HDI (39%). In four samples (17%) no isocyanates were detected. These four samples were all base coat hardeners. Product samples have not been analyzed quantitatively. Since the water-based clear coat hardener also contained a variety of the above mentioned compounds the application of this hardener was assigned 'application of PU lacquer'.

Factor analysis

To explore correlation in the occurrence of different isocyanates, exploratory factor analysis was performed on binary variables for each compound (above / below LOD) that was found more than once (19 compounds). This yielded 3 factors that explained 84.5% of the total variance (Table 2.1.4). HDI based compounds loaded on factor 1: 'HDI factor', TDI based compounds loaded on factor 2: 'TDI factor' and MDI based compounds loaded on factor 3: 'MDI factor'. IPDI isomers loaded on both the HDI and TDI factors. Since theoretically IPDI is more likely to coincide with paint related compounds from the HDI factor and since the factor loadings were slightly higher on the HDI factor, we choose to assign the IPDI isomers to the HDI factor. Monoisocyanates loaded on the TDI

factor or MDI factor. PhI loaded on both factors and was assigned to the TDI factor for further analyses. Assignment of PhI to the MDI factor in any of the further analyses did not influence the results.
Sum concentrations for the personal and stationary samples were calculated for different groups of compounds to give insight in concentrations based on different aggregation methods. Exposure measures were aggregated according to: the 3 factors, chemical structure (monomers, oligomers and thermal degradation products), and a total NCO measure was calculated (Table 2.1.5).

Table 2.1.4: Clusters of correlating compounds and percentage of explained variance as determined by factor analysis (factor loadings after orthogonal varimax rotation between brackets).

HDI factor 44.1%[b]		**TDI factor 27.0%[b]**		**MDI factor 13.4%[b]**	
Biuret	(0.85)	2,6-TAI	(0.77)	4,4-MDI	(0.77)
Diisocyanurate	(0.80)	4,2-TAI	(0.66)	4,4-MAI	(0.66)
Uritidone	(0.77)	2,4-TAI	(0.54)	PIC	(0.33)
Unknown polyHDI	(0.73)	PhI*	(0.52)	PhI*	(0.51)
Isocyanurate	(0.70)	2,6-TDI	(0.40)		
1,6-HDI	(0.69)	MIC	(0.33)		
1,6-HAI	(0.55)	2,4-TDI	(0.28)		
IPDI*	(0.43)	IPDI*	(0.30)		
IPDI isomer*	(0.38)	IPDI isomer*	(0.33)		

*Present in multiple factors
[b]Percentage of variance explained

Table 2.1.5: Descriptive statistics of sum measures in personal samples in car body repair shops and industrial painting companies. Number of detectable samples and median (range) concentration (in $\mu g/m^3$ NCO) for the samples above the LOD for sum concentrations.

	Car body repair shop workers N=475		**Industrial spray painters N=36**	
Compound	n>LOD	Median (range)	n>LOD	Median (range)
HDI factor	256	8.55 (0.002-1124)	35	6.67 (0.01-2643)
TDI factor	111	0.07 (0.001-5.38)	11	0.02 (0.004-0.65)
MDI factor	12	0.10 (0.02-0.54)	0	-
TDP*	103	0.12 (0.001-4.64)	17	0.17 (0.01-3.95)
Monomers	217	0.42 (0.002-15.5)	34	0.11 (0.01-28.8)
Oligomers	217	27.92 (0.02-1122)	29	14.21 (0.12-2614)
Total	293	5.13 (0.01-1124)	35	6.68 (0.01-2643)

*TDP: thermal degradation products

Tasks
The task-based frequency of detectable samples and exposure range (samples above LOD) of each factor for all personal samples is presented in Figure 2.1.1.

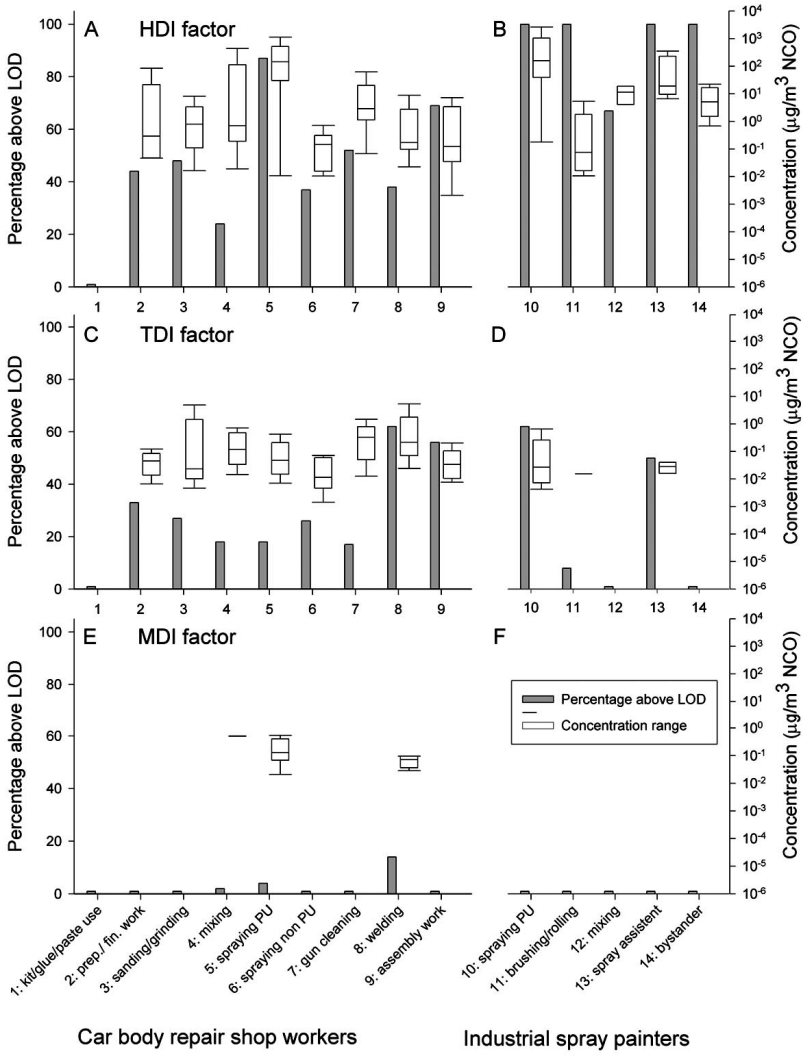

Figure 2.1.1: Task-based personal exposure to the sum concentrations of the HDI factor (A,B), TDI factor (C,D) and MDI factor (E,F) for car body repair shop workers (A,C,E) and industrial spray painters (B, D, F). Grey bar = percentage above LOD, white box plot = exposure range for samples above the LOD in µg/m^3 NCO (minimum, P_{25}, median, P_{75}, maximum).

Exposure to the HDI factor was found frequently during paint related tasks. Frequency of detectable samples was higher in the industrial painting companies. PU spray painting resulted in the highest exposures with industrial spray samples in the high range of car body repair spray samples. Other paint related tasks, without aerosol formation, like mixing and cleaning the spray gun resulted in lower exposures to the HDI factor. Besides spray assistants, other workers in the same area were also exposed.
Exposure to the TDI factor was found regularly. However, compared to the HDI factor, levels were lower and less contrast in levels between tasks was observed. Exposure to the MDI factor was found incidentally during welding and spraying of non-PU lacquers. Levels were lower than levels of the TDI factor.
A t-test showed that in car body repair shops, task-based sampling time was significantly (p=0.004) longer for detectable (any compound) samples (mean: 9.9 min) than for non-detectable samples (mean: 6.4 min). In industrial painting companies the only non-detectable task-based sample (any compound) was collected during a shorter sampling time (3.8 min) than the other samples (range: 7.0 - 40.8 min).

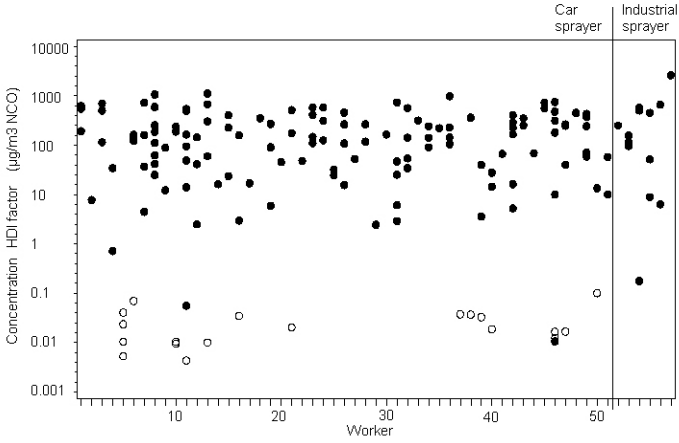

Figure 2.1.2: Scatter plot of the concentrations of the HDI factor (y-axis) during (repeated) spray painting of PU lacquers measurements for each worker (x=axis). ○ = non detectable (=all compounds below LOD), ● = detectable.

Figure 2.1.2 shows exposure levels to the HDI factor during PU spray painting for each worker in both industries (n=57). Variation within workers was large while the range was similar for workers. Variance components obtained by a mixed model confirmed a high within person variance of 9.1 compared to a between person variance of 1.6. This results in a range within which 95% of the estimates for an individual fall ($_{ww}R_{0.95}$) of 140,000 and a range within which 95% of the individual mean exposures fall: ($_{bw}R_{0.95}$) of 145. Including company type, lacquer type and water-based clear coat simultaneously into the model as

fixed components showed that lacquer type is the only significant predictor of exposure level during PU spraying. Spraying PU color top coat and PU clear coat both lead to significantly different exposure levels compared to spraying PU base coat ($p<0.01$). Company type (car body repair shop or industrial painting company) and the use of water base clear coats did not have a significant effect on exposure levels. The corrected (for the company type and water-based clear coat) geometric mean levels and 95% confidence intervals were 71 $\mu g/m^3$ (11-457), 79 $\mu g/m^3$ (12-504) and 4.6 $\mu g/m^3$ (0.6-35) for spraying PU color top coat, clear coat and base coat, respectively. Including all fixed effects into the model resulted in a reduction of 11 % for the within worker variance component ($_{ww}R_{0.95}$=68,000) and 19% for the between worker variance component ($_{bw}R_{0.95}$=82).

Stationary samples
Results of stationary samples are summarized in Table 2.1.6. Detectable exposure in offices was found in 64% of car body repair shops and 100% industrial painting companies. Exposures to all factors in car body repair shops and the HDI and TDI factor in industrial painting companies were in the same range and levels were very low compared to personal samples. Stationary samples near spray painting and drying of paint showed mainly exposure to the HDI factor. Two samples taken near mixing and near welding did not show any detectable isocyanate levels (data not shown).

Comparison with exposure limits
Currently, in The Netherlands an occupational exposure limit (OEL) exists for monomers only. None of the samples in this study was above the short-term exposure limit (STEL) of 70 $\mu g/m^3$ NCO for HDI. For oligomers no exposure limits exist in The Netherlands. Bello et al. (2004) gives a summary of existing airborne OELs worldwide. Bayer Corporation has established a Manufacturer's Guideline Limit, which was later adopted by the Oregon State OSHA, as a 15 min STEL of 220 $\mu g/m^3$ NCO for the sum of biuret and isocyanurate only. The frequency of samples above this limit in the present study was 1% during mixing and 26% during spraying in the car body repair shops. In industrial painting companies the frequency of samples above this limit was 46% during PU spraying and 25% during assisting spray painters. The United Kingdom Health and Safety Executive (UK-HSE) combines all monomers and polyisocyanates into a 15 min STEL single isocyanate standard of 70 $\mu g/m^3$ NCO. The frequency of samples above this limit for total NCO was 6% for preparatory/finishing work, 1% for mixing and 54% for PU spray painting in car body repair shops and 69% during PU spray painting and 25% during assisting a spray painter in industrial painting companies.

Table 2.1.6: Descriptive statistics of sum measures in stationary samples in car body repair shops and industrial painting companies. Number of detectable samples and median (range) concentration (in μg/m^3 NCO) for the samples above the LOD for sum concentrations.

	Car body repair shops						**Industrial painting companies**			
	Office n= 28		Near spray painting n=4		Near drying n=16		Office n= 2		Near spray painting n=3	
	n> LOD	Median (range)	n> LOD	Median (range)	n> LOD	Median (range)	n> LOD	Median (range)	n> LOD	Median (range)
HDI factor	10	0.04 (0.001-0.13)	3	10.47 (0.07-10.48)	12	0.49 (0.01-2.67)	1	0.005	3	20.79 (3.01-31.57)
TDI factor	16	0.03 (0.004-0.25)	1	0.01 -	5	0.01 (0.01-0.03)	2	(0.02-0.06)	0	-
MDI factor	1	0.009 -	0	-	0	-	0	-	0	-
TDP*	15	0.03 (0.004-0.25)	0	-	5	0.01 (0.01-0.03)	2	(0.02-0.06)	0	-
Monomers	8	0.01 (0.001-0.05)	3	0.11 (0.01-0.20)	10	0.05 (0.01-0.23)	1	0.004	3	0.17 (0.11-0.29)
Oligomers	7	0.09 (0.01-0.10)	3	10.27 (0.07-10.36)	8	0.50 (0.23-2.61)	0		3	20.62 (2.91-31.28)
All compounds	18	0.04 (0.004-0.35)	3	10.47 (0.08-10.48)	13	0.48 (0.01-2.68)	2	(0.03-0.06)	3	20.79 (3.01-31.57)

*TDP=thermal degradation products

Discussion

The aim of this study was to assess which compounds contribute to isocyanate exposure in car body repair shops and industrial painting companies and to identify tasks with high risk of isocyanate exposure. This is the first study in which the occurrence of a wide range of individual isocyanate compounds, including monomers, oligomers and products of thermal degradation has been assessed separately on a large-scale.

From the 23 analyzed isocyanate compounds 20 could be detected. The results indicate that despite their relatively low vapor pressure, oligomers of HDI are present more frequently and exposure levels are higher than for all other compounds in both car body repair shops and industrial painting companies. The dominance of oligomers of HDI over HDI can be explained by the replacement of the monomer by its oligomers. This is also found in other studies on isocyanate exposure during spray painting (14, 20, 23). No IPDI is found in industrial painting companies suggesting that IPDI is merely present in car lacquers. In the United States, IPDI and its oligomers appear to be increasingly used in auto body coatings (15, 24). In this study IPDI oligomers were not analyzed. However, material safety data sheets indicate that IPDI oligomers are present in part of the different brands and types of lacquers used in The Netherlands and may constitute 2.5-12.5% while IPDI monomer may constitute less than 2.5%.
HDI monomer levels in the present study do not exceed the current Dutch exposure limit. Nevertheless, exposure to oligomers of HDI occurs in much higher concentrations and exposures above the exposure limits of Oregon State OSHA (USA) and HSE (UK) are found during paint related tasks. However the validity of these OELs is under debate and further clinical, epidemiological and animal research is needed to elucidate disease mechanisms and clarify exposure-response relationships before more reliable exposure limits can be constructed (25).

Exploratory factor analysis reveals that, in practice, compounds with the same mother compounds tend to cluster. This is not surprising, for clustering of compounds is likely to be determined by the exposure source. Since the clusters give informative insight in exposure sources and task-based exposure patterns it was decided to use the 3 factors in task-based analyses. When comparing different tasks it should be noted that a longer sampling time is associated with detectable samples. Therefore the comparison of levels is preferred over frequencies.
The HDI factor contains all compounds that were found in product samples and 1,6-HAI. Since this compound is not found in any of the product samples it may be formed during the application of lacquers. The dominance of the HDI factor in both frequency and levels shows that paint is, not surprisingly, the most important source and major contributor of isocyanate exposure in both car body repair shops and industrial painting companies. In addition, this factor is mainly present during paint related tasks. Exposure patterns for the HDI factor are

similar for car body repair shops and industrial painting companies with highest levels during PU spray painting. In industrial painting companies levels during PU spray painting are in the higher range of the levels found in car body repair shops. In industrial painting companies higher exposure levels during PU spray painting were anticipated, for working conditions are less controlled by ventilation. Surprisingly, the mixed effect model indicates that not company type but the use of base coats probably accounts for the lower exposure levels during PU spray painting found in car body repair shops. Additionally, no significant effect was seen for the use of a new water-based clear coat, which indeed still contains isocyanates. The presence of (low level) exposure to workers outside the direct vicinity of the spray painter, as well as detectable isocyanate levels in stationary samples suggest regular bystander exposure. Sources of bystander exposure might be PU spray painting, curing outside the spray booth or the opening of the spray booth door.
The TDI and MDI factor contain, in addition to mono isocyanates, TDI and its amino isocyanates and MDI and its amino isocyanates, respectively. Since TDI and MDI are never found during the use of kits, glues and pastes (in which these compounds are present) but are found in combination with thermal degradation products, the exposure source for these factors is probably a thermal degradation process. Mono isocyanates can be formed by thermal degradation of different monomers explaining PhI loading on both factors. Very little contrast in exposure to these two factors is observed between the tasks. Moreover, in the office samples, exposures in the same range are found as in the personal samples. Therefore, it is not clear whether the exposure source of thermal degradation products is welding or whether other, unidentified, activities may contribute. However, the relatively lower abundance and variety of thermal degradation products in industrial painting companies suggest that less processes of thermal degradation are present in this industry. Since for thermal degradation products comparable frequencies and levels were found in impingers and on filters, the TDI and MDI factors are underestimated relatively more than the HDI factor by excluding filters.

Accumulating exposure to different isocyanate compounds into NCO sum measures is general practice. However, while health surveys, specific inhalation challenges and animal studies suggest that isocyanate oligomers, thermal degradation products and diisocyanates have similar health effects (26-32), animal studies indicate that relative potencies of different isocyanate compounds are variable (33-36). Although with the number of different compounds the use of a sum measure or possibly a marker compound is inevitable, it is desirable to also have information on the compounds behind these measures.

A shortcoming of the present study is that only short-term task-based samples have been taken. Short-term levels are more strongly influenced by exposure peaks than 8-hour levels, resulting in high variability in exposure levels. During spray painting within worker variability ($_{ww}S^2$) is large compared to between worker variability ($_{bw}S^2$) suggesting that variability in task-based exposure

during spray painting over time is more prominent than differences in mean exposures between workers. However, next to true variability over time this component also constitutes of sampling and analysis error. Measured concentrations can vary greatly with the location of the sampler on the body (37) and by spraying direction and orientation. Additionally, even when the individual analysis error per compound is below 20%, when 9 compounds are added up the resulting error can be substantial. Kromhout et al. (1993) give an overview of within and between worker components of 8-hour occupational exposure to chemical agents from different job titles throughout industry (38). Although the ${}_{bw}R_{0.95}$ of 145 in the present study falls well within the reported range, the ${}_{ww}R_{0.95}$ of 140,000 is higher than the maximum ${}_{ww}R_{0.95}$ of 10,000 for 8-hour measurements. Measurement time probably accounts for this difference. Conversely, the mix of tasks performed on different days and by different workers will introduce variability in full shift exposure levels that is not captured by task-based exposure measurements.
Despite the introduction of large variability, task-based sampling has many advantages like a more direct understanding of the sources of high exposure, exposure levels can be estimated for a whole range of task combinations and increased efficiency of the sampling campaign by focusing on high risk tasks (39). In addition, although the relative importance of intensity, duration and frequency of exposure in relation to disease development and aggravation is not well understood, new exposure standards for isocyanates appear to be aimed at short-term high-level excursions rather than chronic low-level exposure (24). Short-term exposure peaks, which may be an important contributor to disease development or aggravation, are more easily identified using task-based measurements. However when caused by an unusual or unforeseen exposure source like an incident or maintenance, peak exposure can also be missed by task-based sampling.

Another restriction of the present study is that only exposure outside the respirator has been measured. Sampling inside a facemask is complicated because of interference with the worker and respirator. A study by Rosenberg and Tuomi (1984) indicates that if a combination of a charcoal and dust filter is used almost 100% of the HDI and biuret is absorbed and in case of a charcoal filter almost 100% of the HDI and 70% of biuret is absorbed (14). This results in protection factors from 2 to 5 (20). However, the validity of these figures may be questionable since they are based on a small-scale study that may be outdated. A re-evaluation is justified. Possibly, exposure during cleaning of the spray gun, mixing or even tasks without direct exposure to isocyanates may result in higher actual exposures due to the absence of protection of inhalation filtering devices. Biomonitoring might give more informative insight in actual internal dose.

Additionally the low response rate for car body repair shops may introduce selection bias. A small questionnaire on the reply form that was completed by 41 non-participating and 116 participating companies revealed that there was no

difference in the presence of a spray booth. However, non-participating non-branch members were small (mean: 1.4 workers, sd=0.8) compared to non-participating branch members (mean: 6.6 workers sd=3.3), participating non branch members (mean: 8.6 workers sd=9.5) and participating branch members (mean: 9.5 workers sd=7.1). This implies that indications exist that the population is somewhat biased towards larger companies. However, no obvious effect of shop size on exposure levels could be observed.

Comparing isocyanate levels and frequency of detects between different studies is problematic. The field of isocyanate sampling and analysis is an active area of research for a number of reasons. New calibration standards are required because of the shift from monomers to oligomers and the new focus on thermal degradation products; decreasing exposure limits bring about the need for more sensitive methods; the high reactivity of isocyanates demands for a derivatization step immediately upon sampling; both aerosols and vapors need to be collected efficiently (12, 40, 41). Consequently several methods based on different reagents, sample collection and analysis methods have been and still are used resulting in variable compounds being measured, measurement times, LODs and units. The method used in the present study was chosen because of the efficiency of impingers to collect paint aerosols and its ability to differentiate between and quantify isocyanate compounds including thermal degradation products. However, aspiration characteristics of impingers are much less described than aspiration characteristics of filter samplers.
Sparer et al. (2004) gives a thorough summary of PU spray painting levels of previous isocyanate sampling studies converted to the $\mu g/m^3$ NCO metric. Despite differences in methods, conditions and analyzed compounds, exposure to total NCO during spray painting in the present study (<LOD – 1124 $\mu g/m^3$ NCO in car body repair shops and 0.2-2643 $\mu g/m^3$ NCO in industrial painting companies) is in the same range as the summarized results. Because of the large proportion of non detectable samples it is only possible to compare exposure ranges.
Limited studies give task-based estimates for other tasks in car body repair shops or other spray paint industries. An Australian study found exposures around 1 $\mu g/m^3$ NCO (never exceeding 2 $\mu g/m^3$ NCO) during mixing and gun cleaning and 6-19 $\mu g/m^3$ NCO during sanding of painted cars (18). In a recent study in the United States exposures of <LOD-108.7 $\mu g/m^3$ NCO for near spray activities, <LOD-118.3 $\mu g/m^3$ NCO for mixing and <LOD-36.1 $\mu g/m^3$ NCO for sanding are reported (15). In the present study, which is the first to assess non-spraying tasks in Europe, levels are mostly in the same range as the latter. A small-scale study on airborne thermal degradation products in car body repair shops showed exposure (sampled in impingers) to MIC, HDI, TDI and MDI (<0.5-4 $\mu g/m^3$ NCO) during welding and no exposure during grinding (42). These compounds and levels are consistent with the compounds and levels found in the present study.

When comparing the present study to other studies where hygiene conditions have been described it seems that hygiene conditions are more controlled in the present study (18, 24). Spray booths with down draft ventilation systems, which result in lower exposure levels (23, 24), are widespread. All booths are manufacturer built and filters are changed when pressure drops. Spraying of base coats outside the booth is always done in a spray bay with local exhaust ventilation. Surprisingly, exposure levels during PU spraying and other paint related tasks do not seem to be lower than those reported in the other studies. In addition, no significant difference in exposure levels between car body repair shops and industrial spray painters, where hygiene conditions were also less controlled, could be demonstrated. This indicates that ventilation may not be an important determinant of isocyanate exposure.
Work in progress involves a more detailed evaluation of the exposure data to study exposure determinants and the effect of control measures as well as the exploration of the application of these data in combination with questionnaire data on job title and daily activities to estimate exposure in the epidemiological study.

References

1. Lesage J, Goyer N, Desjardins F, Vincent JY, Perrault G. Workers' exposure to isocyanates. Am Ind Hyg Assoc J 1992;53(2):146-53.
2. Bernstein JA. Overview of diisocyanate occupational asthma. Toxicology 1996;111(1-3):181-9.
3. 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(3):213-28.
4. Wisnewski AV, Redlich CA. Recent developments in diisocyanate asthma. Curr Opin Allergy Clin Immunol 2001;1(2):169-75.
5. Di Stefano F, Verna N, Di Giampaolo L, Schiavone C, Di Gioacchino D, Balatsinou L, et al. Occupational asthma due to low molecular weight agents. Int J Immunopathol Pharmacol 2004;17(2 Suppl):77-82.
6. Redlich CA, Karol MH. Diisocyanate asthma: clinical aspects and immunopathogenesis. Int Immunopharmacol 2002;2(2-3):213-24.
7. Streicher RP, Reh CM, Key-Schwartz RJ, Schlecht PC, Cassinelli ME, O'Connor PF. Determination of airborne isocyanate exposure: considerations in method selection. Am Ind Hyg Assoc J 2000;61(4):544-56.
8. Bernstein DI, Korbee L, Stauder T, Bernstein JA, Scinto J, Herd ZL, et al. 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(3):387-96.
9. Kimber I. The role of the skin in the development of chemical respiratory hypersensitivity. Toxicol Lett 1996;86(2-3):89-92.
10. Kimber I, Dearman RJ. Chemical respiratory allergy: role of IgE antibody and relevance of route of exposure. Toxicology 2002;181-182:311-5.
11. Karlsson D, Dahlin J, Skarping G, Dalene M. Determination of isocyanates, aminoisocyanates and amines in air formed during the thermal degradation of polyurethane. J Environ Monit 2002;4(2):216-22.
12. Molander P, Levin JO, Ostin A, Rosenberg C, Henriks-Eckerman ML, Brodsgaard S, et al. Harmonized Nordic strategies for isocyanate monitoring in workroom atmospheres. Journal of Environmental Monitoring 2002;4(5):685-687.
13. Henriks-Eckerman ML, Valimaa J, Rosenberg C, Peltonen K, Engstrom K. Exposure to airborne isocyanates and other thermal degradation products at polyurethane-processing workplaces. J Environ Monit 2002;4(5):717-21.

14. Rosenberg C, Tuomi T. Airborne isocyanates in polyurethane spray painting: determination and respirator efficiency. Am Ind Hyg Assoc J 1984;45(2):117-21.
15. Woskie SR, Sparer J, Gore RJ, Stowe M, Bello D, Liu Y, et al. Determinants of isocyanate exposures in auto body repair and refinishing shops. Ann Occup Hyg 2004;48(5):393-403.
16. Carlton GN, England EC. Exposures to 1,6-hexamethylene diisocyanate during polyurethane spray painting in the U.S. Air Force. Appl Occup Environ Hyg 2000;15(9):705-12.
17. Maitre A, Leplay A, Perdrix A, Ohl G, Boinay P, Romazini S, et al. Comparison between solid sampler and impinger for evaluation of occupational exposure to 1,6-hexamethylene diisocyanate polyisocyanates during spray painting. Am Ind Hyg Assoc J 1996;57(2):153-160.
18. Pisaniello DL, Muriale L. The use of isocyanate paints in auto refinishing--a survey of isocyanate exposures and related work practices in South Australia. Ann Occup Hyg 1989;33(4):563-72.
19. Myer HE, O'Block ST, Dharmarajan V. A survey of airborne HDI, HDI-based polyisocyanate and solvent concentrations in the manufacture and application of polyurethane coatings. Am Ind Hyg Assoc J 1993;54(11):663-70.
20. 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(3):299-310.
21. Bobeldijk I, Karlsson D, Pronk A, Gonsalves J, Hekman M, van de Lagemaat D, et al. LC-MS/MS determination of airborne isocyanates: a short interlaboratory comparison. In preparation.
22. Rappaport SM. Assessment of long-term exposures to toxic substances in air. Ann Occup Hyg 1991;35(1):61-121.
23. Goyer N. Performance of painting booths equipped with down-draft ventilation. Am Ind Hyg Assoc J 1995;56(3):258-265.
24. Sparer J, Stowe MH, Bello D, Liu Y, Gore RJ, Youngs F, et al. Isocyanate exposures in autobody shop work: the SPRAY study. J Occup Environ Hyg 2004;1(9):570-81.
25. Bello D, Woskie SR, Streicher RP, Liu Y, Stowe MH, Eisen EA, et al. Polyisocyanates in occupational environments: a critical review of exposure limits and metrics. Am J Ind Med 2004;46(5):480-91.
26. Vandenplas O, Cartier A, Lesage J, Perrault G, Grammer LC, Malo JL. Occupational asthma caused by a prepolymer but not the monomer of toluene diisocyanate (TDI). J Allergy Clin Immunol 1992;89(6):1183-8.
27. Vandenplas O, Cartier A, Lesage J, Cloutier Y, Perreault G, Grammer LC, et al. Prepolymers of hexamethylene diisocyanate as a cause of occupational asthma. J Allergy Clin Immunol 1993;91(4):850-61.
28. 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(4):1183-93.
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(1):48-55.
30. Lastbom L, Colmsjo A, Johansson R, Karlsson D, Melin J, Nordqvist Y, et al. Effects of thermal degradation products from polyurethane foams based on toluene diisocyanate and diphenylmethane diisocyanate on isolated, perfused lung of guinea pig. Scand J Work Environ Health 2003;29(2):152-8.
31. Littorin M, Welinder H, Skarping G, Dalene M, Skerfving S. Exposure and nasal inflammation in workers heating polyurethane. Int Arch Occup Environ Health 2002;75(7):468-74.
32. Jakobsson K, Kronholm-Diab K, Rylander L, Hagmar L. Airway symptoms and lung function in pipelayers exposed to thermal degradation products from MDI-based polyurethane. Occup Environ Med 1997;54(12):873-9.
33. 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(3):154-64.
34. Pauluhn J. Pulmonary irritant potency of polyisocyanate aerosols in rats: comparative assessment of irritant threshold concentrations by bronchoalveolar lavage. J Appl Toxicol 2004;24(3):231-47.
35. 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(4-5):257-69.
36. 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(2-3):147-60.
37. Goller JW, Paik NW. A comparison of iron oxide fume inside and outside of welding helmets. Am Ind Hyg Assoc J 1985;46(2):89-93.

38. Kromhout H, Symanski E, Rappaport SM. A comprehensive evaluation of within- and between-worker components of occupational exposure to chemical agents. Ann Occup Hyg 1993;37(3):253-70.
39. Seixas NS, Sheppard L, Neitzel R. Comparison of task-based estimates with full-shift measurements of noise exposure. Am Ind Hyg Assoc J 2003;64(6):823-9.
40. Streicher RP, Kennedy ER, Lorberau CD. Strategies for the simultaneous collection of vapours and aerosols with emphasis on isocyanate sampling. Analyst 1994;119(1):89-97.
41. Streicher RP, Reh CM, Key-Schwartz R, Schlecht PC, Cassinelli ME, O'Connor PF. Selecting isocyanate sampling and analytical methods. Appl Occup Environ Hyg 2002;17(3):157-62.
42. Karlsson D, Spanne M, Dalene M, Skarping G. Airborne thermal degradation products of polyurethene coatings in car repair shops. J Environ Monit 2000;2(5):462-9.

Chapter 2.2

Dermal, inhalation and internal exposure to 1,6-HDI and its oligomers in car body repair shop workers and industrial spray painters

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Abstract

Objectives were to study inhalation and dermal exposure to hexamethylene diisocyanate (HDI) and its oligomers as well as personal protection equipment (PPE) use during task performance in conjunction with urinary hexamethylene diamine (HDA) in car body repair shop workers and industrial spray painters.
Personal task-based inhalation samples (n=95) were collected from 6 car body repair shops and 5 industrial painting companies using impingers with di-n-butylamine (DBA) in toluene. In parallel, dermal exposure was assessed using nitril rubber gloves. Gloves were submerged into DBA in toluene after sampling. Analysis for HDI and its oligomers was performed by LC-MS/MS. Urine samples were collected from 55 workers (n=291) and analyzed for HDA by GC-MS.
Inhalation exposure was strongly associated to tasks during which aerosolization occurs. Dermal exposure occurred during tasks that involve direct handling of paint. In car body repair shops associations were found between detectable dermal exposure and glove use (odds ratio (OR) 0.22, 95% confidence interval (CI) 0.09-0.57) and inhalation exposure level (OR 1.34, 95%CI 0.97-1.84 for a 10-fold increase). HDA in urine could be demonstrated in 36% and 10% of car body repair shop workers and industrial painting company workers respectively. In car body repair shops, the frequency of detectable HDA was significantly elevated at the end of the working day (OR 2.13, 95%CI 1.07-4.22 for 3-6 PM vs. 0-8 AM). In both branches HDA was detected in urine of ~25% of the spray painters. In addition HDA was detected in urine of a large proportion of non-spray painters in car body repair shops.
In conclusion, although (spray) painting with lacquers containing isocyanate hardeners results in the highest external exposures to HDI and oligomers, workers that do not perform paint related tasks may also experience a considerable internal dose.

Introduction

In industrialized countries isocyanates are one of the most common causes of occupational asthma (1-3). As part of an epidemiological study on isocyanate exposure and related health effects in The Netherlands, we recently assessed inhalation exposure to a range of isocyanates for end users of polyurethane (PU) lacquers in car body repair shops and industrial painting companies (4). Although inhalation exposure is probably the most important route through which allergic sensitization is achieved, there is some limited evidence that dermal contact may result in respiratory sensitization (5) as well as disease aggravation in humans (6). Animal studies have shown that topical exposure to isocyanates can result in respiratory sensitization (7-9) and vice versa (10). During the process of spray painting direct contact with lacquers is likely to occur (11, 12). The presence of contaminated surfaces and skin exposure to isocyanates in car body repair shops was confirmed by a qualitative study (13). However, no established methods exist for quantitative assessment of dermal exposure to isocyanates.

The field of external isocyanate sampling and analysis is complex because isocyanates are highly reactive and may be present as mono-, di- and poly-isocyanates, both in vapor and particulate form (14, 15). In the present paper all polymeric isocyanates, which are indicated with different terms (polyisocyanates, oligomers, adducts) in the literature, will be indicated as oligomers. In addition large variability in exposure levels and the widespread use of control measures like filtering respirators during high risk tasks complicate the interpretation of external concentrations with respect to a total internal dose (4). Therefore biomonitoring of corresponding amines in hydrolyzed urine has been suggested as a measure of total internal diisocyanate exposure received by all routes of exposure.
Few, mostly small, studies on urinary hexamethylene diamine (HDA) in relation to inhalation exposure data have been conducted. Elevated HDA levels were found in hexamethylene diisocyanate (HDI) monomer production and manufacturing workers (16) as well as subjects inhalatory exposed to HDI monomer in a test chamber (17, 18). In addition, several studies exist on toluene diisocyanate (TDI) and methylene bisphenyl diisocyanate (MDI) exposure in relation to corresponding amines (19-24). Although in most product formulations monomeric diisocyanates have been replaced by their oligomers, knowledge on bio-transformation and metabolites reflecting isocyanate oligomer exposure is lacking. Besides one test chamber study on HDI biuret and urinary HDA, no (field) studies on exposure to isocyanate oligomers in relation to corresponding amines in urine exist (25).

In the present study a method for the quantitative short-term task-based assessment of dermal exposure to isocyanates was developed. Determinants of dermal exposure were studied. In addition, task-based inhalation and dermal exposure measurements, taken in parallel, were used to identify tasks with

elevated exposure. Furthermore, urine samples were taken during the measurement day and analyzed for HDA. These samples were compared to tasks performed on the measurement day to gain insight in HDA in relation to tasks and exposure. This study is the first to quantitatively assess dermal exposure and combine dermal and inhalation exposure measurements with biomonitoring.

Methods

Population
Exposure assessment was carried out as part of an epidemiological study on isocyanate exposure and health effects in car body repair shop workers and industrial spray painters in The Netherlands. The study currently involves 520 workers from 98 companies. Car body repair shops were enrolled using databases from the branch organization (written information to 763 companies; positive response rate: 11%) and the Chamber of Commerce (written information to 93 companies; positive response rate: 9%). Industrial painting companies were contacted through shipyards resulting in companies specialized in painting of mainly ships and harbor equipment (telephone contact with 16 companies, positive response rate: 69%). Measurements were performed in a random selection of these companies.

Sampling strategy
Within the epidemiological study large-scale task-based inhalation exposure assessment has been carried out. Preliminary data from this study showed that paint related tasks lead to highest exposures (4). For dermal exposure is only expected during manual handling of paint we chose to include mainly paint related tasks in the present study. Additionally welding was included since heating of PU-containing materials may give rise to emission of the original monomers and other isocyanate species. Because the inhalation study demonstrated that HDI and its oligomers are the most dominant compounds, these were used as exposure measures.
In six car body repair shops 68 task-based inhalation and dermal samples were taken in parallel during the following tasks: mixing PU lacquer, spraying PU lacquer, cleaning spray gun and welding. Additionally, in five industrial painting companies 27 task-based inhalation and dermal samples were taken in parallel during the following tasks: mixing PU lacquer, spraying PU lacquer, rolling/brushing PU lacquer and assisting a spray painter. Coinciding with the personal exposure measurements, data on work circumstances and PPE use was collected.
In addition to exposure measurements, all workers in the company were asked to collect urine samples during 24 hours starting from pre-shift on the measurement day until pre-shift on the next day. Only workers of whom at least three urine samples were collected were included in the biomonitoring study. Coinciding with urine collection workers were asked to complete a short

questionnaire on activities and PPE use during the measurement day. In total, 45 car body repair shop workers and 10 industrial paint shop workers participated in the urine collection from which 239 and 52 urine samples were collected, respectively. Workers on whom external exposure measurements were performed did not always participate in the biomonitoring study.
Sampling was carried out during July-November 2004.

Inhalation exposure measurements
Task-based personal air samples were collected at 1 l/min using midget impingers, containing 10 g (=11.5 ml) 0.01 M di-n-butylamine (DBA) in toluene, attached to the lapel (26). Gillian personal sampling pumps were calibrated before and after sampling with a rotameter and average flows were used for calculations. When spray painting involved several layers, the pump was stopped during intermitting times. The use of impingers in combination with the volatile toluene results in limited sampling times and therefore eliminates the possibility of taking 8-hour samples.

Dermal sampling method
A dermal sampling procedure was set up using nitril rubber gloves (Romed powder free NT 810) without a reagent for sampling. After sampling gloves were immediately submerged into 0.01M DBA in toluene and removed after an extraction time. Prior to field sampling two laboratory experiments were conducted to determine extraction time and the effect of measurement time.
Toluene causes decomposition or leaching of compounds from the gloves resulting in interference with the liquid chromatography and tandem mass spectrometry (LC-MS/MS) analysis. Suitable extraction times were determined as follows. A drop of hardener without solvent was applied to 4 nitril rubber gloves that were immediately submerged into flasks containing 200 ml 0.01M DBA in toluene. Gloves were removed after 8, 24, 72 and 168 hours and analyzed.
Since gloves do not contain a reagent to capture the isocyanates during sampling the effect of measurement time on the recovery of different masses of isocyanates was studied. A dilution series of HDI (Sigma-Aldrich, Zwijndrecht, The Netherlands) and a mixture of HDI oligomers (Poly(hexamethylene diisocyanate); Sigma-Aldrich, Zwijndrecht, The Netherlands) in toluene was applied separately on nitril rubber gloves. The following masses were used: 2.5, 10 and 40 µg HDI (1.25, 5 and 20 µg NCO) or oligomer mixture (NCO content unknown) in 20 µl toluene. Gloves were submerged into 200 ml 0.01 M DBA in toluene after 0, 10 and 30 minutes and removed after 24 hours. In addition both dilution series were directly added to 200 ml 0.01 M DBA in toluene. The whole experiment was conducted in duplicate.

Dermal exposure measurements
During field sampling, task-based dermal samples were collected in parallel with inhalation samples using nitril rubber gloves for sampling. Workers wore the gloves underneath normal PPE during the task. Both gloves were submerged

into one flask containing 200 ml 0.01M DBA in toluene immediately after the task was finished. Measurement times were equal to air sampling times except for when air sampling was stopped in between separate layers of spray painting.

Urine sampling
Urine samples were collected by the workers during 24 hours starting from pre-shift on the measurement day until the morning of the next day. Printed instructions on the procedure and hygiene of urine collection and name-labeled urine containers (500 ml) for each worker were delivered at the company on the day before the measurement day. A sticker was adhered to each container on which the worker registered date and time of urine collection. Urine samples were stored at room temperature and collected on the day following the measurement day.

Analysis and quantification of air and dermal samples
Immediately upon sampling, isocyanate groups derivatize with DBA in the impingers in case of inhalation samples or in the sampling flasks in case of dermal samples. After sampling, samples were stored at 4°C.
Compounds were separated by reversed phase high performance liquid chromatography (RP-HPLC) ionized with electrospray in the positive ionization mode and detected with tandem mass spectrometric detection (MS-MS). All compounds were quantified in a single analytical run. Analyzed compounds in the field samples were HDI and oligomers of HDI (uretidone, isocyanurate, biuret, diisocyanurate, unknown oligomer of HDI).
A deuterated internal standard (derivatized with DBA) and derivatized external standard were used for calibration of HDI (26). D9-DBA derivatized external standards were available for the quantification of biuret, isocyanurate and diisocyanurate. For uretidone and an unknown oligomer of HDI no standards were available. Based on the structure of the molecule it was decided to use the biuret calibration for uretidone and consequently express uretidone in biuret equivalents. The unknown oligomer of HDI was expressed in diisocyanurate equivalents.

To be able to interpret the contribution of individual compounds, all concentrations are expressed in $\mu g /m^3$ isocyanate groups (N=C=O-group: NCO group) in air or total mass (μg) NCO group on two gloves (irrespective of sampling time) calculated as the concentration of the compound divided by its molecular weight times the number of NCO groups times the molecular weight of NCO (42): $(C_{compound} / MW_{compound})*N_{NCO}*MW_{NCO}$.
The limit of detection (LOD) for inhalation samples depends on compound and measurement time. The maximum LOD (calculated with the minimum measurement time of 1 min, standard volume of 11.5 ml and standard flow of 1 litre/min) in this study is roughly 0.1 $\mu g/m^3$ NCO for HDI and 1.4-37.7 $\mu g/m^3$ for oligomers of HDI. These LOD's decrease linearly when measurement time increases.

The LOD for dermal samples depends merely on the compound and is 0.3 µg on both gloves for HDI and 0.3-5.3 µg on two gloves for oligomers of HDI (based on 200 ml derivatization solution).

In the samples from the laboratory experiments for the dermal sampling method HDI and isocyanurate were analyzed. Isocyanurate was selected because this is the most common oligomer found in the inhalation study (4). Setting the dilution series that was directly added to DBA in toluene at 100%, the results of these samples are expressed as mean percentage recovery for each duplicate.

Analysis and quantification of urine samples
Urine samples were frozen until sample preparation was carried out. Aliquots of 2 ml of each sample were at first mixed with 3 ml 6 M hydrochloric acid [HCl] (37%, Merck, Darmstadt, Germany) and then spiked with 50 µl internal standard solution (1,7-diaminoheptane [HpDA] (purity 98%, Aldrich, Taufkirchen, Germany, at 1.0 mg/L in water). Samples were heated for 16 hours (overnight) at 100°C. After cooling to room temperature, the acid hydrolysate was basified by the addition of 4 ml fresh saturated solution of sodium hydroxide [NaOH] (purity 99%, Merck, Darmstadt, Germany). The samples were then extracted with 3 ml toluene (purity 99,5%, Merck, Darmstadt, Germany). Two ml dried organic phase by Na_2SO_4 (p.a. Merck, Darmstadt, Germany) were used in derivatization by adding 25 µl pentafluoropropionic anhydride (purity 99%, Aldrich, Taufkirchen, Germany). The vials were closed tightly, shaken for 1 min. The derivatization was stopped by adding 3 ml 1 M phosphate buffer (tri-potassium phosphate p.a., Riedel-de-Haen, Taufkirchen, Germany, pH 7.5). After centrifugation, the organic phase was supplemented with 100 µl n-decane (purity 98%), Fluka, Taufkirchen, Germany) as keeper and then evaporated with nitrogen to a residual volume of about 100 µl.

Two µl of this solution containing HDA amide derivative were analyzed by gas chromatography / mass spectrometry in selected ion-monitoring mode on a Agilent mass spectrometry detector MSD HP 5973 connected to a gas chromatograph HP 6890, equipped with an autosampler (GC/MS: Agilent, Waldbronn, Germany). The separation was performed on a capillary column HP-5MS (30 m x 0,25 mm) with a film thickness of 0,25 µm. The column was held at 100°C for 2 min, ramped at 10° C/min to 280°C. Injections were performed in the splitless mode under helium at a flow rate of 1.2 ml/min. Under these conditions, the retention times for 1,6-hexane diamine (HDA) (purity 98%, Aldrich, Taufkirchen, Germany) and HpDA were 9.63 min and 10.68 min, respectively. The specific ions, i.e., m/z 176 and 232 for HDA and m/z 176 and 303 for HpDA, were selected as quantifier and qualifier, respectively. The quantification was achieved by comparison with a calibration curve in the range of 5 to 100 µg/L. The limit of detection for HDA calculated from a signal-to-noise ration of 3:1 was found to be 3.0 µg/L. Urinary creatinine on each sample was determined in grams per liter (g/L) using HPLC (Merck-Hitachi, Darmstadt,

Germany) (27). HDA concentration was then expressed in µg per g (µg/g) of creatinine.

Each urine sample was analyzed twice. The mean was reported when the difference relative to the arithmetic mean was less than 5% (>90% of samples). In case the difference was more than 5% the analysis was repeated at least twice.
To compare our method with another described method measuring HDA diurethane derivative, part of the samples was assayed with both procedures (28). The resulting regression between data from both sources was linear and the correlation coefficient was greater than 0.95.

Statistical analysis
SAS statistical software (SAS System for Windows, version 8.02; SAS Institute, Cary, NC) was used for data analysis. Due to the large proportion of samples with non-detectable concentrations, exposure distributions were (severely) truncated to the left for the majority of the individual compounds. Therefore external as well as internal exposure is described by the frequency of detects and the minimum, median and maximum concentration for samples above LOD.
The association between both dermal exposure and urinary HDA and covariates was studied by means of logistic regression (PROC GENMOD). To account for correlation between repeated measurements on the same worker the generalized estimating equations (GEE) method was used (29). Consequently, all presented odds ratios (ORs) are corrected for correlation among repeated measurements. The binary response variable for dermal exposure (detected / non detected, diisocyanates or oligomers) was modeled as a function of glove use (yes/no) and inhalation exposure level (10 fold increase, µg/m^3). For this analysis inhalation concentrations below LOD were replaced by LOD/2. The binary response variable for HDA in urine (detected / non detected) was modeled as a function of a categorized time interval variable (8-12 AM, 0-3 PM, 3-6 PM, 6-12 PM and 0-8 AM on the next day versus 0-8 AM on the measurement day).

Results

Dermal sampling method
Isocyanates could be determined in the samples from which gloves were removed after 8 and 24 hours. Longer removal periods resulted in samples in which too many glove compounds were present that interfered with the LC-MS/MS analysis. The extraction time was therefore set to a minimum of 8 and maximum of 24 hours.
Figure 2.2.1 shows the mean percentage recovery for different masses of HDI and isocyanurate after 0, 10 and 30 minutes on the glove before submersion into DBA in toluene (isocyanates directly added to DBA in toluene= 100%). The recovery of HDI ranged form 75 to 95% and a slightly declining time trend could

be observed. For isocyanurate the recovery was around 100% without a clear time trend. The percentage difference relative to the arithmetic mean was below 17% for the HDI and isocyanurate duplicates.

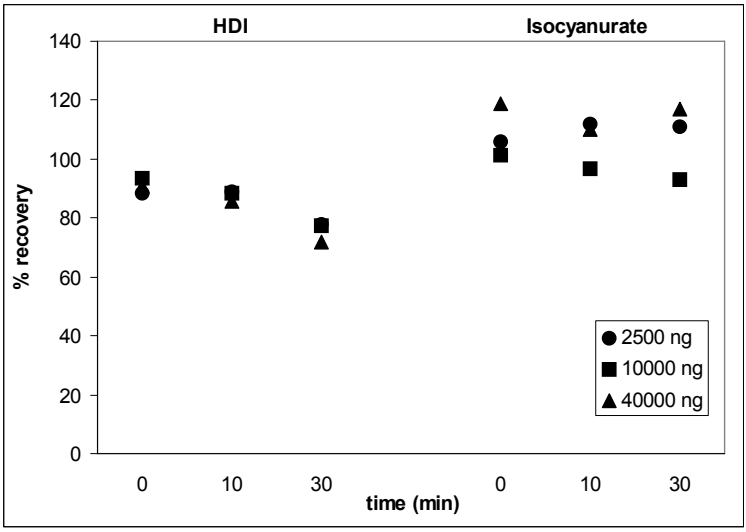

Figure 2.2.1: Percentage recovery (mean) for HDI and isocyanurate(2500, 10000 and 40000 ng) after 0, 10 and 30 minutes on a nitril rubber glove before submersion in DBA solution (dilution series directly added to DBA solution= 100%).

Inhalation and dermal exposure

Table 2.2.1 gives an overview of the inhalation exposure levels to HDI and the sum of its oligomers separately. Oligomers of HDI dominated over the monomer during all tasks. In both branches inhalation exposure was highest during tasks where paint is aerosolized: spraying and assisting the spray painter. During other tasks the frequencies of detectable samples as well as exposure levels were lower.

Table 2.2.2 shows dermal exposure mass to HDI and the sum of its oligomers separately. As in case of inhalation exposure, oligomers of HDI dominated over the monomer during all tasks. In both car body repair shops and industrial painting companies dermal exposure was found most frequently during tasks where direct exposure with lacquers might occur: mixing, applying and cleaning. Detectable oligomer exposure was found more frequently in industrial painting companies but exposure ranges were larger in car body repair shops. Less contrast in dermal exposure masses was observed between the tasks than in inhalation exposure levels.

Table 2.2.1: Task-based inhalation exposures in car body repair shops and industrial painting companies. Percentage of samples above the limit of detection (LOD) and exposure median and range (µg/m^3 NCO) for samples above LOD.

	Sampling time			1,6-HDI		Oligomers
	n	(min) Median (range)	%> LOD	Median (range)	%> LOD	Median (range)
Car body repair shops						
Mixing PU lacquer	15	4 (2-7)	20	1.0 (0.2-2.7)	27	1.4 (0.3-33.1)
Spraying PU lacquer	31	8 (3-40)	65	2.1 (0.2-6.5)	87	116.3 (2.5-728.4)
Cleaning spray gun	19	3 (1-8)	0	-	32	11.1 (1.6-45.3)
Welding	3	22 (5-29)	33	0.04	33	0.1
Industrial painting company						
Spraying PU lacquer	10	25 (7-33)	100	3.7 (0.03-28.8)	100	199.6 (6.4-2613.8)
Rolling/ brushing PU lacquer	11	25 (7-41)	100	0.02 (0.01-0.1)	46	0.7 (0.1-5.3)
Mixing PU lacquer	3	8 (4-10)	67	0.5 (0.01-1.0)	67	10.8 (1.6-20.0)
Assisting spray painting	3	31 (29-40)	100	0.3 (0.09-4.4)	100	14.2 (6.3-347.7)

Table 2.2.2: Task-based dermal exposure mass on 2 gloves in car body repair shops and industrial painting companies. Percentage of samples above the limit of detection (LOD) and exposure median and range (µg NCO) for samples above LOD.

	Sampling time			1,6-HDI		Oligomers
	n	(min) Median (range)	%> LOD	Median (range)	%> LOD	Median (range)
Car body repair shops						
Mixing PU lacquer	15	4 (2-7)	47	2.2 (0.3-20.1)	53	207.3 (19.5-2848.5)
Spraying PU lacquer	31	8 (3-40)	39	1.3 (0.3-10.2)	42	133.2 (6.5-1507.0)
Cleaning spray gun	19	3 (1-8)	42	1.3 (0.3-2.0)	32	33.8 (15.5-315.8)
Welding	3	22 (5-29)	0	-	0	-
Industrial painting company						
Spraying PU lacquer	10	25 (7-33)	10	0.5	90	43.7 (3.8-209.8)
Rolling/ brushing PU lacquer	11	25 (7-41)	0	-	91	15.7 (3.5-153.9)
Mixing PU lacquer	3	8 (4-10)	0	-	100	63.0 (3.5-95.3)
Helper spray painting	3	31 (29-40)	0	-	33	0.7

ORs for the association between detectable isocyanate levels on the sampling gloves and glove use (observed by a field worker) as well as inhalation exposure level separately for both branches are given in Table 2.2.3. In car body repair shops the association for glove use was highly significant. When including both covariates in the same model the effect of glove use and inhalation exposure remain similar. In industrial painting companies no positive association could be found with inhalation levels and no OR for glove use could be computed since all workers wore gloves.

Urinary HDA
On the sampling days 239 urine samples were collected from 45 car body repair shop workers and 52 urine samples were collected from 10 industrial painting company workers (Table 2.2.4). On average 5 urine samples per worker were collected (standard deviation: 1.5) in both branches.

Questionnaires were used to categorize workers into mutually exclusive categories based on tasks performed: spray painting, paint handling (no spraying) and welding. Workers who did not perform any of these tasks were classified as bystanders in case they indicated to have been near a spray painting job, as office in case they spent a full day in the office and as no bystander/office when the worker neither reported to have been near a spray painting job nor spent a full day in the office. Eleven car body repair shop workers who neither worked with paint nor welded and of whom no specific information on bystanding or office work was available were indicated as unspecified.
Figures 2.2.2 A,B,C and D show the HDA curves for workers with at least one positive HDA sample for different groups of workers. In car body repair shops HDA could be detected in urine of ~25% of the spray painters, ~50% of the welders, ~50% of the bystanders and ~25% of other workers. Additionally, no obvious differences in urine levels could be observed between the different groups of workers. For the industrial spray company workers HDA could be detected in urine of ~25% of the spray painters and none of the bystanders or office workers.

Table 2.2.5 shows the fraction of detectable urine samples, the OR for having a positive sample (reference = 0-8 AM) as well as the HDA concentration median and range for different time intervals of the measurement day in car body repair shops. Four urine samples (4 workers) were excluded since they were collected after 8 AM on the next day. The frequency of detectable urine sample was significantly elevated in the late afternoon (3-6 PM) when compared to early morning (0-8 AM).

From 37 workers urine was collected before 8 AM on the measurement day. Four out of 29 workers that did not spray on the preceding day had detectable HDA levels before 8 AM. In contrast 2 out of 8 workers that did spray on the day before started with detectable HDA levels (OR 2.0, CI 0.3-14.2).

Table 2.2.3: Odds ratios for the univariate association between detectable task-based dermal exposure and glove use and inhalation exposure level separately during all measured tasks.

	Car body repair shops	**Industrial painting companies**
Gloves vs no gloves	0.22 (0.09-0.57)	-*
Inhalation exposure level (µg/m^3 NCO)**	1.34 (0.97-1.84)	0.97 (0.68-1.38)

* No OR calculated since all workers used gloves
** OR for a 10-fold increase in inhalation exposure levels

Table 2.2.4: Overview of urine samples taken in car body repair shops and industrial painting companies.

	Car body repair shop				Industrial painting companies			
	N workers	% workers pos. HDA	N urine samples	% samples pos. HDA	N workers	% workers pos. HDA	N urine samples	% samples pos. HDA
Spray painting	15	27%	74	19%	4	25%	18	22%
Paint handling	1	100%	6	67%	2	0%	12	0%
Welding	8	50%	44	27%	-	-	-	-
Other tasks - bystander	4	50%	22	41%	3	0%	19	0%
Other tasks - office	1	0%	5	0%	1	0%	3	0%
Other tasks - no byst./office	5	20%	25	4%	-	-	-	-
Other tasks - unspecified	11	36%	63	14%	-	-	-	-
Total	45	36%	239	21%	10	10%	52	8%

Table 2.2.5: Characteristics of urine samples from car body repair shop workers per time interval (0 = midnight before working day): fraction of samples above LOD, OR of being above the LOD vs 0-8 AM working day (corrected for repeated measurements) and concentration range and median in samples above LOD.

Time interval	Fraction above LOD	OR (95% CI) detectable samples	Median level (range) detects (μg HDA/gC)
0-8 AM	6/38	-	13.1 (6.8-50.2)
8-12 AM	7/41	1.10 (0.85-1.44)	7.3 (2.3-67.8)
0-3 PM	4/35	1.06 (0.59-1.91)	21.5 (2.7-150.2)
3-6 PM	7/30	2.13 (1.07-4.22)	18.0 (1.9-89.9)
6-12 PM	12/45	2.00 (0.91-4.40)	20.6 (1.9-61.7)
0-8 AM next day	12/46	2.04 (0.91-4.59)	8.1 (3.2-65.6)

Information on task-based use of PPE during the measurement day was also collected through the questionnaires. During spray painting respiratory protection was widespread but gloves were used by 40% and 75% of the workers in car body repair shops and industrial painting companies respectively. Less than 50% of workers in both industries reported to have used respiratory protection during mixing of paint. The same applies for glove use during mixing of paint.

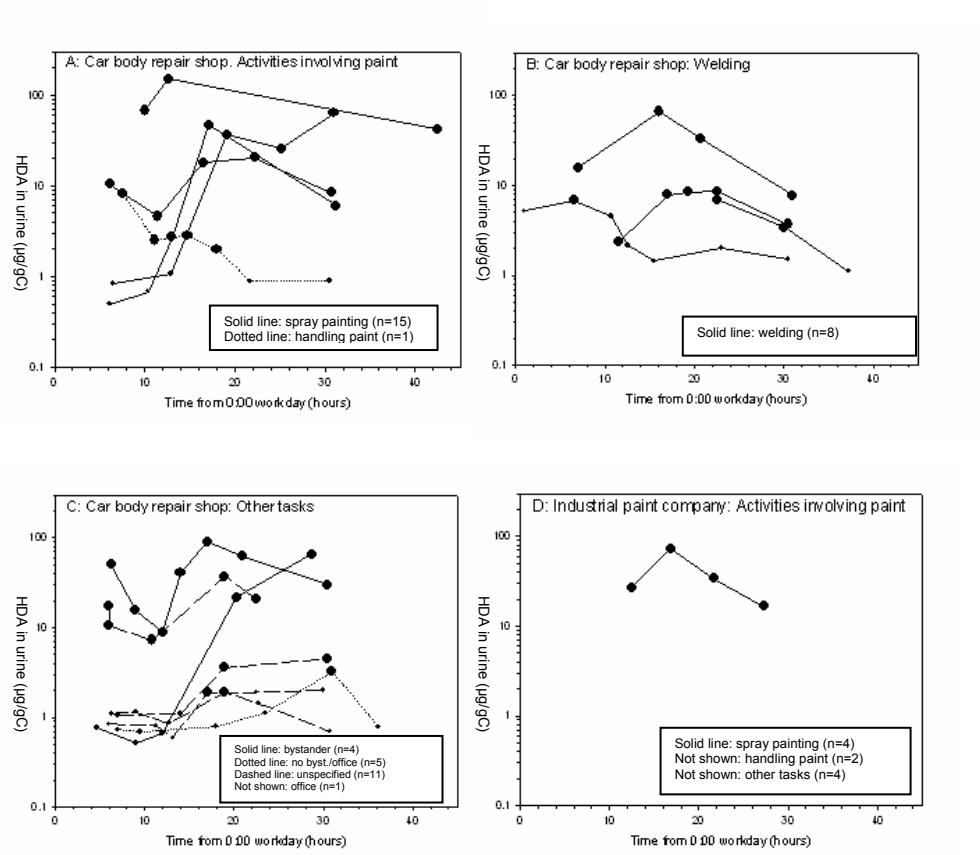

Figure 2.2.2: HDA in urine from car body repair shop workers (A, B and C) and industrial spray painters (D) per activity during the working day (0:00 = midnight before working day). Car body repair shop workers: A: activities involving paint, B: welding, C: other tasks. Industrial painting companies: D: activities involving paint. Large dots (●) indicate samples above LOD, small dots (•) indicate samples below LOD. Only workers with at least one positive urine sample are shown.

Discussion

In this study we focused on task-based inhalation and dermal exposure measurements of HDI and its oligomers in conjunction with urinary HDA levels in car body repair shop workers and industrial spray painters. This study is the first field study to quantitatively assess dermal exposure as well as to combine dermal and inhalation exposure measurements of HDI and oligomers with biomonitoring.

In the literature no existing methods on quantitative assessment of dermal isocyanate exposure could be found. Due to the high reactivity of isocyanates, a

reagent is necessary to capture the isocyanates. However, the reagent used for inhalation sampling (DBA) is not suitable for direct application on patches, sampling gloves or wipes because of toxicity and volatility. Moreover, the importance of a good sense of touch during spray painting prevents the use of cotton gloves or patches on the fingers. The present study shows that it is possible to quantitatively assess task-based dermal exposure using nitril rubber gloves, which are submerged into DBA immediately upon sampling, as a sampling matrix. The different masses of HDI and isocyanurate were efficiently collected form the gloves. In addition the recovery after 30 minutes on the glove before submersion into DBA in toluene was above 75% for both compounds.

The field samples show that dermal exposure occurs during a substantial fraction of all tasks that involve direct handling of paint. In car body repair shops detectable dermal exposure was negatively associated with glove use and positively associated with inhalation exposure level. Differences in frequency of detects as well as exposure levels between paint related tasks are small compared to inhalation exposure, which is mainly found during tasks where aerosol formation occurs. Several mass transport processes as described in the conceptual model for assessment of dermal exposure may be involved (30). The association between inhalation exposure level and the occurrence of dermal exposure in car body repair shops suggests aerosol deposition. In addition, emission (splashing or spilling of exposure source) and transfer of isocyanates (from surface contaminant) may occur during all paint related tasks.
To date information on the role of dermal exposure in respiratory sensitization and disease aggravation is lacking. Yet, allergic contact dermatitis as well as skin irritation as a result of dermal exposure have been reported by isocyanate workers (31-36).

Summarizing, task-based external exposure estimates indicate that in car body repair shops and industrial painting companies mainly (spray) painters are exposed to HDI and its oligomers through inhalation as well as dermal contact. Interpretation of these short-term exposure levels with respect to a total internal dose is complicated by large variability in levels within tasks, PPE use and the lack of knowledge on the relevance of dermal exposure (4).
In this context biomonitoring of HDA may give better insight into total HDI uptake. The half life of HDA after inhalation is a few hours which makes it possible to study the effect of recent exposure (17, 18). Knowledge on the metabolism of oligomers of HDI is lacking. Biomonitoring indeed demonstrates HDA in urine of 36% and 10% of all car body repair shop and industrial painting company workers, respectively. Detectable HDA is found in urine of only about 25% of the spray painters in both branches. Similar results were found in an earlier study (37). No obvious differences were found between groups of workers performing different tasks in car body repair shops. Since highest external exposures were found during spray painting tasks, this indicates that PPE may be better and even sufficient among spray painters mainly in industrial paint companies. However spray painters perform multiple tasks on a working

day during which both respiratory and dermal exposure may occur and task-based PPE use may be variable. Therefore this study is not suitable for analyzing the efficacy of PPE. An experimental design would be more appropriate for this purpose.

Surprisingly, in car body repair shops urinary HDA is not confined to spray painters but is also detected in a large proportion of the workers that neither handled paint nor reported bystander exposure. A possible explanation is a different source of HDA, which is for example also used as a raw material for the production of Nylon 66 (38). Another explanation is a longer half-life of HDI oligomers than of the monomer resulting in detectable HDA levels on days following HDI oligomer exposure. Although not significant, the OR of 2.0 for a worker being HDA positive before 8 AM when having sprayed on the preceding day may be indicative of an effect from earlier exposures. Yet, in car body repair shops the frequency of detectable samples is significantly elevated in the late afternoon indicating that HDA in urine was at least partly a result of exposures during the measurement day. More likely, non-spray painters without personal protection may receive unprotected 'bystander' exposure on the general work floor that is not picked up by task-based measurements. Possibly exposure peaks are generated when spray painting takes place outside the spray booth or when the spray booth is opened after the painted object has been cured. Long clearing times of air isocyanate levels after spraying have been described (37). Furthermore dermal exposure may occur from contaminated surfaces. Another explanation is inhalation exposure through contaminated dust. Although the levels of analyzed compounds in welding samples were very low, possibly other HDI based components may be released as a result of thermal degradation during welding or grinding.
Differences between car body repair shops and industrial painting companies responsible for the smaller proportion of positive bystanders and other workers remain unclear.

Like external exposure measurements, the use of HDA for internal exposure assessment of HDI based compounds is subject to difficulties. Large inter-individual variability exists, possibly as a result of acetylator status (38). A test chamber study found net increases of HDA ranging from 0.4 to 101 μg/g creatinine (GM 16.2, GSD 3.5) four hours after exposure to a HDI biuret aerosol of 58.2 (GM, GSD 1.6) $\mu g/m^3$ NCO (25). In addition the short half-life of HDI brings about that the urine samples only represent exposure over the past hours and HDA levels will vary greatly within a person over a working day (17, 25). Moreover the LOD for aliphatic amines is relatively high resulting in low sensitivity (39). Besides large variability between persons the test chamber study by Liu et al. (2004) showed that although urinary HDA was present after exposure to a biuret aerosol, the correlation between external exposure and the biomarker was weak (25). Thus, HDA might be more indicative of monomer exposure. However, knowledge on metabolites that better reflect isocyanate

oligomer exposure is lacking and to date HDA is the only usable biomarker of HDI based compounds.

In conclusion, in this study we succeeded to quantitatively assess dermal exposure using nitril rubber gloves. External exposure measurements indicate that mostly workers performing (spray) painting tasks are inhalatory and dermally exposed to mainly oligomers of HDI. Biomonitoring indeed demonstrates HDA in urine of a proportion of the workers. Due to large inter and intra-person variability, urinary HDA as an exposure measure for isocyanates is not easily interpretable. However, the results demonstrate that although (spray) painting results in highest external exposures, workers that do not perform paint related tasks may receive considerable doses of isocyanates. These findings need to be taken into account when using external exposure assessment data for risk assessment or epidemiological purposes.

References

1. Latza U, Baur X. Occupational obstructive airway diseases in Germany: Frequency and causes in an international comparison. Am J Ind Med 2005;48(2):144-52.
2. Redlich CA, Karol MH. Diisocyanate asthma: clinical aspects and immunopathogenesis. Int Immunopharmacol 2002;2(2-3):213-24.
3. Wisnewski AV, Redlich CA. Recent developments in diisocyanate asthma. Curr Opin Allergy Clin Immunol 2001;1(2):169-75.
4. Pronk A, Tielemans E, Skarping G, Bobeldijk I, van Hemmen J, Heederik D, et al. Inhalation exposure to isocyanates of car body repair shop workers and industrial spray painters. Ann Occup Hyg 2006;50(1):1-14.
5. Kimber I, Dearman RJ. Chemical respiratory allergy: role of IgE antibody and relevance of route of exposure. Toxicology 2002;181-182:311-5.
6. 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(4):1183-93.
7. Scheerens H, Buckley TL, Muis TL, Garssen J, Dormans J, Nijkamp FP, et al. Long-term topical exposure to toluene diisocyanate in mice leads to antibody production and in vivo airway hyperresponsiveness three hours after intranasal challenge. Am J Respir Crit Care Med 1999;159(4 Pt 1):1074-80.
8. Rattray NJ, Botham PA, Hext PM, Woodcock DR, Fielding I, Dearman RJ, et al. Induction of respiratory hypersensitivity to diphenylmethane-4,4'-diisocyanate (MDI) in guinea pigs. Influence of route of exposure. Toxicology 1994;88(1-3):15-30.
9. Karol MH, Hauth BA, Riley EJ, Magreni CM. Dermal contact with toluene diisocyanate (TDI) produces respiratory tract hypersensitivity in guinea pigs. Toxicol Appl Pharmacol 1981;58(2):221-30.
10. Ebino K, Ueda H, Kawakatsu H, Shutoh Y, Kosaka T, Nagayoshi E, et al. Isolated airway exposure to toluene diisocyanate results in skin sensitization. Toxicol Lett 2001;121(1):79-85.
11. Hughson GW, Aitken RJ. Determination of dermal exposures during mixing, spraying and wiping activities. Ann Occup Hyg 2004;48(3):245-55.
12. Delgado P, Porcel J, Abril I, Torres N, Teran A, Zugasti A. Potential dermal exposure during the painting process in car body repair shops. Ann Occup Hyg 2004;48(3):229-36.
13. Liu Y, Sparer J, Woskie SR, Cullen MR, Chung JS, Holm CT, et al. Qualitative assessment of isocyanate skin exposure in auto body shops: a pilot study. Am J Ind Med 2000;37(3):265-74.
14. Streicher RP, Kennedy ER, Lorberau CD. Strategies for the simultaneous collection of vapours and aerosols with emphasis on isocyanate sampling. Analyst 1994;119(1):89-97.
15. Streicher RP, Reh CM, Key-Schwartz R, Schlecht PC, Cassinelli ME, O'Connor PF. Selecting isocyanate sampling and analytical methods. Appl Occup Environ Hyg 2002;17(3):157-62.

16. Maitre A, Berode M, Perdrix A, Stoklov M, Mallion JM, Savolainen H. Urinary hexane diamine as an indicator of occupational exposure to hexamethylene diisocyanate. Int Arch Occup Environ Health 1996;69(1):65-8.
17. Brorson T, Skarping G, Nielsen J. Biological monitoring of isocyanates and related amines. II. Test chamber exposure of humans to 1,6-hexamethylene diisocyanate (HDI). Int Arch Occup Environ Health 1990;62(5):385-9.
18. Tinnerberg H, Skarping G, Dalene M, Hagmar L. Test chamber exposure of humans to 1,6-hexamethylene diisocyanate and isophorone diisocyanate. Int Arch Occup Environ Health 1995;67(6):367-74.
19. Kaaria K, Hirvonen A, Norppa H, Piirila P, Vainio H, Rosenberg C. Exposure to 4,4'-methylenediphenyl diisocyanate (MDI) during moulding of rigid polyurethane foam: determination of airborne MDI and urinary 4,4'-methylenedianiline (MDA). Analyst 2001;126(4):476-9.
20. Lind P, Dalene M, Skarping G, Hagmar L. Toxicokinetics of 2,4- and 2,6-toluenediamine in hydrolysed urine and plasma after occupational exposure to 2,4- and 2,6- toluene diisocyanate. Occup Environ Med 1996;53(2):94-9.
21. Lind P, Dalene M, Tinnerberg H, Skarping G. Biomarkers in hydrolysed urine, plasma and erythrocytes among workers exposed to thermal degradation products from toluene diisocyanate foam. Analyst 1997;122(1):51-6.
22. Tinnerberg H, Dalene M, Skarping G. Air and biological monitoring of toluene diisocyanate in a flexible foam plant. Am Ind Hyg Assoc J 1997;58(3):229-35.
23. Kaaria K, Hirvonen A, Norppa H, Piirila P, Vainio H, Rosenberg C. Exposure to 2,4- and 2,6-toluene diisocyanate (TDI) during production of flexible foam: determination of airborne TDI and urinary 2,4- and 2,6-toluenediamine (TDA). Analyst 2001;126(7):1025-31.
24. Sakai T, Morita Y, Roh J, Kim H, Kim Y. Improvement in the GC-MS method for determining urinary toluene-diamine and its application to the biological monitoring of workers exposed to toluene-diisocyanate. Int Arch Occup Environ Health 2005.
25. Liu Y, Berode M, Stowe MH, Holm CT, Walsh FX, Slade MD, et al. Urinary hexane diamine to assess respiratory exposure to hexamethylene diisocyanate aerosol: a human inhalation study. Int J Occup Environ Health 2004;10(3):262-71.
26. Karlsson D, Dahlin J, Skarping G, Dalene M. Determination of isocyanates, aminoisocyanates and amines in air formed during the thermal degradation of polyurethane. J Environ Monit 2002;4(2):216-22.
27. Muller H. HPLC-Methode zur simultanen bestimmung von harnstoff, kreatinin und harnsäure in serum und urin. Fresenius Z Anal Chem 1988;332:464-7.
28. Lewalter J, Skarping G, Ellrich D, Schoen U. Hexamethylene diisocyanate (HDI) and hexamethylenediamine (HDA). In: Angerer J, Schaller K, editors. Analyses of hazardous substances in biological materials. Weinheim: Wiley-VCH; 2003. p. 119-31.
29. Zeger SL, Liang KY. Longitudinal data analysis for discrete and continuous outcomes. Biometrics 1986;42(1):121-30.
30. Schneider T, Vermeulen R, Brouwer DH, Cherrie JW, Kromhout H, Fogh CL. Conceptual model for assessment of dermal exposure. Occup Environ Med 1999;56(11):765-73.
31. Goossens A, Detienne T, Bruze M. Occupational allergic contact dermatitis caused by isocyanates. Contact Dermatitis 2002;47(5):304-8.
32. Estlander T, Keskinen H, Jolanki R, Kanerva L. Occupational dermatitis from exposure to polyurethane chemicals. Contact Dermatitis 1992;27(3):161-5.
33. Daftarian HS, Lushniak BD, Reh CM, Lewis DM. Evaluation of self-reported skin problems among workers exposed to toluene diisocyanate (TDI) at a foam manufacturing facility. J Occup Environ Med 2002;44(12):1197-202.
34. Larsen TH, Gregersen P, Jemec GB. Skin irritation and exposure to diisocyanates in orthopedic nurses working with soft casts. Am J Contact Dermat 2001;12(4):211-4.
35. Frick M, Bjorkner B, Hamnerius N, Zimerson E. Allergic contact dermatitis from dicyclohexylmethane-4,4'-diisocyanate. Contact Dermatitis 2003;48(6):305-9.
36. Frick M, Isaksson M, Bjorkner B, Hindsen M, Ponten A, Bruze M. Occupational allergic contact dermatitis in a company manufacturing boards coated with isocyanate lacquer. Contact Dermatitis 2003;48(5):255-60.
37. Williams NR, Jones K, Cocker J. Biological monitoring to assess exposure from use of isocyanates in motor vehicle repair. Occup Environ Med 1999;56(9):598-601.
38. Brorson T, Skarping G, Sandstrom JF, Stenberg M. Biological monitoring of isocyanates and related amines. I. Determination of 1,6-hexamethylene diamine (HDA) in hydrolysed human urine after oral administration of HDA. Int Arch Occup Environ Health 1990;62(1):79-84.

39. Rosenberg C, Nikkila K, Henriks-Eckerman ML, Peltonen K, Engstrorm K. Biological monitoring of aromatic diisocyanates in workers exposed to thermal degradation products of polyurethanes. J Environ Monit 2002;4(5):711-6.

Chapter 3

Exposure-response associations

Chapter 3.1

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

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Abstract

Associations between oligomeric isocyanate exposure, sensitization and respiratory disease have received little attention, despite the extensive use of isocyanate oligomers. Objectives of this study were to investigate exposure-response relationships of respiratory symptoms and sensitization in a large population occupationally exposed to isocyanate oligomers during spray painting.

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.

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.

In conclusion, 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.

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 $\mu g/m^3$ 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 vapor 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 3.1.1 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.

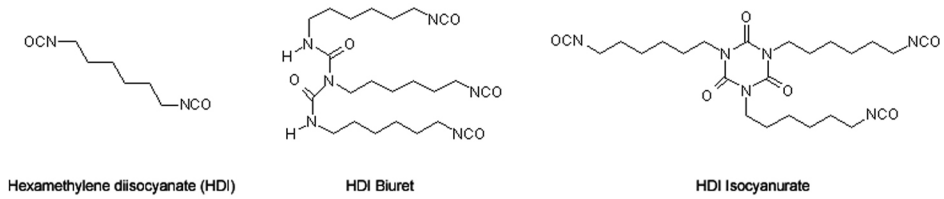

Figure 3.1.1: Chemical structures of hexamethylene diisocyanate (HDI) and two HDI oligomers.

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 mono-isocyanates, 5 amino-isocyanates, 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 μg NCO*m^{-3}*hr*$month^{-1}$;
n = 1,2, .. 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;
$(Median\ NCO\ concentration)_n$ = Median inhalatory isocyanate concentration during task n expressed in μg NCO/m^3.

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 (12). Briefly, personal samples were taken using midget impingers for sampling, di-n-butylamine as a reagent and LC-MS/MS for analysis. Diisocyanates, several mono-isocyanates, amino-isocyanates 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 in car body repair shops and industrial painting companies 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 exposure measurements were not available and walk-through surveys indicated that the spray-painting environment was very similar in these industries.

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 information on the exposure measurements is given in Chapter 2.2.

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 (HDI_L-HSA) (13) and vapor-phase (HDI_V-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 3.1.1 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 on the EIA procedures and establishment of cut-off values are provided in Chapter 4.1.

Table 3.1.1: 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_LHSA	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

Physiological testing
Bronchial hyperresponsiveness (BHR) was assessed in a subset of 229 workers. Selection of this subset is described in chapter 3.2. 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 European Respiratory Society 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 µmoles).

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 3.1.2.
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 μg NCO*m^{-3}*hr*$month^{-1}$ respectively). The minimum, 25th percentile, median, 75th percentile and maximum of the total exposure distribution were 0, 1.7, 165, 33821, and 66464 μg NCI*m^{-3}*hr*$month^{-1}$ respectively. Pearson correlation coefficients between the exposure estimates for total NCO, HDI, biuret and isocyanurate were very high (≥ 0.95).

Table 3.1.2: 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 pack years, 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/harbor 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 (μg NCO*m^{-3}*hr*$month^{-1}$):			
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)

Prevalence of symptoms and positive serology

Exposed workers more often reported respiratory symptoms than office workers (Table 3.1.3).

Table 3.1.3: 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

Asthma-like symptoms were significantly (p≤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 was 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 3.1.3). 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 3.1.4 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.

Table 3.1.4: Association between respiratory symptoms and positive IgE and IgG sensitization: Prevalence Ratio (95% confidence interval) 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 prevalence ratio

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 (1.7-3382 μg NCO*m^{-3}*hr*$month^{-1}$ or a difference in exposure of approximately a factor 2000) (Table 3.1.5). 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 3.1.5).

Table 3.1.5: Association between respiratory symptoms and specific IgE and IgG sensitization and exposure: Prevalence Ratio (95% confidence interval) 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

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 3.1.2A) and COPD-like symptoms (plot not shown) the smoothed plots corroborate log-linear relations. For work-related chest tightness (Figure 3.1.2B) 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 3.1.2C).
Interestingly, the prevalence of atopy was lower at high exposure levels. Figure 3.1.2D shows a sharp reduction for the prevalence of atopy at isocyanate exposures above ~1000 μg NCO*m^{-3}*hr*$month^{-1}$ (p spline ≤ 0.05). Atopic subjects were significantly less exposed than non-atopic subjects (geometric mean: 24.1 and 57.9 μg NCO*m^{-3}*hr*$month^{-1}$ respectively, p≤0.05).

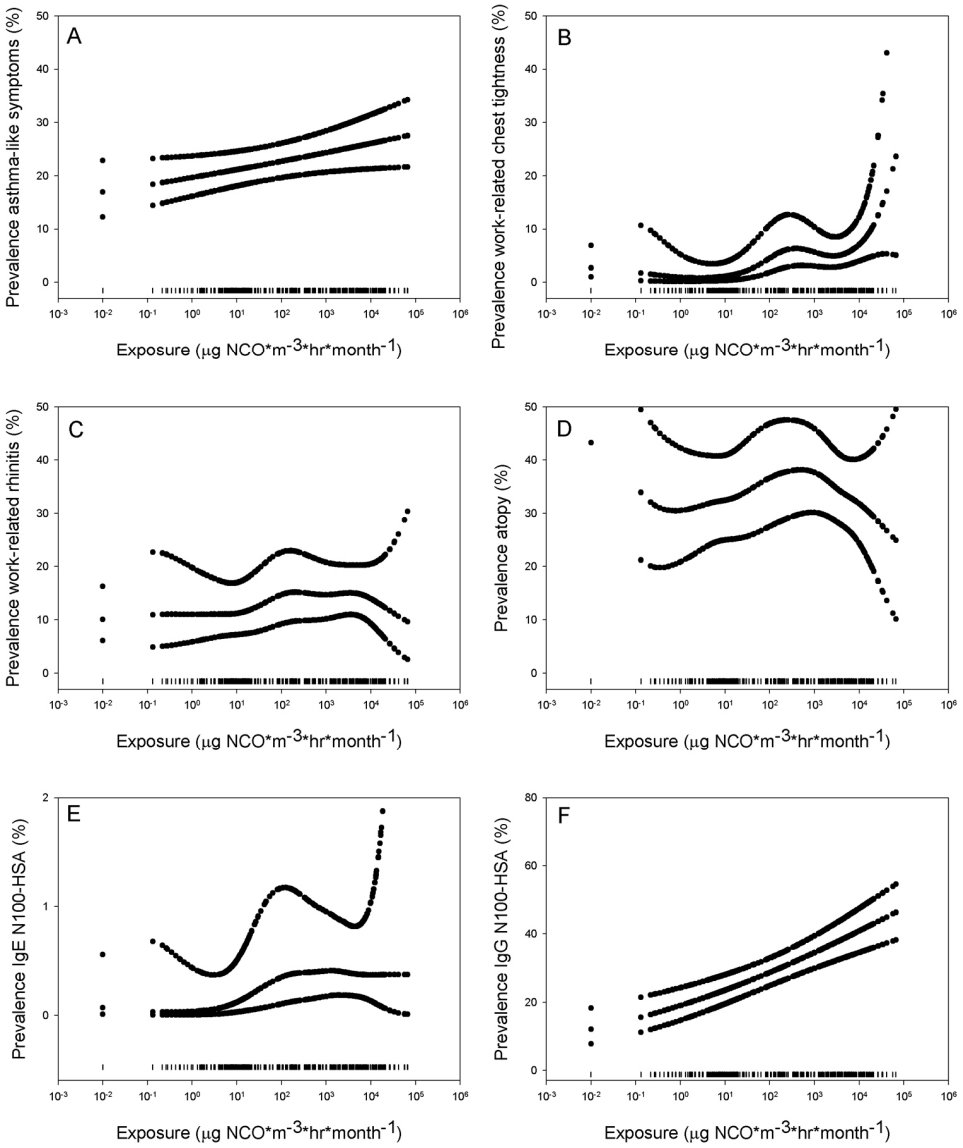

Figure 3.1.2: Association between log-transformed exposure to isocyanates (µg NCO*m^{-3}*hr*$month^{-1}$) and selected health end points. Penalized smoothed spline plots are given with smoothed 95% confidence intervals for: A: Asthma-like symptoms, spline: $p > 0.10$, B: Work-related chest tightness, 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.

Exposure was also associated with N100-HSA-specific IgE. The smoothed plot shows a very slight increase (Figure 3.1.2E). 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 μg NCO/m^3 were found which did not exceed the Dutch short-term exposure limit for HDI (70 μg NCO/m^3). In contrast, HDI oligomer levels ranged up to 3760 μg NCO/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.

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(3):213-28.
2. Bernstein JA. Overview of diisocyanate occupational asthma. Toxicology 1996;111(1-3):181-9.
3. Wisnewski AV, Redlich CA. Recent developments in diisocyanate asthma. Curr Opin Allergy Clin Immunol 2001;1(2):169-75.
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(2):146-53.
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(12):891-901.
7. Bello D, Woskie SR, Streicher RP, Liu Y, Stowe MH, Eisen EA, et al. Polyisocyanates in occupational environments: a critical review of exposure limits and metrics. Am J Ind Med 2004;46(5):480-91.
8. McDonald JC, Keynes HL, Meredith SK. Reported incidence of occupational asthma in the United Kingdom, 1989-97. Occup Environ Med 2000;57(12):823-9.
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(5):451-8.
10. Ameille J, Pauli G, Calastreng-Crinquand A, Vervloet D, Iwatsubo Y, Popin E, et al. Reported incidence of occupational asthma in France, 1996-99: the ONAP programme. Occup Environ Med 2003;60(2):136-41.
11. Bronchitis MRCCotAoC. 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, et al. Inhalation exposure to isocyanates of car body repair shop workers and industrial spray painters. Ann Occup Hyg 2006;50(1):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(6):337-40.
14. Wisnewski AV, Stowe MH, Cartier A, Liu Q, Liu J, Chen L, et al. Isocyanate vapor-induced antigenicity of human albumin. J Allergy Clin Immunol 2004;113(6):1178-84.
15. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, et al. Standardisation of spirometry. Eur Respir J 2005;26(2):319-38.
16. Sterk PJ, Fabbri LM, Quanjer PH, Cockcroft DW, O'Byrne PM, Anderson SD, et al. 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(1):91-5.
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(10):890-7.
20. White WG, Morris MJ, Sugden E, Zapata E. Isocyanate-induced asthma in a car factory. Lancet 1980;1(8171):756-60.
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(3):169-79.
22. Jones RN, Rando RJ, Glindmeyer HW, Foster TA, Hughes JM, O'Neil CE, et al. Abnormal lung function in polyurethane foam producers. Weak relationship to toluene diisocyanate exposures. Am Rev Respir Dis 1992;146(4):871-7.
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(4):627-33.
24. Bernstein DI, Korbee L, Stauder T, Bernstein JA, Scinto J, Herd ZL, et al. 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(3):387-96.
25. Ott MG, Klees JE, Poche SL. Respiratory health surveillance in a toluene di-isocyanate production unit, 1967-97: clinical observations and lung function analyses. Occup Environ Med 2000;57(1):43-52.
26. Meredith SK, Bugler J, Clark RL. Isocyanate exposure and occupational asthma: a case-referent study. Occup Environ Med 2000;57(12):830-6.
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(5):517-21.
28. Ulvestad B, Melbostad E, Fuglerud P. Asthma in tunnel workers exposed to synthetic resins. Scand J Work Environ Health 1999;25(4):335-41.
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(1):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(4):1183-93.
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(2):104-11.
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(3):299-310.
33. Talini D, Monteverdi A, Benvenuti A, Petrozzino M, Di Pede F, Lemmi M, et al. Asthma-like symptoms, atopy, and bronchial responsiveness in furniture workers. Occup Environ Med 1998;55(11):786-91.
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(6):503-10.
35. Cullen MR, Redlich CA, Beckett WS, Weltmann B, Sparer J, Jackson G, et al. 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(3):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(8):662-8.
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(7):582-8.
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(11):1096-100.
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(4-5):257-69.
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(3):231-47.
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(2-3):147-60.

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(3):154-64.
43. Raulf-Heimsoth M, Baur X. Pathomechanisms and pathophysiology of isocyanate-induced diseases--summary of present knowledge. Am J Ind Med 1998;34(2):137-43.
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(3):357-64.

Chapter 3.2

Bronchial hyperresponsiveness and lung function are associated with measured isocyanate exposure in spray painters

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Abstract

Associations have been observed between exposure to mainly hexamethylene diisocyanate (HDI) oligomers, (work-related) respiratory symptoms and isocyanate specific sensitization in a population of workers in the spray painting industry. The aim was to assess associations between exposure and objective measures such as bronchial hyperresponsiveness (BHR), baseline spirometry and exhaled NO (eNO) in a subset of that population.
Methacholine challenge and eNO measurements were performed in 229 workers. Questionnaires and blood samples were obtained. Personal exposure was estimated by combining personal task-based inhalatory exposure measurements and time activity information. Specific IgE and IgG to HDI were assessed in serum by ImmunoCAP assay and enzyme immunoassays using various HDI-human serum albumin (HSA) -conjugates.
A positive association was found between total isocyanate exposure and BHR (prevalence ratio (PR) and 95% confidence interval (CI) interquartile range increase in exposure: 1.8 (1.1-3.0)). Exposure related obstructive lung function changes independent of BHR were also found (FEV1, FEV1/FVC and flow parameters associated with exposure, $p<0.05$). Sensitized (specific IgE and IgG) workers were more often hyperresponsive. This was statistically significant for IgG positives assessed by ImmunoCAP assay only (PR (95% CI): 3.1 (1.1-8.2)). Although eNO was not associated with exposure, workers with IgG to the oligomeric HDI conjugates were suggested to have higher levels of eNO.
These findings strongly support our earlier observations that respiratory health effects are associated with isocyanate levels in workers exposed to mainly HDI oligomers in the spray painting industry.

Introduction

Isocyanates are among the most common causes of occupational asthma (1-3). Besides allergic asthma, isocyanate exposure may also cause irritant induced asthma, hypersensitivity pneumonitis and possibly accelerated lung function decline (4).
Diisocyanates, which are characterized by two highly reactive NCO-groups, are used as polymerizing agents in polyurethane products (5). Lately, isocyanate oligomers with considerably lower vapor pressures are being increasingly used to reduce inhalatory exposure (6). Exposure-response information is scarce for isocyanate oligomers because exposure assessment of mixtures of isocyanate oligomers is complex. In addition, these mixtures are frequently used in small scale end-user activities, which are difficult to study.
We recently demonstrated an association between exposure to isocyanates, (work-related) respiratory symptoms and isocyanate specific sensitization in a large population of spray painters exposed to mainly hexamethylene diisocyanate (HDI) oligomers (7).
To investigate the nature of respiratory health effects in this population more objective health effect measures were assessed in a subset of that population. The aim of this study was to evaluate associations between exposure and bronchial hyperresponsiveness (BHR) as a hallmark of asthma, baseline lung function parameters, and exhaled NO (eNO) as a marker of respiratory inflammation. We also explored associations between these respiratory effect measures and symptoms and serological anti-HDI responses.

Methods

Population and study design
The present study was carried out in a subset of a population of workers in the spray painting industry (7). At the time of selection results for specific IgE and IgG against diisocyanates measured by ImmunoCAP assay (Phadia, Uppsala, Sweden) were available from the baseline population. All workers from companies with at least one worker with detectable specific antibodies were invited to participate in the present study.
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. The institutional review board for human studies of University Medical Centre Utrecht approved of the protocols and written consent was obtained from all participants.

Questionnaire and exposure estimates
Questionnaire items were previously described (7). For statistical analyses respiratory symptoms suggestive of COPD and asthma were combined: "COPD-like symptoms" included chronic cough, chronic phlegm or shortness of breath; "asthma-like symptoms" included wheezing or chest tightness. Workers were

classified into the following categories based on task information: 'office workers', 'spray painters' and 'others' (7). The latter category consisted of mostly mechanics and metal workers.
Task-based personal inhalation exposure to 23 isocyanate compounds was assessed using midget impingers for sampling, di-n-butylamine as a reagent and LC-MS/MS for the analysis (8). Personal exposure estimates were obtained by combining over 500 measurements performed in the population under study and time activity information (7).

Serology
Blood samples were stored and processed as previously described (7). Briefly, specific IgE and IgG to HDI were assessed in serum by ImmunoCAP assay and enzyme immunoassay using vapor (HDI_V-HSA) and liquid (HDI_L-HSA) phase HDI-human serum albumin (HSA) and HSA-conjugates prepared with commercial products containing oligomeric HDI (N3300-HSA and N100-HSA). Specific IgE to common aeroallergens was assessed using the Phadiatop (Phadia, Uppsala, Sweden) as a measure of atopy. Cut-off values were used to dichotomize serological outcomes as previously described (7).

Spirometry and Methacholine challenge
At least 2 maximal expiratory flow-volume maneuvers were obtained to assess baseline lung function. From 31 workers only one technically acceptable maneuver could be obtained. The largest FEV1 and FVC were recorded. Other lung function parameters were obtained from the maneuver with the largest sum of FEV1+FVC as described by Miller et al. (9). Reference values were calculated using equations from the European Respiratory Society (ERS) (10).
BHR was assessed by methacholine challenge according to the ERS Guidelines (11). 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 μmoles). If necessary, bronchoconstriction was treated with inhalation of Albuterol.

Exhaled NO (eNO)
A NIOX MINO® hand held device was used to measure eNO according to American Thoracic Society/ERS recommendations (12). NO free air was inhaled to total lung capacity though the device and then exhaled through the device for 10 sec. During exhalation the flow rate was kept constant at 50 ± 5 ml/sec by a

built in flow control unit. The last 3 sec portion of exhaled air was analyzed by an electrochemical sensor in the device.

Statistical analyses
SAS v9.1 statistical software was used. Prevalence ratio's (PR) and 95% confidence intervals (95%CI) were calculated by log-binomial regression (PROC GENMOD) to describe associations for dichotomous health outcomes (BHR, symptoms, and serology). Because exposure was strongly skewed to the right, log-transformed exposure data were used. PRs per unit increase in exposure were converted to PRs per interquartile range (IQR) increase in exposure as found in the baseline study; IQR: 1.7-3382 μg $NCO*m^{-3}*hr*month^{-1}$, or approximately a factor 2000 (7). In the present study a similar IQR of 0.3-2799 μg $NCO*m^{-3}*hr*month^{-1}$ was found. 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 (13) but limited to three. Associations for continuous health outcomes (lung function parameters, log transformed eNO) were assessed by linear regression (PROC GENMOD). Unless stated otherwise all associations were adjusted for current smoking, age, gender and atopy. Associations for lung function parameters were additionally adjusted for length and race.

Results

Population characteristics
In total 229 workers from 38 companies participated in the present study. Of all companies invited, 90% participated and the worker participation rate within these companies was 66%. Of the workers who did not participate, 27% refused, 22% were not present at the time of the study and 51% had left the company (50% left for another company in the same industry, 25% left the industry and for 25% this was not known).
General characteristics of the study population are shown in Table 3.2.1. Estimated median total NCO exposure levels were higher in the spray painter category than among other workers, with a wide range in both categories. Exposure mainly consisted of HDI oligomers, and HDI monomer contributed for a small fraction to total NCO.

Table 3.2.1: General population Characteristics, work history and isocyanate exposure.

	Office workers	Spray painters	Others
N	20	91	118
Gender: % male	80	98	99
Age: AM (SD)	44.3 (11.9)	39.6 (9.7)	40.2 (10.4)
Smoking			
Smoker %	29.4	45.5	38.8
Smoker past yr %	0	1.1	5.2
Ever smoked %	41.2	20.5	23.3
Never smoked %	29.4	33.0	32.8
Packyears, AM (SD)	6.2 (8.0)	9.1 (13.5)	8.8 (13.9)
Branch type			
Car body repair shop %	65.0	52.8	88.1
Furniture paint shop %	5.0	11.0	4.2
Boat/harbor equipment paint shop %	0	5.5	0.9
Airplane paint shop %	30.0	30.8	6.8
Work history (AM (SD))			
Number of years worked	18.6 (12.4)	18.3 (9.7)	20.7 (10.8)
Number of years in branch	17.0 (13.6)	17.5 (9.5)	19.5 (11.1)
Number of years as spray painter	3.0 (6.1)	16.8 (9.9)	4.7 (8.9)
Isocyanate exposure (μg NCO*m^{-3}*hr*month^{-1})			
Total isocyanate, Median (minimum-maximum)	0	4,530 (15.4-66,464)	5.6 (0-3,785)
HDI, Median (minimum-maximum)	0	36.2 (1.3-472)	0.7 (0-354)

AM (SD): Arithmetic mean (standard deviation)

Symptoms, serology and associations with exposure

Spray painters significantly less often reported 'chest tightness before starting work' than office workers (adjusted PR (95% CI): 0.2 (0.04-0.8). No statistically significant differences were found for any of the other symptoms ($p<0.05$) (Table 3.2.2). Asthma-like symptoms were borderline significantly associated with exposure (adjusted PR per IQR increase in exposure (95% CI): 1.3 (0.9-1.7). COPD-like symptoms and work-related chest tightness prevalence increased with increasing exposure but associations were not significant (adjusted PR IQR: (95% CI): 1.1 (0.6-1.5) and 1.7 (0.7-4.2)) respectively.

Specific IgE sensitization was uncommon (up to ~4.4%) and not significantly associated with exposure. The prevalence of specific IgG sensitization was higher (up to 47%) and depended on the serological assay used. Specific IgG sensitization assessed by HDI_L-HSA, HDI_V-HSA and N100-HSA was more common in highly exposed individuals (adjusted PR's IQR: 1.2, 1.7 and 1.9 respectively, $p<0.1$). Individuals with high exposure were significantly less often atopic (adjusted PR IQR: (95% CI): 0.7 (0.6-0.9)).

Table 3.2.2: Prevalence of reported symptoms (n=229) and serological outcomes (n=223): atopy, and specific IgE and IgG sensitization against HDI.

	Office workers	Spray painters	Others
Respiratory symptoms %			
Chronic cough	15.0	13.2	14.4
Chronic phlegm	20.0	13.2	13.7
Shortness of breath	10.0	8.9	12.7
Wheezing	15.0	35.2	25.4
Frequent wheezing (>1w)	10.5	11.1	6.8
Shortness of breath during wheezing	10.5	14.4	14.4
Chest tightness	25.0	14.3	13.7
Chest tightness before start work	15.0	3.3*¶	6.8
Work-related allergic symptoms %			
Work-related rhinitis	35.0	15.9^	22.2
Work-related chest tightness	5.0	8.0	4.3
Work-related conjunctivitis	20.0	13.6	10.3
Clusters of symptoms %			
COPD-like symptoms	25.0	26.4	27.4
Asthma-like symptoms	26.3	35.6	29.1
Serology			
Atopy (Phadiatop)	55.0	39.3^§	39.5^§
Specific IgE			
HDI-ImmunoCAP	0	2.3	1.8
HDI_L-HSA	0	2.3	4.4
HDI_V-HSA	0	0	1.8
N3300-HSA	0	1.1	1.8
N100-HSA	0	3.4	3.5
Specific IgG			
HDI-ImmunoCAP	5.0	11.2	10.5
HDI_L-HSA	40.0	47.2	43.0
HDI_V-HSA	5.0	19.1	14.0
N3300-HSA	10.0	22.5	17.5
N100-HSA	0	38.2	23.7

Significantly different from 'office worker' category: * p< 0.05, ^ p<0.10 after correction for atopy, current smoking, age and gender
¶ adjusted for atopy, current smoking and gender
§adjusted for age

Bronchial hyperresponsiveness, spirometry and eNO

From 14 workers BHR and baseline spirometry were not determined because of no acceptable maneuver (6), ß-blocker usage (3), other medical reasons (3) and refusal (2). One person stopped during challenge because of health complaints. In 11 workers, eNO could not be determined because of problems with exhaling at a constant flow (3), health complaints (1), refusal (3) and device errors (4).

None of the office workers showed bronchial hyperresponsiveness (BHR20) (Table 3.2.3) despite the fact that many office workers had asthma-like symptoms and were atopic. In contrast 20% of the spray painters were

hyperresponsive. When using a fall in FEV1 of 15% as a cut-off level (BHR15), a prevalence of 15, 29 and 22% was found for office workers, spray painters and other workers respectively. A total of 18 workers met the GOLD criteria for COPD (FEV1/FVC < 70%); 12 fell in the GOLD1 category and 6 in the GOLD2 category (4 spray painters and 2 other workers). Geometric mean eNO levels were between 17.0 and 19.9 ppb without significant differences between job title categories (Table 3.2.3).

Table 3.2.3: Baseline spirometry (% reference value, n=215), FEV1/FVC<70% (n=215), bronchial hyperresponsiveness (BHR20, n=214) and exhaled NO (ppb, n=218).

	Office workers	Spray painters	Others
Lung function parameters, AM (SD)			
FEV1	103.6 (14.8)	101.6 (14.1)	104.6 (13.3)
FVC	103.6 (16.5)	106.02 (11.4)	103.9 (13.9)
FEV1/FVC	103.6 (8.5)	98.7 (11.3)*¶	103.6 (10.0)
MMEF	92.9 (22.7)	83.3 (29.8)ǂ	94.8 (28.7)
PEF	126.0 (25.8)	114.9 (20.2)*¶	121.8 (21.7)
MEF75	112.7 (23.3)	100.5 (26.7)ǂ	110.5 (26.7)
MEF50	98.4 (26.0)	87.4 (31.5)ǂ	96.5 (29.2)
MEF25	77.7 (25.7)	72.6 (31.5)ǂ	87.6 (35.5)
FEV1/FVC < 70% (%)	5.0	15.3	3.6
BHR20 (%)	0	20.0	14.7
Exhaled NO, GM (GSD)	19.9 (1.8)	17.0 (1.9)	17.3 (1.8)

* Significantly different from 'office workers', ǂ significantly different from 'others' category (P<0.05). AM (SD): Arithmetic mean (standard deviation), GM (GSD): Geometric standard deviation)

Associations with symptoms and serology

Workers with asthma-like symptoms had significantly more BHR and significantly lower baseline FEV1, FEV1/FVC and MMEF. COPD-like symptoms or work-related rhinitis were only (borderline) significantly associated with BHR (7). Statistically significantly increased exhaled NO levels were found in workers with reported asthma-like symptoms and work-related rhinitis and conjunctivitis (adjusted PR (95% CI): 1.7 (1.1-2.5), 1.6 (1.1-2.4) and 1.9 (1.1-3.6) respectively).

Overall sensitized (specific IgE and IgG) workers were more often hyperresponsive (Table 3.2.4). This was statistically significant for IgG measured with the ImmunoCAP assay only. Exhaled NO was not associated with a specific IgE antibody response to isocyanates, but was increased in workers with specific IgG against N3300-HSA or N100-HSA (Table 3.2.4).

Table 3.2.4: Associations between BHR and specific sensitization (Prevalence ratio (95% Confidence Interval)) and between exhaled NO and specific sensitization (ß log eNO (ppb) (p-value)) adjusted for current smoking, age, gender and atopy.

	BHR20	Log eNO (ppb)
Specific IgE		
HDI-ImmunoCAP	1.62 (0.30-8.85)*	0.312 (0.24)
HDI_L-HSA	1.69 (0.53-5.45)*	0.182 (0.37)
HDI_V-HSA	2.32 (0.52-10.31)*	-0.043 (0.91)
N3300-HSA	1.96 (0.39-9.93)*	0.32 (0.30)
N100-HSA	2.38 (0.75-7.57)*	0.190 (0.35)
Specific IgG		
HDI-ImmunoCAP	3.07 (1.14-8.22)	0.153 (0.20)
HDI_L-HSA	0.76 (0.40-1.45)	0.040 (0.58)
HDI_V-HSA	1.06 (0.45-2.49)	0.064 (0.52)
N3300-HSA	1.28 (0.61-2.69)	0.169 (0.07)
N100-HSA	1.03 (0.52-2.05)	0.156 (0.05)

* adjusted for current smoking, gender and atopy

Associations with exposure

Hyperresponsiveness was strongly associated with exposure expressed as total NCO (Table 3.2.5). Exposure-response relationships explored using smoothed spline plots revealed similar log-linear associations for both BHR20 and BHR15. The likelihood of being hyperresponsive increased gradually with increasing exposure without a clear indication for an exposure threshold (Figure 3.2.1A).

COPD according to the GOLD criteria (FEV1/FVC<70%) was also strongly associated with exposure (Table 3.2.5). Other determinants of FEV1/FVC<70% were age and smoking, which were not associated with BHR. No significant association between exposure and exhaled NO was found for log eNO on a continuous scale or dichotomized with 75^{th} or 90^{th} percentiles as cut-off points.

To explore associations between exposure and potentially different asthma phenotypes, BHR20 was combined with other parameters. The combination with FEV1/FVC <70% or eNO resulted in strong and (borderline) significant associations with exposure (Table 3.2.5). Especially asthma-like symptoms with BHR20 were also strongly associated with exposure in a log-linear fashion (Table 3.2.5, Figure 3.2.1B).

Individuals with high exposure had a lower FEV1, FEV1/FVC, and flow volume parameters (Table 3.2.6). For all parameters but MEF25, associations were significantly different between atopic and non-atopic individuals ($p<0.05$). Surprisingly, after stratification, these associations between lung function and exposure were especially found in non-atopic workers. When hyper-responsive individuals were excluded from the analyses, similar associations between exposure and lung function were found. Smoothed spline plots showed that at medium to high exposure levels exposure-response relationships in atopic individuals run parallel to those observed in non-atopic individuals. However, low exposed atopic individuals showed a systematically lower lung function. Consequently, associations within atopic individuals were not statistically significant (data not shown). Excluding the workers with only one technically

acceptable baseline maneuver did not affect the association between lung function parameters or BHR and exposure.

Table 3.2.5: Association between (combined) health end-points and exposure adjusted for current smoking, age, gender and atopy (PR (95% CI)).

	n	PR (95% CI)
BHR20	33	1.8 (1.1-3.0)
eNO (ppb) ≥ 90th percentile	22	0.9 (0.5-1.6)*
FEV1/FVC < 70%	18	2.3 (1.1-4.9)
BHR20 + eNO (ppb) ≥ 90th percentile	6	5.2 (0.9-31.0)*
BHR20 + FEV1/FVC < 70%	10	4.4 (1.1-17.1)**
BHR20 + Asthma-like symptoms	19	2.3 (1.02-5.0)
BHR20 + COPD-like symptoms	15	1.4 (0.7-3.1)
BHR20 + Work-related chest tightness	3	0.9 (0.1-5.9)***
BHR20 + Work-related rhinitis	10	2.0 (0.7-5.7)****
BHR20 + Work-related conjunctivitis	7	3.4 (0.7-16.2)****

Adjusted for smoking, age, gender and atopy, * adjusted for atopy, ** adjusted for smoking
*** adjusted for smoking, age and atopy, **** adjusted for smoking, gender and atopy

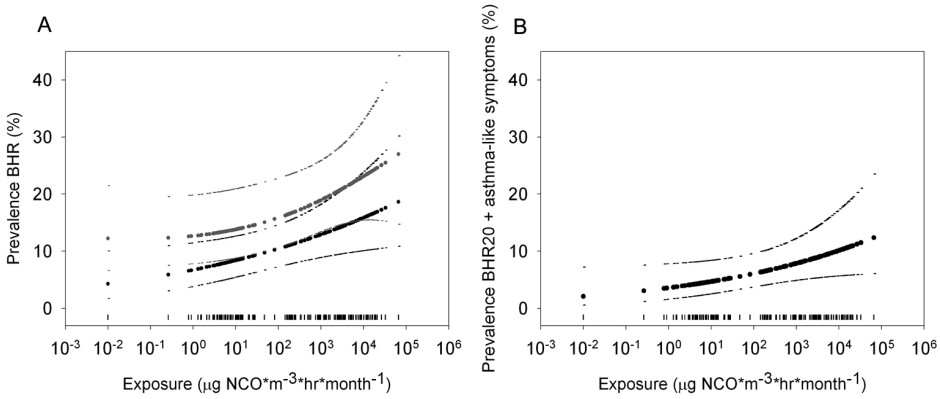

Figure 3.2.1: Association between log-linear exposure to isocyanates and health end-points. Penalized smoothed spline plots are given with smoothed 95% confidence intervals for. A: BHR20 (black) and BHR15 (grey), splines: non significant (ns), B: BHR20 combined with asthma-like symptoms, spline: ns. Data rugs at the bottom of each graph indicate the distribution of data points.

Table 3.2.6: Association between lung function parameters and exposure (ß log exposure (p-value)) adjusted for current smoking, age, gender, height and atopy.

	All	Non-atopics	Atopics
FEV1	-0.004 (0.60)	-0.017 (0.08)	0.012 (0.27)*
FVC	0.015 (0.08)	0.010 (0.37)	0.02 (0.10)
FEV1/FVC	-0.321 (0.004)	-0.543 (0.0002)	-0.088 (0.61)*
PEF	-0.026 (0.31)	-0.088 (0.01)	0.045 (0.22)*
MMEF	-0.037 (0.03)	-0.071 (0.003)	0.0014 (0.95)*
MEF75	-0.043 (0.13)	-0.109 (0.01)	0.032 (0.42)*
MEF50	-0.037 (0.07)	-0.076 (0.01)	0.010 (0.72)*
MEF25	-0.024 (0.02)	-0.038 (0.002)	-0.010 (0.51)

* Atopics and non atopics significantly different (p<0.05)

Discussion

In this study a positive association was found between exposure to isocyanate oligomers and bronchial hyperresponsiveness. In addition, indications of exposure related obstructive lung function changes (FEV1, FEV1/FVC and flow parameters) independent of BHR were found. These findings support our earlier observations (7) that respiratory symptoms are associated with isocyanate exposure level in workers exposed to mainly HDI oligomers in the spray painting industry.

Overall, the prevalence of symptoms and sensitization in the present study was similar to the baseline study (7). Office workers had more symptoms compared to the baseline study and were more often atopic. This may have influenced associations between exposure and (work-related) respiratory symptoms, which were less strong and often not statistically significant compared to the baseline study. The lengthy and more invasive tests in the present study may have led to a participation bias of atopic and symptomatic office workers since these have short working hours and can often not be replaced. In addition, in both studies a negative association between atopy and exposure to isocyanates was found, which was earlier interpreted as indicative of a healthy worker effect (7). Despite these possible biases, which would both lead to an underestimation of exposure-response relationships, associations between isocyanate exposure and respiratory effects were found.

A clear association between exposure to isocyanates and BHR was observed. BHR is closely related to variable airway obstruction and is usually considered a hallmark of asthma in epidemiological studies (14). The associations between BHR and both exposure and reported asthma-like symptoms are suggestive of isocyanate-induced asthma in this population. BHR and asthma-like symptoms (wheezing or chest tightness) taken together appeared even more strongly related to exposure. Few studies have assessed BHR on the population level in isocyanate exposed workers. A study by Redlich et al. showed a similar pattern of BHR across job title categories in car body repair shop workers, which did not reach statistical significance due to small sample size (15). A larger study in furniture painters did not find more bronchial hyperresponsiveness in painters than in controls (assembly and wood workers) (16). Yet, this is the first study in which quantitative exposure assessment has been conducted and in which exposure-response relationships are investigated.
The positive, but not significant associations between specific IgE and BHR may indicate that IgE is a mediator in the development of isocyanate asthma. However, specific IgE sensitization occurs only in very few individuals and as a result, just a small proportion of BHR20 may be attributed to IgE mediated responses in this study. Moreover, specific IgE was found in only 1 of the 19 workers with both BHR20 and asthma-like symptoms and in none of the 9 workers with both BHR20 and FEV1/FVC<70%. These results do not point towards specific IgE as an important mediator in the development of asthma in

this population. Other mechanisms, like cell-mediated allergic or irritant mechanisms may be involved (4, 17). Exacerbations of pre-existing asthma due to isocyanate exposure may explain the findings to some extent, although the clear exposure-related difference in prevalence of BHR pleas against an important role for exacerbations of pre-existing asthma.

The association between IgG to HDI-ImmunoCAP and BHR20 in the absence of an association between HDI-ImmunoCAP and exposure is remarkable and needs further exploration. Strikingly, the presence of specific IgG antibodies also seemed associated with elevated eNO levels. Exhaled NO is thought to reflect eosinophilic inflammation as seen in allergic airway disease (18, 19) and has been associated with BHR in atopic asthmatics (20). While eNO seems to be a direct marker of a classic allergen reaction immediately after exposure (21, 18), BHR probably reflects the continuous process of airway wall remodeling (22). A specific inhalation study has shown significantly more eNO increase in isocyanate-responders, of whom 25% showed diisocyanate specific IgE antibodies (23). We showed that eNO is associated with symptoms and BHR in this study, but found no clear association with isocyanate exposure. Interestingly, BHR in the presence of high eNO levels was strongly associated with exposure. Combining BHR and eNO may have resulted in a distinct subgroup of allergic asthmatics (22). Although specific IgE to any of the isocyanate conjugates was not found in any of the six workers with BHR and high eNO levels all of these individuals were atopic.

Exposure to isocyanates was also associated with obstructive lung function changes. Accelerated lung function decline has been demonstrated in adults with self-reported asthma (24) and a recent study suggests that occupational asthma results in faster rates of decline than non-occupational asthma (25). Although these changes may be observed in specific subgroups of asthmatics (26), differences in lung function are usually not observed in the population at large in healthy working populations exposed to occupational allergens. The distinction between asthma and COPD, which are both phenotypic heterogeneous diseases characterized by obstructive airflow limitation and inflammatory changes in the respiratory tract (27), is difficult. BHR and lung function decline may be present in both and the present study was not directed to confirm a diagnosis of asthma or COPD. BHR in the presence of FEV1/FVC < 70% was strongly associated with exposure to isocyanates. This may reflect asthma related chronic effects. Yet, the association between lung function parameters and exposure was also found in non-atopic workers without BHR. Since asthma was probably not manifest in these workers, other mechanisms are likely involved. Lung function decline caused by isocyanates has been previously described. Early longitudinal studies in TDI or foam manufacturing units demonstrated accelerated FEV1 loss among exposed workers but this was not reproduced in later studies in similar industries (28). In spray painters, isocyanate exposure related effects on lung function have also been shown (29, 30). Interestingly, we primarily found effects on lung function in non-atopic workers. Since smoking was not

associated with atopy, it seems unlikely that smoking is responsible for the observed differences between atopic and non-atopic subjects.
It should be noted that bronchodilators were not administered before baseline spirometry. This may have resulted in overestimation of chronic respiratory effects (31), especially since spirometry was performed on a working day at the company.

In conclusion, this study provides evidence of associations between exposure to isocyanates and asthma but also points towards chronic respiratory effects which are more confined to chronic bronchitis with or without bronchus obstruction. The mechanisms behind the development of these respiratory effects are unclear. Different phenotypical subgroups with respect to BHR, FEV1/FVC<70%, antibodies, and increased eNO were observed. These may reflect different health end-points, underlying mechanisms, and disease stages.

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(3):213-28.
2. Bernstein JA. Overview of diisocyanate occupational asthma. Toxicology 1996;111(1-3):181-9.
3. Wisnewski AV, Redlich CA. Recent developments in diisocyanate asthma. Curr Opin Allergy Clin Immunol 2001;1(2):169-75.
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(2):146-53.
6. Wisnewski Aea. Polyisocyanates and their prepolymers. In: Bernstein IC-Y, M; Malo, JL; Bernstein, DI, editor. Asthma in the workplace. New York: Taylor & Francis Group; 2006. p. 481-504.
7. Pronk A, Preller L, Raulf-Heimsoth M, Jonkers IC, Lammers JW, Wouters IM, et al. Respiratory symptoms, sensitization and exposure-response associations in HDI exposed spray painters. Am J Respir Crit Care Med 2007; Epub ahead of print.
8. Pronk A, Tielemans E, Skarping G, Bobeldijk I, van Hemmen J, Heederik D, et al. Inhalation exposure to isocyanates of car body repair shop workers and industrial spray painters. Ann Occup Hyg 2006;50(1):1-14.
9. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, et al. Standardisation of spirometry. Eur Respir J 2005;26(2):319-38.
10. Quanjer PH, Tammeling GJ, Cotes JE, Pedersen OF, Peslin R, Yernault JC. Lung volumes and forced ventilatory flows. 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;16:5-40.
11. Sterk PJ, Fabbri LM, Quanjer PH, Cockcroft DW, O'Byrne PM, Anderson SD, et al. 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.
12. ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005. Am J Respir Crit Care Med 2005;171(8):912-30.
13. Hastie T, Tibshirani RJ. Generalized additive models. New York: Chapman & Hall; 1990.
14. Becklake MR, Malo JL, Chan-Yeung M. Epidemiological approaches in occupational asthma. In: Bernstein IL, Chan-Yeung M, Malo JL, Bernstein DI, editors. Asthma in the workplace. New York: Taylor and Francis Group; 2006.

15. Redlich CA, Stowe MH, Wisnewski AV, Eisen EA, Karol MH, Lemus R, et al. Subclinical immunologic and physiologic responses in hexamethylene diisocyanate-exposed auto body shop workers. Am J Ind Med 2001;39(6):587-97.
16. Talini D, Monteverdi A, Benvenuti A, Petrozzino M, Di Pede F, Lemmi M, et al. Asthma-like symptoms, atopy, and bronchial responsiveness in furniture workers. Occup Environ Med 1998;55(11):786-91.
17. Raulf-Heimsoth M, Baur X. Pathomechanisms and pathophysiology of isocyanate-induced diseases--summary of present knowledge. Am J Ind Med 1998;34(2):137-43.
18. Simpson A, Custovic A, Pipis S, Adisesh A, Faragher B, Woodcock A. Exhaled nitric oxide, sensitization, and exposure to allergens in patients with asthma who are not taking inhaled steroids. Am J Respir Crit Care Med 1999;160(1):45-9.
19. Frank TL, Adisesh A, Pickering AC, Morrison JF, Wright T, Francis H, et al. Relationship between exhaled nitric oxide and childhood asthma. Am J Respir Crit Care Med 1998;158(4):1032-6.
20. Jatakanon A, Lim S, Kharitonov SA, Chung KF, Barnes PJ. Correlation between exhaled nitric oxide, sputum eosinophils, and methacholine responsiveness in patients with mild asthma. Thorax 1998;53(2):91-5.
21. Olin AC, Alving K, Toren K. Exhaled nitric oxide: relation to sensitization and respiratory symptoms. Clin Exp Allergy 2004;34(2):221-6.
22. Henriksen AH, Lingaas-Holmen T, Sue-Chu M, Bjermer L. Combined use of exhaled nitric oxide and airway hyperresponsiveness in characterizing asthma in a large population survey. Eur Respir J 2000;15(5):849-55.
23. Barbinova L, Baur X. Increase in exhaled nitric oxide (eNO) after work-related isocyanate exposure. Int Arch Occup Environ Health 2006;79(5):387-95.
24. Lange P, Parner J, Vestbo J, Schnohr P, Jensen G. A 15-year follow-up study of ventilatory function in adults with asthma. N Engl J Med 1998;339(17):1194-200.
25. Anees W, Moore VC, Burge PS. FEV1 decline in occupational asthma. Thorax 2006;61(9):751-5.
26. Portengen L, Hollander A, Doekes G, de Meer G, Heederik D. Lung function decline in laboratory animal workers: the role of sensitisation and exposure. Occup Environ Med 2003;60(11):870-5.
27. Welte T, Groneberg DA. Asthma and COPD. Exp Toxicol Pathol 2006;57 Suppl 2:35-40.
28. 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(12):891-901.
29. 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(3):299-310.
30. 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(2):104-11.
31. Sterk PJ. Let's not forget: the GOLD criteria for COPD are based on post-bronchodilator FEV1. Eur Respir J 2004;23(4):497-8.

Chapter 4

Specific sensitization

Chapter 4.1

IgE and IgG sensitization to hexamethylene diisocyanate (HDI) in spray painters: a comparison of ImmunoCAP® and enzyme immunoassays with various HDI-albumin conjugates

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Abstract

Studies on the role of allergen-specific IgE and IgG in isocyanate asthma have produced conflicting results. One of the reasons may be the use of different types of immunoassays for serologic analyses. Objectives were to compare various immunoassays for IgE and IgG to hexamethylene diisocyanate (HDI), in a large population of spray painters exposed mainly to HDI oligomers.
HDI-specific IgE and IgG were measured in 581 sera by ImmunoCAP® and enzyme immunoassays (EIA) with HDI-human serum albumin (HSA) conjugates prepared with HDI in the liquid (HDI_L) or vapor phase (HDI_V), or with HDI-oligomers (N100, N3300). Concordance and correlations between assays and associations with exposure and respiratory health parameters were assessed.
Highest prevalences were found in EIAs with HDI_L- and N100-HSA, both for IgE (3%) and IgG (45%). Concordance and correlation between most assays were moderate to high, and inhibition assays revealed a marked cross-reactivity between the various HDI-conjugates. Results of EIAs with N100-HSA showed the strongest associations with exposure levels. Associations with health end-points were not always clear but also seemed strongest for EIAs with N100-HSA.
In conclusion, on a population level most immunoassays produced similar results, but also showed marked differences in reactivity and associations with exposure and health effects. In spray painters with mainly exposure to HDI oligomers, EIAs with HSA-conjugated HDI oligomers provide the best combination of sensitivity and specificity. These results indicate that exposure type should be taken into account when choosing optimal immunoassays for anti-isocyanate antibodies. The diagnostic value of assays using oligomeric isocyanates should be further explored.

Introduction

Isocyanates are among the most common causes of occupational asthma in industrialized countries (1-3). First symptoms generally occur after a latency period of months to years of exposure, in a small proportion of the exposed population. Once asthma has developed, symptoms might occur at very low exposure levels. These features suggest immune-mediated sensitization, as in typical IgE-mediated allergic disease (4). Yet, to date no clear association with isocyanate-specific IgE antibodies has been demonstrated (4). In most case-control studies specific IgE has been found in a minority of specific inhalation challenge confirmed cases (5-9). According to some studies, isocyanate-specific IgG may have a higher diagnostic value. It might also be primarily a marker of exposure, without a clear role in the pathogenesis (10).
Like other low molecular weight allergens, isocyanates presumably act as haptens, being conjugated to human proteins in vivo via their highly reactive NCO-groups (3). It is not well known, however, what are the predominant structures of isocyanate-protein conjugates causing sensitization in exposed workers (4). As a consequence, there has been debate regarding the validity of applied serologic assays for the assessment of isocyanate-specific antibodies. Some studies used the commercially available ImmunoCAP® method in which isocyanates are coupled to a carrier matrix (11, 12). Most have employed enzyme or radio-immunoassays (EIA or RIA) with 'in house' prepared isocyanate-protein conjugates (5-9). Usually, these have been prepared by liquid phase reactions of diisocyanate monomers added to a solution of human serum albumin (HSA). Alternatively, conjugates can be prepared by incubating an HSA solution with diisocyanate vapor. This is a procedure that may better reflect conjugation reactions in the human airways (13, 14). Another complicating factor may be the increased industrial application of less volatile diisocyanate oligomers. Although little is known about their immunogenicity and cross-reactivity, conjugates produced with oligomers may be more representative of the immunogenic agents formed in vivo during work-related exposure (15, 16).
In this study we therefore compared immunoassays for isocyanate sensitization using sera from a large-scale epidemiological study among spray painters with predominantly exposure to HDI oligomers (17). Specific IgE and IgG were measured by ImmunoCAP® and by EIAs with a series of different HDI-human serum albumin (HSA) conjugates, including HSA coupled to monomeric HDI in the liquid and vapor phase, and HSA coupled to the HDI oligomers. We investigated: (a) concordance of positive tests, and quantitative correlations between positive test results; (b) cross-reactivity of the various conjugates, as shown by inhibition assays; and (c) relations of IgE or IgG results with either exposure levels and with respiratory health data.

Methods

Subjects
Serum samples were obtained from 581 workers in the spray-painting industry with mainly exposure to oligomers of HDI (17). Blood samples were processed within 8 hours and serum aliquots stored at -20°C until analysis. Specific IgE and IgG to HDI were assessed by ImmunoCAP® assays and EIAs with as test antigens various HDI-HSA conjugates (Table 4.1.1). As a control group 150 sera were randomly selected from a similar respiratory health study among bakery workers (18).

Table 4.1.1: Assays and HDI-conjugates used in specific IgE and IgG anti-HDI analyses.

Test conjugate	Source*	Carrier	Phase isocyanate	HDI/HSA molar ratio	Anti HDI test system
HDI-ImmunoCAP (K77)	Phadia	ImmunoCAP (as solid phase)	#	#	ImmunoCAP
HDI_L-HSA	IRAS	HSA	Liquid	29 : 1	EIA
HDI_V-HSA	Yale	HSA	Vapor	10 : 1	EIA
$N3300_{0.1\%}$-HSA	Yale	HSA	Liquid	8 : 1	EIA
$N3300_{1.0\%}$-HSA	Yale	HSA	Liquid	8 : 1	EIA
$N100_{0.1\%}$-HSA	Yale	HSA	Liquid	6 : 1	EIA
$N100_{1.0\%}$-HSA	Yale	HSA	Liquid	6 : 1	EIA

* Source: Phadia, Sweden, and the laboratories of the Institute for Risk Assessment Sciences (IRAS), The Netherlands, and Yale School of Medicine, US.
# No information from manufacturer available

Preparation of conjugates
Liquid phase HDI-HSA conjugates (HDI_L-HSA) were prepared at IRAS, essentially as described by Dewair and Baur (19). HDI (Sigma-Aldrich, Steinheim, Germany) was dissolved at 1.5% (v/v) in 10 mL dioxane, and added drop wise to 100 mL of a 1% solution of HSA (Sigma; product no. A5843-5G) in 0.05 M KCl, 0.05 M Na-borate buffer while stirring for 3 hrs at room temperature. The reaction was terminated with 2 mL of ethanolamine and the supernatant after centrifugation (1 hr; 2,100 g) was dialyzed against phosphate-buffered saline (PBS; three cycles), and de-ionized water, and stored at -20°C.
Vapor-induced HDI-HSA (HDI_V-HSA) conjugates were prepared at Yale School of Medicine as previously described (14). HDI (Sigma) vapor with a concentration of 20-200 ppb was passively generated in a closed circuit system and monitored with an Autostep monitor (GMD, Pittsburgh, PA). A sterile 0.5% solution of HSA (Sigma) in tissue culture grade PBS (Gibco-BRL; Grand Island, NY) was exposed overnight in open 60 mm petri dishes (Becton Dickinson, Franklin Lakes, NJ), in a sterilized vapor exposure unit. After exposure, the HSA-HDI solutions were filtered (0.2 μm; Corning; Corning, NY), and stored in aliquots at –20°C.
Oligomeric HDI-HSA conjugates were prepared at Yale School of Medicine as follows. Commercial HDI oligomer preparations of biuret (Desmodur N-100) and isocyanurate (Desmodur N-3300; Bayer; Pittsburgh, PA), were mixed 1:1 with

acetone (JT.Baker; Phillipsburg, NJ) and added to a 0.5 % HSA solution in tissue culture-grade PBS to achieve a final concentration of either 0.1% or 1% (v/v) HDI. After incubation in an end-over-end roller (2 hr, 37°C), the solutions were 0.2 μm filtered, dialyzed four times against tissue-culture grade PBS, using Spectra/Por cellulose membranes sterilized by irradiation (Spectrum Labs, Inc.; Los Angeles, CA), and refiltered (0.2 μm). Resulting biuret and isocyanurate conjugates were designated $N100_{0.1\%}$-HSA, $N100_{1.0\%}$-HSA, $N3300_{0.1\%}$-HSA, and $N3300_{1.0\%}$-HSA, respectively.
Protein content of all conjugates was assessed by Lowry analysis, with bovine serum albumin as the standard, or derived from the OD_{280}, assuming an OD_{280} of 5.3 for a 1% HSA solution. The substitution ratio (HDI/HSA molar ratio; Table 4.1.1) was derived from the % free NH_2 groups measured by reaction with trinitrophenylbenzene sulphonic acid (TNBS), as described previously (13, 14).

ImmunoCAP® assay
HDI-specific IgE and IgG were measured by ImmunoCAP® (Phadia CAP no. K77; Uppsala, Sweden), using the UniCAP 250 system, following the supplier's instructions. For IgE tests a positive reaction was defined at a cut-off level of 0.35 kU/l. The cut-off for IgG was set at 5 mg/l, being the mean + 3 standard deviations in sera from 20 healthy non-exposed controls.

EIAs for HDI-specific IgE antibodies
Microtiter plates (Greiner, Frickenhausen, Germany) were coated overnight at 4°C with HDI-HSA conjugates and non-treated HSA at 10 μg/ml in PBS-azide. Aliquots of 0.1 ml per microwell were used in all EIA steps. Sera diluted 1/5 in PBS-Tween-0.2% gelatin (PBTG) were incubated for 2 h at 37°C, and IgE antibody binding detected by 1 hr incubations at 37°C with mouse monoclonal anti-human IgE (1/16,000; Sanquin Reagents, Amsterdam, NL), rabbit anti-mouse Ig/biotin (1/5,000; DAKO, Glostrup, DK), and avidin-horse radish peroxidase (HRPO; 1/2,000; DAKO), and finally 30 min at 20°C with orthophenylenediamine (OPD) as the peroxidase substrate (20). After stopping the reaction with 0.05 mL 2M HCl, the optical density (OD) was read at 492 nm.
Replicate aliquots of each serum were tested simultaneously in a row of microwells coated with the various HDI-HSA conjugates or non-treated HSA, and in each plate one row of microwells was incubated with PBTG instead of serum, to control for non-specific binding of detecting reagents. Since OD_{492} values in these 'no serum' control wells did not differ significantly between wells coated with any of the HSA-HDI conjugates or with HSA, OD_{492} values in HDI-HSA coated wells with test sera were only adjusted by subtracting the OD_{492} in an HSA-coated well incubated with the same serum.

EIA for specific IgG antibodies
Design and procedure of IgG EIAs were similar to IgE EIAs but coating concentrations were 20 μg/mL, sera were tested at 1/100 dilution, and IgG binding was detected with HRPO-labeled rabbit anti-human IgG (1/2,000; DAKO) for 1h at 37°C, and OPD. Again, OD values in 'no serum control' wells

showed no significant differences between wells coated with HDI-HSA or HSA. OD values for sera in HDI-HSA coated wells were adjusted only for the OD in the corresponding HSA-coated well.

Inhibition assays
IgE ImmunoCAP inhibition was performed with five sera giving the highest IgE anti-HDI levels in the screening tests (from 1.4 to 9.0 kU/L). In the inhibition tests, 0.01 mL portions of diluted HDI-HSA conjugates were added to the 0.050 mL aliquots of (undiluted) serum with which the CAP assays was routinely performed, just before the start of the assay. Dilutions were chosen such that during the assay the (inhibitor) conjugate concentrations were 25, 5 and/or 1 μg/mL.
Cross-reactivity of HDI-conjugates was also studied by IgE and IgG inhibition EIAs. All test wells of a microplate were coated with the same HDI-conjugate (10 μg/mL and 20 μg/mL in the IgE and IgG EIA, respectively) Serial dilutions (50 μL) of the various HDI-conjugates and non-modified HSA were added in various concentrations. Directly after addition of the inhibitor dilution series, 50 μL diluted serum was added in the optimal concentration and mixed, and the IgE or IgG EIA protocol was followed as described above. Inhibition was expressed by plotting the measured (HSA-adjusted) ODs in microwells with inhibitor as % of the mean (adjusted) OD values in the 'no inhibitor' control wells containing diluted serum in PBTG alone.

Data analysis
Reproducibility of tests at various cut-off values was assessed by calculating kappa values (κ) for concordance of results after re-analyses of sera. Kappa values were also used to assess concordance between positive and negative tests results in different IgE or IgG assays. In each pair-wise comparison of assays, quantitative agreement was calculated as Pearson correlation coefficients (r) of log transformed adjusted OD values for samples with a positive test result in both assays.

Results

Specific IgE

Cut-off levels: HDI-specific IgE levels as measured in the various assays are plotted in Figure 4.1.1. Only 8 sera (1.3%) were positive (>0.35 kU/L) in the ImmunoCAP assay. The optimal cut-off for EIAs was determined by completely retesting all sera (n=80) with an adjusted OD of ≥0.05 on at least one of the HDI-conjugates. Reproducibility, calculated for cut-off levels from 0.05 to 0.3, was optimal for a cut-off of 0.1 (κ = 0.78-0.86). In all EIAs inter-test correlation was high for positive reactions confirmed in a second test (Pearson r > 0.9 for log-transformed OD values).

With the cut-off set at 0.1 the prevalence of positive IgE EIA reactions was generally low like in the ImmunoCAP, but higher for EIAs with HDI_L- and N100-HSA (2.4- 2.9%), and lower for the N3300- (0.9-1.4%) and the HDI_V-EIA (0.5%).

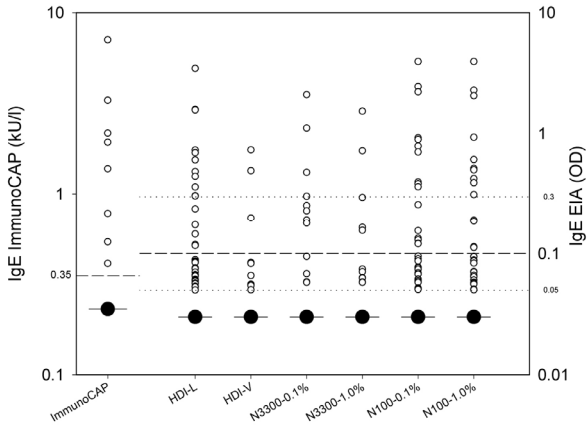

Figure 4.1.1: HDI-specific IgE in sera from 581 spray painters assessed by ImmunoCAP (kU/l) and EIAs with 6 different HDI-HSA-conjugates (HSA-adjusted OD). In the ImmunoCAP assay a cut-off level of 0.35 kU/l was used. For EIAs adjusted OD levels from 0.05 to 0.3 were compared and a cut-off level of 0.1 was chosen. Solid circles represent the >90% of the sera with results below 0.35 kU/L or and adjusted OD <0.05.

Comparison of IgE assays: Figure 4.1.2 shows illustrations of the comparison of IgE ImmunoCAP results with the OD values in the EIAs with HDI_L-HSA and $N3300_{0.1\%}$-HSA. The number of concordant positive reactions was very similar in both comparisons. However, 9 of the 17 sera with a positive reaction in the HDI_L-EIA had a negative ImmunoCAP compared to only 1 of 8 in the $N3300_{0.1\%}$-EIA, resulting in a better concordance for the latter (κ=0.63 vs 0.87). In

contrast, the correlation between OD values for sera with two positive tests was higher for the comparison of the HDI_L- EIA with ImmunoCAP.

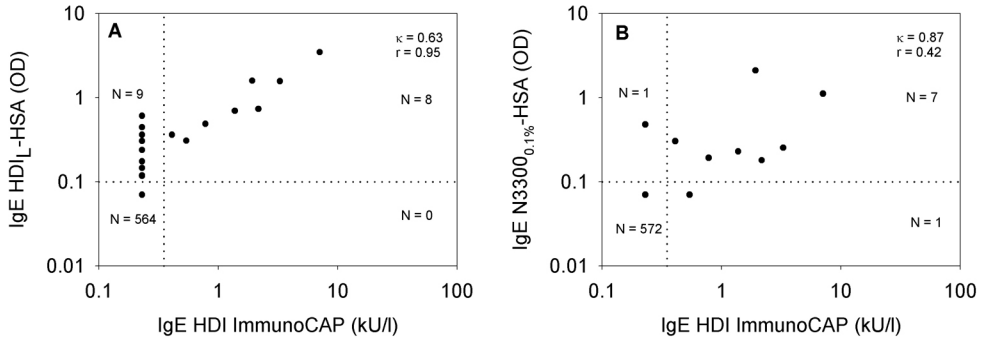

Figure 4.1.2: Comparison of the IgE HDI ImmunoCAP results with IgE anti-HDI measured by EIAs with (A) HDI_L-HSA and (B) $N3300_{0.1\%}$-HSA. Cut-off levels are indicated by dotted lines. Kappa values (κ) for the concordance of positive and negative results, and correlation coefficients (r) for the correlation between positive results are given.

Table 4.1.2: Comparison of HDI-specific IgE values measured by ImmunoCAP and EIAs with six different HDI-HSA conjugates: kappa values (κ) for the concordance of positive and negative results, and correlation coefficients (r) for the correlation between positive results (n) are given.

		HDI_V-HSA	**$N3300_{0.1\%}$-HSA**	**$N3300_{1.0\%}$-HSA**	**$N100_{0.1\%}$-HSA**	**$N100_{1.0\%}$-HSA**	**ImmunoCAP**
HDI_L-HSA	κ:	0.29	0.63	0.45	0.78	0.83	0.63
	r (n):	0.74 (3)	0.64 (8)	0.57 (5)	0.91 (13)	0.94 (13)	0.95 (8)
HDI_V-HSA		κ:	0.54	0.75	0.31	0.35	0.54
		r (n):	0.83 (3)	0.81 (3)	0.83 (3)	0.63 (3)	0.39 (3)
$N3300_{0.1\%}$-HSA			κ:	0.77	0.66	0.72	0.87
			r (n):	>0.99 (5)	0.68 (8)	0.64 (8)	0.42 (7)
$N3300_{1.0\%}$-HSA				κ:	0.47	0.52	0.61
				r (n):	0.60 (5)	0.52 (5)	0.40 (4)
$N100_{0.1\%}$-HSA					κ:	0.93	0.66
					r (n):	0.98 (14)	0.73 (8)
$N100_{1.0\%}$-HSA						κ:	0.72
						r (n):	0.77 (8)

Concordance between the various ImmunoCAP and EIA results was moderate to high (κ: 0.54-0.87; Table 4.1.2). The low sensitivity of the HDI_V–HSA EIA was reflected by a lower concordance with other assays. Most other assay comparisons revealed moderate to high levels of concordance. Correlations between quantitative results were also moderate to high (r from ~0.5 to >0.9). Best concordance and correlation was found for EIAs with the same oligomer coupled to HSA in different concentrations (0.1 and 1.0%). These results

compared equally well as results from reproducibility tests with the same HDI conjugate EIA ($\kappa > 0.75$, $r > 0.98$).

Specific IgG

Cut off levels: HDI-specific serum IgG reactions are summarized in Figure 4.1.3. In the IgG ImmunoCAP, 7.9% of the sera exceeded the cut-off level of 5 mg/l. The cut-off value for the EIAs was based on retesting a random sample of 145 sera on the whole panel of conjugates. Kappa values were calculated for cut-off values between 0.15 and 0.6 and a cut-off of 0.3 was set. With this cut-off value, kappa values for repeated tests ranged from approximately 0.70 for the HDI_L and HDI_V EIA to >0.80 for EIAs with HDI oligomers, and OD values of confirmed positive reactions showed a high inter-test correlation (r >0.9).
A higher prevalence of HDI-specific IgG was found in the EIAs than in the ImmunoCAP assay: from 13-18% for the HDI_V and N3300-EIA, 25-28% for the N100-EIAs and 44% in the EIA with HDI_L-HSA.

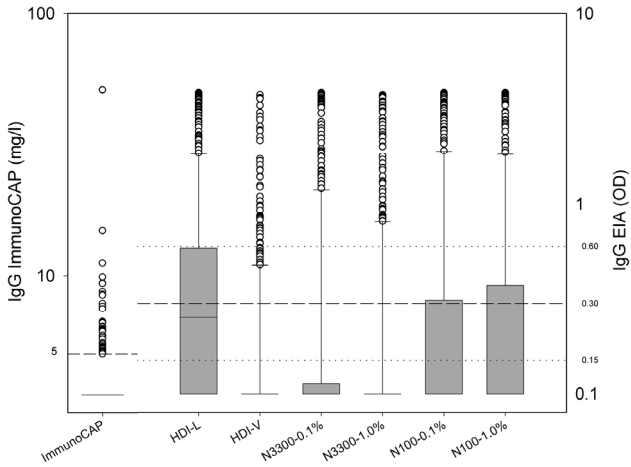

Figure 4.1.3: HDI-specific IgG assessed in 581 sera by ImmunoCAP (mg//L) and EIAs with six different HDI-HSA-conjugates (HSA-adjusted OD values). Box plots indicate the 25th, 50th and 75th percentiles (boxes), 90th percentile (upper whisker), and sera with higher values are plotted separately. For the IgG ImmunoCAP assay a cut-off level of 5 mg/l was used (dashed line). For IgG EIAs adjusted ODs between 0.15 and 0.6 (dotted lines) were compared and a cut-off level of 0.3 (dashed line) was chosen.

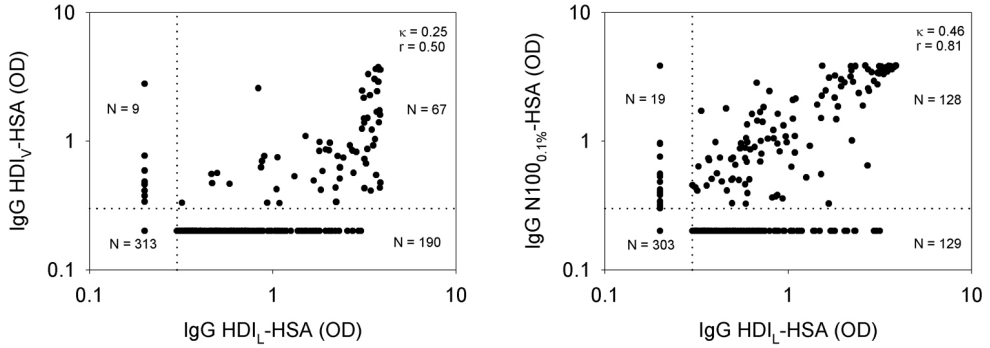

Figure 4.1.4: Comparison of HDI-specific IgG levels in 581 sera assessed by EIAs with (A) HDI_L-HSA vs HDI_V-HSA and (B) HDI_L-HSA vs $N100_{0.1\%}$-HSA. Cut-off levels are indicated by dotted lines. Kappa values (κ) for concordance and correlation coefficients (r) for sera with positive results in both tests, as in Fig. 4.1.2.

Comparison of assays: Two comparisons of IgG EIAs are illustrated in Figure 4.1.4. Many sera positive in the HDI_L-EIA were negative in the other EIAs, resulting in weak to moderate concordance (κ= 0.25 and 0.46). Yet, correlation between positive results was moderate (HDIv: r = 0.5) to high ($N100_{0.1\%}$: r = 0.81).

Table 4.1.3: Comparison of HDI-specific IgG values assessed by ImmunoCAP and EIAs with six different HDI-HSA conjugates. Kappa values, correlation coefficients and numbers of double-positive sera as in Table 4.1.2.

		HDI_V-HSA	**$N3300_{0.1\%}$-HSA**	**$N3300_{1.0\%}$-HSA**	**$N100_{0.1\%}$-HSA**	**$N100_{1.0\%}$-HSA**	**ImmunoCAP**
HDI_L-HSA	κ:	0.25	0.36	0.30	0.46	0.50	0.09
	r (n):	0.50 (67)	0.61 (94)	0.47 (79)	0.81 (128)	0.82 (139)	0.43 (32)
HDI_V-HSA		κ:	0.57	0.61	0.44	0.45	0.23
		r (n):	0.50 (57)	0.64 (54)	0.44 (60)	0.48 (65)	0.27 (19)
$N3300_{0.1\%}$-HSA			κ:	0.81	0.68	0.65	0.19
			r (n):	0.95 (80)	0.65 (93)	0.65 (95)	0.12 (21)
$N3300_{1.0\%}$-HSA				κ:	0.61	0.56	0.19
				r (n):	0.59 (80)	0.58 (80)	0.15 (18)
$N100_{0.1\%}$-HSA					κ:	0.84	0.15
					r (n):	0.98 (135)	0.11 (24)
$N100_{1.0\%}$-HSA						κ:	0.16
						r (n):	0.28 (27)

Table 4.1.3 summarizes the pair-wise comparisons of all IgG assays. Concordance and correlation between positive results were remarkably low for all comparisons with the ImmunoCAP assay (κ<0.23; r<0.43). Results of HDI_L- and HDI_V-EIAs were at best moderately associated (κ = 0.25, r = 0.50). The

concordance between results of the HDI_L-EIA and the EIAs with HDI oligomers was also moderate (κ = 0.3-0.5) but the correlation much better (r up to 0.82). In contrast, EIAs with HSA-coupled HDI oligomers showed a much better concordance (κ = 0.56-0.68) and correlation (r = 0.59-0.98). Results of EIAs with the same oligomer coupled to HSA in different concentrations (0.1 and 1.0%) compared equally well as results from reproducibility tests with the same IgG EIA (κ > 0.80, r > 0.95).

Inhibition assays
Cross-reactivity was studied by IgE ImmunoCAP inhibition with 5 IgE positive sera and inhibition EIAs with 3 sera with strong IgE and 10 with strong IgG reactions. Figure 4.1.5 shows inhibition curves of one serum with the strongest reaction in the IgE ImmunoCAP (~9 kU/L), which was also strongly positive in most anti-HDI EIAs. IgE reactions in both ImmunoCAP and EIAs could be dose-dependently inhibited by all HDI-conjugates, although with different efficiency. Generally, the N100-HSA conjugates were in all cases the most effective inhibitors, followed by the HDI_L-HSA, while N3300-HSA and HDI-v-HSA were less or much less effective. This pattern was found for all IgE inhibition assays except for one serum with a clearly positive ImmunoCAP reaction that could not be inhibited by any of the HDI-HSA conjugates, in spite of moderately positive reactions in the various IgE EIAs (not shown).

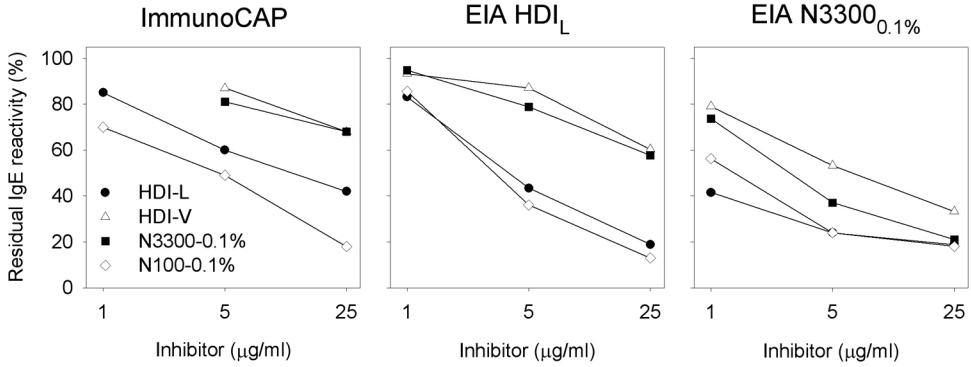

Figure 4.1.5: Cross-reactivity of HDI-conjugates in IgE inhibition assays. Residual IgE binding (% of non inhibited control) is plotted for the reactions in the ImmunoCAP, the EIA with HDI_L-HSA and EIA with $N3300_{0.1\%}$-HSA, after incubation with various concentrations of inhibiting HDI-conjugates.

Similar results were obtained in the IgG inhibition EIAs, although with some more divergent inhibition patterns. All sera with a strong IgG reactions to several coated HDI-HSA conjugates could also in all assays be dose-dependently inhibited with the various conjugates. The most efficient inhibitors were the N100- and HDI_L-HSA conjugates (not shown). Clear exceptions were a few sera with a selective IgG reactivity to only one or two of the HDI-conjugates. Such

reactions could also only be inhibited with those conjugates, and not or much less by other HDI-conjugates.

Relation with exposure categories
As reported previously (17) specific IgE antibodies were not found in office workers of car body repair shops while in spray painters and other workers on the work floor (i.e. mechanics, car washers etc) IgE to HDI was found in up to 4.2%. Specific IgG was found in office workers (Figure 4.1.6), and especially in the HDI_L-EIA with a remarkably high prevalence (32%). We therefore tested sera from an unexposed control population of bakery workers for HDI-specific IgG. Positive reactions with HDI_L-HSA in 17%, and much lower frequencies in the other EIAs were found (Figure 4.1.6). Although the frequencies strongly differed per HDI conjugate, in all assays the prevalence of specific IgG showed a clear gradient with exposure level: lowest in office workers, intermediate in 'other' workers with intermediate levels of exposure, and highest in spray painters.

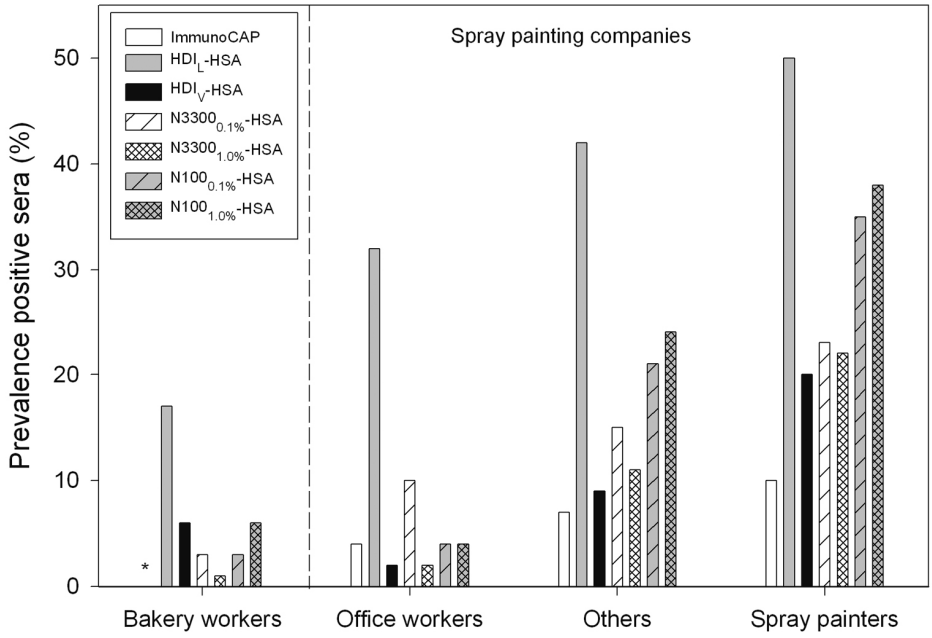

Figure 4.1.6: Prevalence of IgG anti-HDI reactivity assessed by various immunoassays in 145 bakery workers (unexposed controls) and three exposure categories in spray painting companies: office workers (50), other workers (241) and spray painters (290).
* Bakery workers' sera were not analyzed in the IgG anti-HDI ImmunoCAP.

Discussion

In this study, HDI-specific IgE and IgG antibodies were measured in 581 workers from the spray-painting industry, using ImmunoCAP or EIAs with various HDI-HSA conjugates. To our knowledge this is the first study to compare assays for these isocyanate-specific antibodies on such a large scale.
Although in general results of different immunoassays were well correlated, marked differences were also noted. The various IgE and IgG clearly varied in the prevalence of positive reactions, with the highest prevalence found for EIAs with HDI_L- and N100-HSA. Concordance and correlation between results of IgG assays were lower than between reactions measured in IgE assays.
Inhibition studies demonstrated that correlation between immunoassays for both specific IgE and IgG antibodies was largely due to immunologic cross-reactivity. While most sera with a clearly positive reaction to one HDI-conjugate also reacted with several other HDI-conjugates, such reactions could practically always also be inhibited with a range of different conjugates, although with different inhibitory potency. A significant correlation was found between the IgG reactivity of a serum to a certain coated HDI-conjugate, and the efficiency of the same conjugate as fluid phase inhibitor of the IgG anti-HDI antibodies in the same serum (not shown). This indicates heterogeneity in epitope specificity among sera. Previous inhibition studies have also demonstrated variable patterns of IgE cross-reactivity to different diisocyanate-HSA conjugates among sera (21-24).

Besides assay characteristics like sensitivity and reproducibility, other important evaluation criteria may be the associations with exposure levels and/or with health effects. For most assays positive associations between exposure and both IgE and IgG were found (17). For IgE these were, due to the relatively low numbers of positive reactions, only significant for EIAs with N100-HSA conjugates. For IgG the strongest associations were found for EIAs with HDI_V-HSA or the oligomeric conjugates. Other studies have also reported positive associations between isocyanate-specific IgG and exposure categories (8, 13-15, 24). The weaker association found for IgG reactions in the HDI_L-EIA (17) was mainly due to the high prevalence in the control group of office workers. Since specific IgG was also demonstrated in a significant proportion of the unexposed external control group, part of these reactions may be due to non-specific IgG binding to the highly HDI-substituted HDI_L-HSA conjugate (ratio 29:1). Support for such an explanation comes from previous studies indicating that anti-isocyanate antibody binding is dependent on substitution ratios of the test conjugate (14, 19, 24, 26, 27) and that high substitution ratios are a potential cause of increased non-specific IgG binding (21, 24, 27). Although isocyanate-specific IgG is seldom found in unexposed individuals (10), Bernstein et al. (2006) also demonstrated specific IgG to HDI-HSA in 13% of a non-exposed population using a highly substituted conjugate (21).

The role of specific antibodies in the development of isocyanate asthma remains unclear. Case control studies generally report isocyanate specific IgE antibodies

in less than 20% of asthma cases (5-9). This may be attributed to flaws in technical methods for generating biologically relevant isocyanate conjugates (10). We previously showed an association between questionnaire-reported respiratory symptoms and specific anti-HDI IgE, which seemed most pronounced for the N100- and HDI_V-EIAs (17). Yet, the low prevalence of HDI-specific IgE in this open population, and the lack of overt cases of isocyanate asthma do not allow inferences on which IgE EIA would be optimal for the recognition of isocyanate sensitization as the cause of respiratory health problems.

Two previous studies have suggested that specific IgG antibodies are more predictive of isocyanate asthma than specific IgE (5, 6), while others could not demonstrate such associations (8, 28, 29). Like specific IgE, some of our IgG anti-HDI results were also associated with respiratory symptoms. These relations were most pronounced for IgG to N100-HSA, but even weaker than for specific IgE and probably largely attributable to the association between IgG and exposure (17). An unexpected and remarkable finding was the significant association of IgG measured by ImmunoCAP and BHR. This needs to be further explored, especially since BHR was associated with exposure while IgG anti-HDI measured by ImmunoCAP was not (17).

EIAs with N100-HSA showed the best combination of sensitivity and specificity with regard to exposure. Although associations with health end-points were not always clear, they were most pronounced for the N100-EIAs. This may reflect the predominance of HDI oligomer exposure in this population (30). However, the prevalence, specificity and sensitivity of the EIAs with the other oligomer (N3300) were clearly lower than for N100 EIAs. Despite the increasing use of oligomeric isocyanates, few studies have focused on specific antibody responses to these compounds. In a small population of car painters, IgG to N100-HSA was associated with exposure category while no association could be found for IgG to HDI-HSA or IgE to any of these conjugates (15). A larger study in spray painters from a truck factory found no association between specific IgG or IgE to HSA-conjugates prepared with HDI and an HDI trimer and exposure (29).

In conclusion, the immunoassays for specific anti-HDI compared in this study gave largely concordant and correlating results, but also showed marked differences in reactivity and associations with exposure and health effects. In this population exposed to mainly oligomers of HDI, EIAs with N100-HSA, an oligomeric HDI, showed the best combination of sensitivity and specificity with respect to isocyanate exposure. In future studies, the use of different assays should be explored in populations with different isocyanate oligomer exposures. The diagnostic value of the various immunoassays as a marker of isocyanate asthma could not be assessed in this open population study. This should be studied in a case control study with overt asthma cases and well-defined controls, for both of which detailed exposure data must be available.

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(3):213-28.
2. Bernstein JA. Overview of diisocyanate occupational asthma. Toxicology 1996;111(1-3):181-9.
3. Wisnewski AV, Redlich CA. Recent developments in diisocyanate asthma. Curr Opin Allergy Clin Immunol 2001;1(2):169-75.
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. Park HS, Kim HY, Nahm DH, Son JW, Kim YY. Specific IgG, but not specific IgE, antibodies to toluene diisocyanate-human serum albumin conjugate are associated with toluene diisocyanate bronchoprovocation test results. J Allergy Clin Immunol 1999;104(4 Pt 1):847-51.
6. Cartier A, Grammer L, Malo JL, Lagier F, Ghezzo H, Harris K, et al. Specific serum antibodies against isocyanates: association with occupational asthma. J Allergy Clin Immunol 1989;84(4 Pt 1):507-14.
7. Baur X, Dewair M, Fruhmann G. Detection of immunologically sensitized isocyanate workers by RAST and intracutaneous skin tests. J Allergy Clin Immunol 1984;73(5 Pt 1):610-8.
8. Kim H, Kim YD, Choi J. Seroimmunological characteristics of Korean workers exposed to toluene diisocyanate. Environ Res 1997;75(1):1-6.
9. Butcher BT, O'Neil CE, Reed MA, Salvaggio JE. Radioallergosorbent testing of toluene diisocyanate-reactive individuals using p-tolyl isocyanate antigen. J Allergy Clin Immunol 1980;66(3):213-6.
10. Wisnewski AV. Developments in laboratory diagnostics for isocyanate asthma. Curr Opin Allergy Clin Immunol 2007;7(2):138-45.
11. Pezzini A, Riviera A, Paggiaro P, Spiazzi A, Gerosa F, Filieri M, et al. Specific IgE antibodies in twenty-eight workers with diisocyanate-induced bronchial asthma. Clin Allergy 1984;14(5):453-61.
12. Keskinen H, Tupasela O, Tiikkainen U, Nordman H. Experiences of specific IgE in asthma due to diisocyanates. Clin Allergy 1988;18(6):597-604.
13. Ye YM, Kim CW, Kim HR, Kim HM, Suh CH, Nahm DH, et al. Biophysical determinants of toluene diisocyanate antigenicity associated with exposure and asthma. J Allergy Clin Immunol 2006;118(4):885-91.
14. Wisnewski AV, Stowe MH, Cartier A, Liu Q, Liu J, Chen L, et al. Isocyanate vapor-induced antigenicity of human albumin. J Allergy Clin Immunol 2004;113(6):1178-84.
15. Welinder H, Nielsen J, Bensryd I, Skerfving S. IgG antibodies against polyisocyanates in car painters. Clin Allergy 1988;18(1):85-93.
16. Aul DJ, Bhaumik A, Kennedy AL, Brown WE, Lesage J, Malo JL. Specific IgG response to monomeric and polymeric diphenylmethane diisocyanate conjugates in subjects with respiratory reactions to isocyanates. J Allergy Clin Immunol 1999;103(5 Pt 1):749-55.
17. Pronk A, Preller L, Raulf-Heimsoth M, Jonkers IC, Lammers JW, Wouters IM, et al. Respiratory symptoms, sensitization and exposure-response associations in HDI exposed spray painters. Am J Respir Crit Care Med 2007; Epub ahead of print.
18. Suarthana E. Development and validation of diagnostic model for sensitisation to wheat and fungal alpha amylase allergens among Dutch bakery workers. In preparation.
19. Dewair MA, Baur X. Studies on antigens useful for detection of IgE antibodies in isocyanate-sensitized workers. J Clin Chem Clin Biochem 1982;20(6):337-40.
20. Doekes D, 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(1):63-70.
21. 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(3):357-64.
22. Baur X. Immunologic cross-reactivity between different albumin-bound isocyanates. J Allergy Clin Immunol 1983;71(2):197-205.
23. Liss GM, Bernstein DI, Moller DR, Gallagher JS, Stephenson RL, Bernstein IL. Pulmonary and immunologic evaluation of foundry workers exposed to methylene diphenyldiisocyanate (MDI). J Allergy Clin Immunol 1988;82(1):55-61.
24. Wass U, Belin L. Immunologic specificity of isocyanate-induced IgE antibodies in serum from 10 sensitized workers. J Allergy Clin Immunol 1989;83(1):126-35.

25. Lushniak BD, Reh CM, Bernstein DI, Gallagher JS. Indirect assessment of 4,4'-diphenylmethane diisocyanate (MDI) exposure by evaluation of specific humoral immune responses to MDI conjugated to human serum albumin. Am J Ind Med 1998;33(5):471-7.
26. Son M, Lee M, Kim YT, Youn JK, Park H. Heterogeneity of IgE response to TDI-HSA conjugates by ELISA in toluene diisocyanate (TDI) -induced occupational asthma (OA) patients. J Korean Med Sci 1998;13(2):147-52.
27. Spiazzi A, Boccagni P, Germano P, Pezzini A. RAST-detection of specific IgE in diphenylmethane diisocyanate exposed workers: considerations in performance of the test. Allergy 1991;46(3):166-72.
28. Paggiaro PL, Filieri M, Loi AM, Roselli MG, Cantalupi R, Parlanti A, et al. Absence of IgG antibodies to TDI-HSA in a radioimmunological study. Clin Allergy 1983;13(1):75-9.
29. 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(4):627-33.
30. Pronk A, Tielemans E, Skarping G, Bobeldijk I, van Hemmen J, Heederik D, et al. Inhalation exposure to isocyanates of car body repair shop workers and industrial spray painters. Ann Occup Hyg 2006;50(1):1-14.

Chapter 4.2

Exploratory analysis of in vitro production of MCP-1 by blood mononuclear cells incubated with HDI-HSA conjugates

Abstract

A new diagnostic test for isocyanate asthma based on the production of monocyte chemotactic protein-1 (MCP-1) by peripheral blood mononuclear cells (PBMC) stimulated with isocyanate conjugates has been proposed. The aim of this pilot study was to investigate if an MCP-1 assay as reported can be used to detect an early stage of probably isocyanate-related asthmatic symptoms in an occupational survey.
MCP-1 levels were assessed in PBMC culture supernatants from 101 workers in the spray painting industry stimulated with Concavalin A, human serum albumin (HSA) and various hexamethylene diisocyanate (HDI)-HSA conjugates. MCP-1 levels were compared with isocyanate exposure data, with various respiratory health end-points, and with the presence of HDI-specific IgE and IgG.
Preliminary results show that MCP-1 levels in the culture supernatants were highly variable. Although the median MCP-1 level varied between the HSA control and the various HDI-HSA stimulated cell cultures, for the population as a whole no significant differences were found between results obtained with different stimulants. Crude data did not suggest that HDI-induced MCP-1 release differs between the three exposure categories or between individuals with and without asthma-like symptoms, BHR or specific serology. High background levels and large variability found in this study may limit the detection of subtle effects on isocyanate-induced MCP-1 production. Before conclusions can be drawn, determinants of background MCP-1 production should be further explored to gain more insight in the contribution of assay and analysis error and within person variability.

Introduction

Several features of isocyanate asthma point towards immune mediated sensitization (1). However, in contrast to occupational asthma caused by high molecular weight allergens, its diagnosis is often not supported by allergen-specific skin prick tests or specific IgE. Isocyanate-specific IgE antibodies are generally demonstrated in less than 20% of asthma cases confirmed by specific inhalation challenge (2-6). It has been hypothesized that isocyanate asthma may be mediated by non-IgE mediated immunologic or non-immunologic mechanisms. Processes unlike the typical IgE-mediated response have been reported (1, 7-9). In this light a promising new specific cellular diagnostic test for isocyanate asthma was proposed by a study focusing on the production of the cytokine monocyte chemotactic protein-1 (MCP-1) by peripheral blood mononuclear cells (PBMC) stimulated with isocyanate conjugates (8, 10, 11). This test showed greater sensitivity and specificity for the detection of isocyanate asthma than specific antibody reactivity in a clinical case-control study (10).
In our population of spray painters exposed mainly to hexamethylene diisocyanate (HDI) oligomers, associations between exposure and respiratory health effects and specific sensitization were demonstrated (12). Specific IgE antibodies were rare and showed only weak associations with respiratory health end-points, supporting their limited diagnostic use for the recognition of isocyanate asthma. In a subpopulation we investigated isocyanate-specific cellular MCP-1 responses by culturing isolated PBMC with a range of isocyanate-protein conjugates. The aim of this pilot study was to investigate if an MCP-1 assay as reported for cases with diagnosed isocyanate asthma can be used to detect an early stage of probably isocyanate-related asthmatic symptoms. We therefore compared MCP-1 levels in PBMC culture supernatants with isocyanate exposure data, with various respiratory health end-points, and with the presence of HDI-specific IgE and IgG.

Methods

Subjects
The study was carried out in a subset of a population previously described (13). From all 229 workers in that study a blood sample was obtained for PBMC isolation, directly followed by in vitro stimulation with HDI-HSA conjugates. Cell culture supernatants were harvested after 48 hours and stored at -80°C. Serologic results (IgG and/or IgE anti-HDI) were used for selection of 101 workers for whom MCP-1 was measured in the supernatants. At the time of selection only ImmunoCAP (Phadia, Uppsala, Sweden) results for specific IgE and IgG to HDI were available. All workers with positive IgE or IgG serology (n=32) and a random sample of the other workers were selected.
Table 4.2.1 shows the general characteristic of the population. Due to the selection on positive serology, the prevalence of individuals with specific

antibodies was higher than in the source population. Also the prevalence of individuals with BHR and asthma-like symptoms was slightly elevated compared to the original population.

Table 4.2.1: General population characteristics, exposure, respiratory health end-points and specific sensitization.

	Office workers	Spray painters	Others
N	4	42	55
% male	100	100	98
Age	43 (10)	40 (8)	41 (10)
Current smoker	50	45	36
Exposure (μg NCO*m^{-3}*hr*$month^{-1}$)			
Median (minimum-maximum)	0	5187 (182-32.918)	9 (0-3785)
Respiratory end-points			
Asthma-like symptoms %	0	29	25
BHR20 %	0	20	19
Antibodies			
Specific IgE %	0	7	9
Specific IgG %	20	67	62
Phadiatop %	25	45	38

Questionnaire, exposure classification, specific sensitization and BHR
Questionnaire items were previously described (12). For statistical analyses 'wheezing' and 'chest tightness' were combined as "asthma-like symptoms". Workers were classified into the following exposure categories based on task information: 'office workers', 'spray painters' and 'others'. The latter categories consisted of mainly mechanics and metal workers. Exposure was highest in the 'spray painters' category and lowest in 'office workers' (12).
Specific IgE and IgG were assessed in serum by ImmunoCAP assay and enzyme immunoassay using various HDI-HSA conjugates as previously described (14). Specific IgE to common aeroallergens was assessed using the Phadiatop (Phadia, Uppsala, Sweden) as a measure of atopy. Cut-off values were used to dichotomize serological outcomes as previously described (14). Results of the various anti-HDI assays were combined for IgE and IgG separately: sera with a positive result in any of the IgE or IgG assays were designated as anti-HDI IgE- or IgG-positive, respectively.
Bronchial hyperresponsiveness was assessed by methacholine challenge as previously described (13). Airway hyperresponsiveness (BHR20) was defined as a fall in FEV1 of ≥ 20% at a provocative dose of methacholine of ≤ 2.5 mg.

PBMC isolation and stimulation
Blood samples (24 ml) were collected in 8 ml Vacutainer 'Cell Preparation Tubes' (BD Vacutainer® CPT™) with Sodium Heparin. Within 8 hours PBMC were isolated following the supplier's instructions. Cells were washed once in Tris-buffered Hanks balanced saline solution (HBSS-Tris) and twice in HBSS-Tris with 4% fetal calf serum (FCS), and resuspended in RPMI-5% FCS-PS (PS: 100 U/ml penicillin, 100 μg/ml streptomycin). Purified PBMC were >90% viable. PBMC

were plated in round-bottom microtiter plates at 100,000 cells/well in 100 μl RPMI-FCS, and cultured for 48 hrs at 37°C and 5% CO_2 in 100 μl of medium alone (RPMI-FCS) as a blank, Concavalin A (ConA, 50 μg/mL) as a positive control, unmodified HSA (50 μg/ml) as a sham control, and a range of different HDI-HSA conjugates (all 50 μg/ml). Conjugates included HDI-HSA prepared in the liquid phase, the vapor phase and two conjugates prepared with oligomeric HDI (HDI_L-HSA, HDI_V-HSA, N3300-HSA and N100-HSA, respectively (Chapter 4.1)). After culture, plates were centrifuged for 15 min at 1000 g, and supernatants were stored at -80°C until analysis.

MCP-1 measurements
In culture supernatants MCP-1 and a number of other cytokines were measured by Multiplex cytokine assays (15). The limit of detection for MCP-1 was approximately 5 pg/ml. HDI specific MCP-1 release was calculated by subtracting the cytokine concentration in the supernatant from control cultures with non-modified HSA from that in supernatants from HDI-HSA stimulated cells. For plotting and calculation purposes, all resulting values smaller than 5 pg/ml were set at 5 pg/ml.

Preliminary results and discussion

As shown in Figure 4.2.1, MCP1-levels in the culture supernatants showed high variability. Background MCP1-production in control wells with HSA ranged over a factor 1000. Cells stimulated with ConA showed the highest levels, indicating that it is possible to detect stimulated MCP-1 production in this test system. Although the median MCP1 level varied between the HSA control and the various HDI-HSA stimulated cell cultures, for the population as a whole no significant differences were found between results obtained with different stimulants.
In Figure 4.2.2 HSA-adjusted HDI specific MCP-1 release is plotted to show relations with exposure categories and with health end-points. Overall these crude data do not suggest that HDI-induced MCP-1 release differs between the three exposure categories or between individuals with and without asthma-like symptoms, BHR or specific serology. Therefore the results do not confirm a potential role for in vitro MCP-1 production tests as a marker of developing isocyanate asthma in our study.

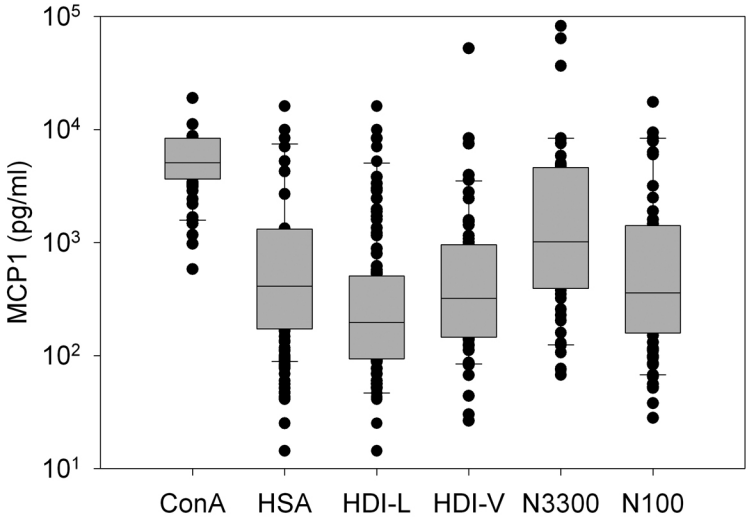

Figure 4.2.1: MCP-1 concentration (pg/ml) in supernatants of PBMC stimulated with ConA, HSA and various HDI-HSA conjugates.

At first sight our results appear to disagree with the results reported by Bernstein et al. (10) who found significantly higher MCP-1 production in isocyanate asthma cases than in controls. However, in that study well-defined cases with a clinical, specific inhalation challenge confirmed diagnosis of isocyanate asthma were compared to a control group. Our study population most probably did not include any such cases with overt isocyanate asthma and workers with symptoms or BHR formed a much more heterogeneous group. If there was any definite isocyanate asthma in these workers, it was likely to be milder than in the cases that have been reported to the clinic.
A role for cellular sensitization leading to enhanced MCP-1 responses in this population can however not be excluded. High background levels and large variability found in this study may limit the detection of subtle effects on isocyanate-induced MCP-1 production. The range of background HSA levels was especially large compared to the increase in MCP-1 production by cells stimulated with HDI-HSA conjugates (HSA-corrected levels). Therefore determinants of background MCP-1 production should be further explored to gain more insight in the contribution of assay and analysis error and within person variability. On the other hand, if HDI-stimulated production of MCP-1 by PBMC would reflect a pathogenic mechanism causally related to the development of asthma, one would have expected to find at least some individuals with a clearly enhanced response in a population at risk of developing the disease.

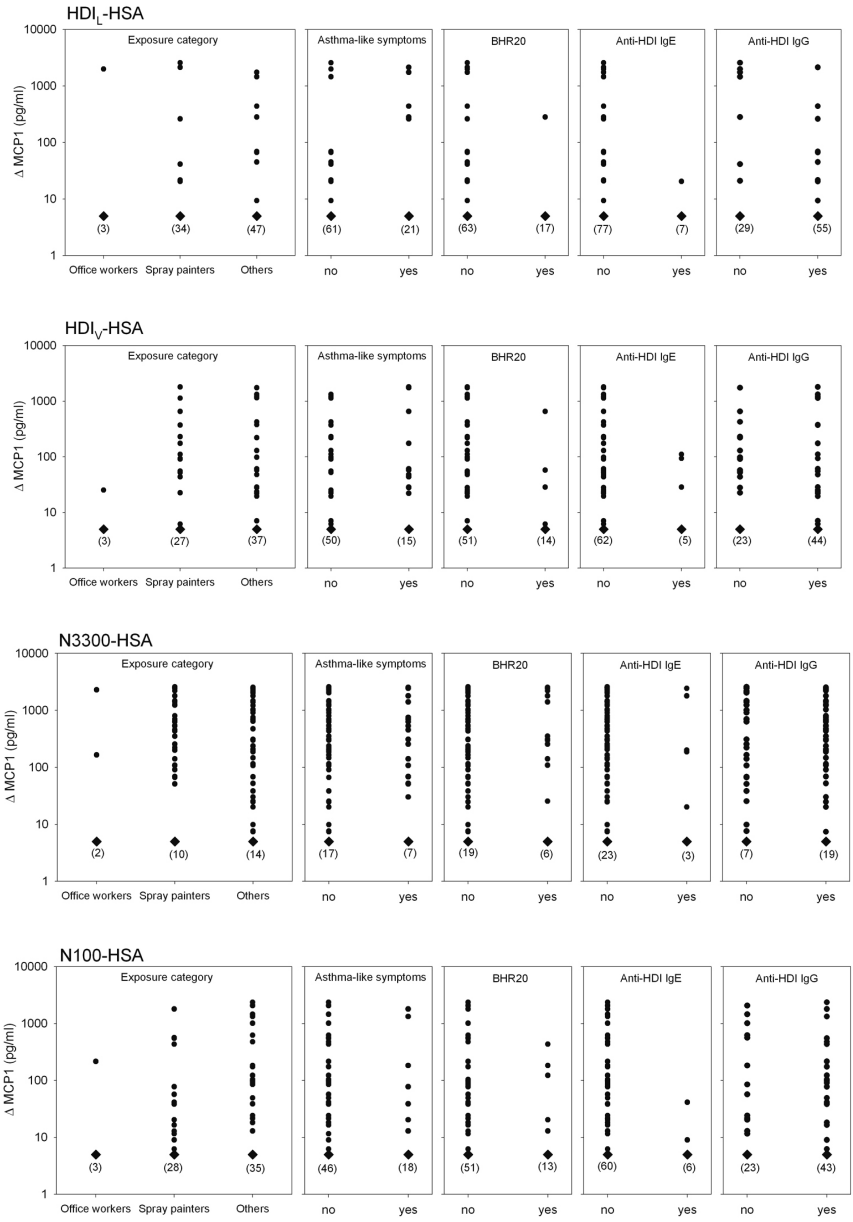

Figure 4.2.2: HDI-specific MCP-1 release (HSA adjusted) in supernatants after stimulation with various HDI-HSA conjugates, shown separately for different exposure categories, for different respiratory health end-points and for spray painters with or without HDI-specific IgE or IgG sensitization. HDI specific MCP-1 release below 5 pg/ml was set at 5 pg/ml. The number of samples at 5 pg/ml (diamond) is indicated in the graph.

References

1. 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.
2. Park HS, Kim HY, Nahm DH, Son JW, Kim YY. Specific IgG, but not specific IgE, antibodies to toluene diisocyanate-human serum albumin conjugate are associated with toluene diisocyanate bronchoprovocation test results. J Allergy Clin Immunol 1999;104(4 Pt 1):847-51.
3. Cartier A, Grammer L, Malo JL, Lagier F, Ghezzo H, Harris K, et al. Specific serum antibodies against isocyanates: association with occupational asthma. J Allergy Clin Immunol 1989;84(4 Pt 1):507-14.
4. Baur X, Dewair M, Fruhmann G. Detection of immunologically sensitized isocyanate workers by RAST and intracutaneous skin tests. J Allergy Clin Immunol 1984;73(5 Pt 1):610-8.
5. Kim H, Kim YD, Choi J. Seroimmunological characteristics of Korean workers exposed to toluene diisocyanate. Environ Res 1997;75(1):1-6.
6. Butcher BT, O'Neil CE, Reed MA, Salvaggio JE. Radioallergosorbent testing of toluene diisocyanate-reactive individuals using p-tolyl isocyanate antigen. J Allergy Clin Immunol 1980;66(3):213-6.
7. Jones MG, Floyd A, Nouri-Aria KT, Jacobson MR, Durham SR, Taylor AN, et al. Is occupational asthma to diisocyanates a non-IgE-mediated disease? J Allergy Clin Immunol 2006;117(3):663-9.
8. Lummus ZL, Alam R, Bernstein JA, Bernstein DI. Diisocyanate antigen-enhanced production of monocyte chemoattractant protein-1, IL-8, and tumor necrosis factor-alpha by peripheral mononuclear cells of workers with occupational asthma. J Allergy Clin Immunol 1998;102(2):265-74.
9. Raulf-Heimsoth M, Baur X. Pathomechanisms and pathophysiology of isocyanate-induced diseases--summary of present knowledge. Am J Ind Med 1998;34(2):137-43.
10. Bernstein DI, Cartier A, Cote J, Malo JL, Boulet LP, Wanner M, et al. Diisocyanate Antigen-stimulated Monocyte Chemoattractant Protein-1 Synthesis Has Greater Test Efficiency than Specific Antibodies for Identification of Diisocyanate Asthma. Am J Respir Crit Care Med 2002;166(4):445-50.
11. Lummus ZL, Alam R, Bernstein JA, Bernstein DI. Characterization of histamine releasing factors in diisocyanate-induced occupational asthma. Toxicology 1996;111(1-3):191-206.
12. Pronk A, Preller L, Raulf-Heimsoth M, Jonkers IC, Lammers JW, Wouters IM, et al. Respiratory symptoms, sensitization and exposure-response associations in HDI exposed spray painters. Am J Respir Crit Care Med 2007; Epub ahead of print.
13. Pronk A, Preller L, Doekes G, Wouters IM, Rooyackers J, Lammers JW, Heederik D. Bronchial hyperresponsiveness and lung function are associated with measured isocyanate exposure in spray painters. Submitted for publication.
14. Pronk A, Wisnewski AV, Raulf-Heimsoth M, Preller L, Heederik D, Wouters IM, Doekes G. IgE and IgG sensitization to hexamethylene diisocyanate (HDI) in spray painters: a comparison of ImmunoCAP® and enzyme immunoassays with various HDI-albumin conjugates. Submitted for publication.
15. de Jager W, te Velthuis H, Prakken BJ, Kuis W, Rijkers GT. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. Clin Diagn Lab Immunol 2003;10(1):133-9.

Chapter 5

General discussion

Chapter 5

General discussion

The main objective of this thesis was to investigate the association between isocyanate exposure and a range of different respiratory health end-points in the spray painting industry. The lack of published data on exposure-response associations might be due to complexities in the exposure assessment of isocyanates. In addition, respiratory health endpoints are diverse and valid immunological markers of specific sensitization are lacking. To overcome these problems, various exposure assessment methods were explored and a range of health end-points were investigated. Exposure to mainly oligomers of HDI was found. Inhalation exposure was clearly associated with respiratory symptoms, sensitization (IgE and IgG) and bronchial hyperresponsiveness (BHR). However, several uncertainties remain. For instance, exposure-response relationships are sensitive to the choice of exposure metric and health end-points. Preferably, the exposure metric should accurately reflect biologically relevant exposure. Otherwise misclassification of exposure may be introduced, which can lead to biased exposure-response estimates. Potential sources of misclassification in this study are evaluated in this chapter. In addition, some difficulties regarding the definition of health end-points and implications of the study are discussed.

Exposure assessment

Detailed exposure assessment was carried out to estimate personal exposure for use in epidemiological analyses. Usually, the cumulative or average exposure is selected as the exposure metric, which is estimated by combining (repeated) 8-hour exposure measurements with job information. One approach is to estimate each individual's exposure by measuring exposure for all workers involved in the epidemiological study. Only a few examples of this strategy exist in the literature because the measurement effort is usually high. Especially intra-individual variability needs to be controlled by taking sufficient repeated measurements per individual (1). To reduce the measurement effort, exposure can also be assessed in, and generalized for exposure categories. This leads to a lower measurement effort at the expense of the precision of the exposure-response relationship, resulting in a larger confidence interval (2). A variant of this approach is the use of exposure models which predict (average) exposure for each individual based on exposure determinants (3, 4).

In this study, a task-based exposure assessment strategy was used because of technical limitations of the applied isocyanate sampling method. For personal sampling midget impingers were used, containing di-n-butylamine in toluene as a reagent for capturing the reactive isocyanates immediately upon sampling.

The choice for this method was based on two important characteristics. First, it covers a wide range of the isocyanates that may be present in this industry. Second, impingers more efficiently derivatize isocyanates in aerosols than filters (5), which is advantageous in spray-painting environments. However, because toluene evaporates rapidly the maximum sampling time was usually around 30-40 minutes and 8-hour samples could not be taken. Short-term averaging times lead to more variation because of the relatively large sampling error and because they are more strongly influenced by exposure peaks. Consequently, a large number of samples had to be taken to estimate average levels accurately. Contrast in exposure between tasks was found with highest exposures observed during spray painting. Still, during spray painting, exposure was highly variable with detectable levels varying over a factor of 1000 (Figure 2.1.2). Within-worker variability was large compared to between-worker variability suggesting that variability in task-based exposure during spray painting over time is more prominent than differences in mean exposures between workers. Determinants of task-based exposure were investigated but no statistically significant determinants were identified. Therefore, personal exposure estimates were calculated on the basis of median task-based exposure levels in combination with task-activity information (time spent on certain tasks). This approach, in which the same exposure estimate is used for workers performing the same task is known to result in a Berkson error (2). This type of error leads to limited or no bias in exposure-response relationships when logistic or log-linear regression models are used. However, precision of the slope of the exposure-response relationship is lost, resulting in wider confidence intervals (2). As a result, weak associations with health effects might have been missed.

An important aspect of the exposure metric is its biological relevance with respect to disease outcome. Misclassification may be introduced when the exposure metric does not reflect true biologically-relevant exposure.
We used an un-weighted total NCO exposure estimate, incorporating 23 different isocyanate compounds quantified in all samples. Overall, HDI oligomers dominated over HDI monomer in terms of both frequency and level of exposure. Health surveys and specific inhalation challenges suggest that isocyanate oligomers have similar health effects as diisocyanate monomers (6-9). Yet, animal studies indicate that the relative potencies of isocyanate monomers may be larger than for oligomers (10-13). For the estimation of each individual's exposure, contributions of the various compounds were not weighted because information was not available for each oligomer. In addition, insight in the relative potency is limited to experimental animal studies. The results of these studies can not easily be extrapolated to humans because exposure levels and conditions generally do not reflect workplace situations (14). Exploratory factor analysis showed high correlation between the presence of HDI and its oligomers in this study, and also exposure estimates calculated with HDI or oligomers separately were highly correlated. The weighting of the compounds would thus mostly have led to a rescaling of the exposure variable and not to different exposure-response estimates.

It has been hypothesized that the risk of a disease involving immune sensitization may be better reflected by an index which quantifies the occurrence of short intense peaks of exposure (15). A working day of a spray painter consists of short, repeating task cycles, some with and some without exposure. The use of personal protection equipment (PPE) in this industry is widespread. Consequently, exposure can probably best be described as a continuous low level exposure with a series of peaks occurring during poor PPE compliance, during spills, or during less protected low exposure level tasks. However, since jobs or individuals with high peak exposure often have high average or cumulative exposure as well (15), it is difficult to disentangle average exposure from the specific pattern of exposure.
Misclassification of actual exposure due to PPE use could theoretically have been reduced by including PPE-use in the model. However, respiratory protection factors have only been established by a small-scale study that may not reflect the present situation (16). In addition, detailed information would be needed on normal task-based use of different types of respiratory protection but also on incidental non-compliance. In this study, such detailed information of high quality was not available.

Another source of misclassification in this study may be incomplete exposure assessment for a particular exposure route (17). Human and animal studies are producing evidence of respiratory sensitization and disease aggravation as a result of dermal isocyanate exposure (18). Although dermal exposure was demonstrated in our study, it was not taken into account in the analysis of exposure-response relationships. Both inhalation and dermal exposure were found during paint related tasks and personal exposure estimates for inhalation and dermal exposure would have been correlated because they are based on time spent on the same tasks. Consequently, dermal exposure may have contributed to the exposure-response associations found in this study. Resulting bias may be substantial, but the effect and magnitude cannot be estimated on the basis of the information available. More information on dermal exposure and the correlation with inhalation exposure is needed.

Health end-point characterization and immunological tests

In a cross-sectional population of actively working individuals like the population under study, very few overt occupational asthma cases can be expected.
In occupational studies on high molecular weight (HMW) allergens, more prevalent parameters have been investigated. Parameters often used include specific sensitization, (work-related) symptoms or BHR, which have been associated with occupational asthma in case-control studies. Epidemiological surveys on HMW allergens have shown that many sensitized individuals are symptomatic, resulting in strong inter-relationships between different health end-points (19-22).

In this study, a similar approach was followed and specific sensitization, BHR, spirometric lung function parameters, and respiratory symptoms were recorded. Interestingly, outcomes were only partly overlapping (Figure 5.1) and associations between various outcomes seemed relatively weak. Especially the low prevalence of specific IgE and its marginal overlap with respiratory health end-points was remarkable, but has been suggested before. Case-control studies have also demonstrated specific IgE in a small proportion of isocyanate asthma cases (23-27), indicating that specific IgE is of limited diagnostic value for the recognition of isocyanate asthma. Some have considered an assay based on the production of *monocyte chemotactic protein-1* (MCP-1) by peripheral blood mononuclear cells (PBMC) stimulated with isocyanate conjugates as a promising new test for the detection of isocyanate asthma (28-30). However, an exploratory investigation of in vitro production of MCP-1 by isocyanate stimulated PBMC from a subset of the workers in our study, did not suggest that MCP-1 production tests could have been used as a marker of developing isocyanate asthma. Yet, high variability in MCP-1 levels was found which should be further explored before conclusions can be drawn.

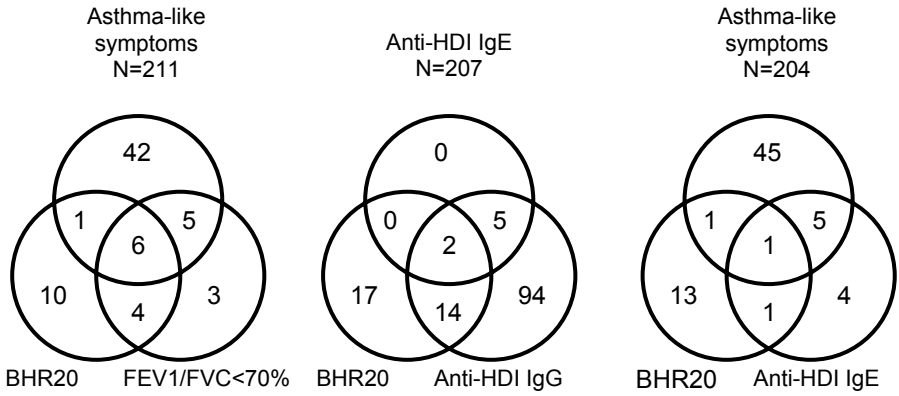

Figure 5.1: Overlap between various health end-points and specific sensitization.

Another difference between HMW allergens and isocyanates is that in addition to occupational asthma, a range of other lower respiratory tract effects have been linked to exposure to isocyanates. This may be reflected by the heterogeneity in respiratory effects found in this study. For instance, not only asthmatic but also COPD-like symptoms and lung function parameters were associated with exposure. These associations might point towards more chronic respiratory effects, like chronic bronchitis. Given the high prevalence of specific IgG antibodies, hypersensitivity pneumonitis (HP) might also come to mind. HP has indeed been reported to occur with low frequency in isocyanate-exposed populations (31, 32). Since HP is a rare disease (33) it seems unlikely that

cases meeting the criteria for a clinical diagnosis (34) were present in our study. However, it can not be excluded that there were some individuals with mild or subclinical HP.
This study was not aimed at establishing a diagnosis of any of the above-mentioned or other respiratory conditions. The heterogeneity in respiratory health endpoints that may have partly overlapping characteristics as well as the lack of immunological markers of specific sensitization complicate the definition of health endpoints for use in epidemiological analyses.

Implications

Despite the extensive use of isocyanate oligomers, this is the first large study in which quantitative exposure-response relationships were investigated in a population exposed to mainly isocyanate oligomers. Significant associations with various self-reported respiratory symptoms, BHR, and lung function parameters were found. Since our population was mainly exposed to HDI oligomers, and HDI monomer levels were low and did not exceed the current internationally accepted Occupational Exposure Limits (OELs), these associations are probably largely attributable to HDI oligomers. OELs for diisocyanate oligomers have only been established by a few countries or institutes and their validity is under debate (14).
The results of this study indicate that isocyanate oligomers can induce respiratory health effects at levels commonly found in the studied spray painting industry. This stresses the importance of regulation and control of oligomer exposure. Associations found may be used as a first step towards a better understanding of exposure-response relationships. However, because this is the first study in which such associations were assessed interpreting of the associations should be done with care.

Based on our experience some suggestions for future epidemiological studies can be made. More insight is needed into when actual exposures take place and isocyanate levels during key moments of exposure should be further investigated. The use of short term (task-based) exposure measurements as used in this study seems suitable for this purpose. However, high variability should be anticipated and the number of samples has to be sufficient. More knowledge on exposure determinants could reduce the measurement effort. To gain more insight in actual exposure, detailed high quality information on the task-based use of respiratory protection should be collected. Before their incorporation in an exposure metric, a re-evaluation of respiratory protection factors seems necessary.
This study, as well as other recent studies (35-38), suggests that in addition to airborne exposure, simultaneous dermal exposure to isocyanates occurs on a large scale within the spray painting industry. Given the growing evidence of the hazards of dermal exposure (18), it does not seem justified to only focus on inhalation exposure. This calls for the validation and use of methods for dermal

exposure as well as their incorporation in epidemiological studies. Existing methods for biological monitoring do not seem useful for estimating total internal exposure to isocyanates in populations exposed to mainly isocyanate oligomers.
More detailed clinical characterization of individuals with adverse respiratory health effects could give more insight in the nature, severity and work-relatedness of respiratory effects. This would allow categorization into more meaningful phenotypes for the assessment of exposure-response associations.

Besides epidemiological studies, future studies might be directed towards gaining more information on reducing exposure. High-risk tasks have been identified in this study. However, variability was high despite a large amount of samples, and no determinants of exposure could be demonstrated. An experimental design, in which some of the variability is controlled, would perhaps be more suitable to study exposure determinants.

References

1. Heederik D, Attfield M. Characterization of dust exposure for the study of chronic occupational lung disease: a comparison of different exposure assessment strategies. Am J Epidemiol 2000;151(10):982-90.
2. Armstrong BG. Effect of measurement error on epidemiological studies of environmental and occupational exposures. Occup Environ Med 1998;55(10):651-6.
3. Preller L, Kromhout H, Heederik D, Tielen MJ. Modeling long-term average exposure in occupational exposure-response analysis. Scand J Work Environ Health 1995;21(6):504-12.
4. Peretz C, de Pater N, de Monchy J, Oostenbrink J, Heederik D. Assessment of exposure to wheat flour and the shape of its relationship with specific sensitization. Scand J Work Environ Health 2005;31(1):65-74.
5. Ekman J, Levin JO, Lindahl R, Sundgren M, Ostin A. Comparison of sampling methods for 1,6-hexamethylene diisocyanate, (HDI) in a commercial spray box. Analyst 2002;127(1):169-73.
6. Vandenplas O, Cartier A, Lesage J, Perrault G, Grammer LC, Malo JL. Occupational asthma caused by a prepolymer but not the monomer of toluene diisocyanate (TDI). J Allergy Clin Immunol 1992;89(6):1183-8.
7. Vandenplas O, Cartier A, Lesage J, Cloutier Y, Perreault G, Grammer LC, et al. Prepolymers of hexamethylene diisocyanate as a cause of occupational asthma. J Allergy Clin Immunol 1993;91(4):850-61.
8. 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(4):1183-93.
9. 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(1):48-55.
10. 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(3):154-64.
11. Pauluhn J. Pulmonary irritant potency of polyisocyanate aerosols in rats: comparative assessment of irritant threshold concentrations by bronchoalveolar lavage. J Appl Toxicol 2004;24(3):231-47.
12. 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(4-5):257-69.
13. 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(2-3):147-60.
14. Bello D, Woskie SR, Streicher RP, Liu Y, Stowe MH, Eisen EA, et al. Polyisocyanates in occupational environments: a critical review of exposure limits and metrics. Am J Ind Med 2004;46(5):480-91.
15. Kriebel D, Checkoway H, Pearce N. Exposure and dose modelling in occupational epidemiology. Occup Environ Med 2007;64(7):492-8.

16. Rosenberg C, Tuomi T. Airborne isocyanates in polyurethane spray painting: determination and respirator efficiency. Am Ind Hyg Assoc J 1984;45(2):117-21.
17. Loomis DP, Savitz DA. Effect of incomplete exposure assessment on epidemiologic dose-response analyses. Scand J Work Environ Health 1994;20(3):200-5.
18. Bello D, Herrick CA, Smith TJ, Woskie SR, Streicher RP, Cullen MR, et al. Skin exposure to isocyanates: Reasons for concern. Environ Health Perspect 2007;115(3):328-335.
19. Houba R, Heederik DJ, Doekes G, van Run PE. Exposure-sensitization relationship for alpha-amylase allergens in the baking industry. Am J Respir Crit Care Med 1996;154(1):130-6.
20. Houba R, Heederik D, Doekes G. Wheat sensitization and work-related symptoms in the baking industry are preventable. An epidemiologic study. Am J Respir Crit Care Med 1998;158(5 Pt 1):1499-503.
21. Hollander A, Heederik D, Doekes G. Respiratory allergy to rats: exposure-response relationships in laboratory animal workers. Am J Respir Crit Care Med 1997;155(2):562-7.
22. Hollander A, Doekes G, Heederik D. Cat and dog allergy and total IgE as risk factors of laboratory animal allergy. J Allergy Clin Immunol 1996;98(3):545-54.
23. Park HS, Kim HY, Nahm DH, Son JW, Kim YY. Specific IgG, but not specific IgE, antibodies to toluene diisocyanate-human serum albumin conjugate are associated with toluene diisocyanate bronchoprovocation test results. J Allergy Clin Immunol 1999;104(4 Pt 1):847-51.
24. Cartier A, Grammer L, Malo JL, Lagier F, Ghezzo H, Harris K, et al. Specific serum antibodies against isocyanates: association with occupational asthma. J Allergy Clin Immunol 1989;84(4 Pt 1):507-14.
25. Baur X, Dewair M, Fruhmann G. Detection of immunologically sensitized isocyanate workers by RAST and intracutaneous skin tests. J Allergy Clin Immunol 1984;73(5 Pt 1):610-8.
26. Kim H, Kim YD, Choi J. Seroimmunological characteristics of Korean workers exposed to toluene diisocyanate. Environ Res 1997;75(1):1-6.
27. Butcher BT, O'Neil CE, Reed MA, Salvaggio JE. Radioallergosorbent testing of toluene diisocyanate-reactive individuals using p-tolyl isocyanate antigen. J Allergy Clin Immunol 1980;66(3):213-6.
28. Bernstein DI, Cartier A, Cote J, Malo JL, Boulet LP, Wanner M, et al. Diisocyanate Antigen-stimulated Monocyte Chemoattractant Protein-1 Synthesis Has Greater Test Efficiency than Specific Antibodies for Identification of Diisocyanate Asthma. Am J Respir Crit Care Med 2002;166(4):445-50.
29. Lummus ZL, Alam R, Bernstein JA, Bernstein DI. Characterization of histamine releasing factors in diisocyanate-induced occupational asthma. Toxicology 1996;111(1-3):191-206.
30. Lummus ZL, Alam R, Bernstein JA, Bernstein DI. Diisocyanate antigen-enhanced production of monocyte chemoattractant protein-1, IL-8, and tumor necrosis factor-alpha by peripheral mononuclear cells of workers with occupational asthma. J Allergy Clin Immunol 1998;102(2):265-74.
31. Vandenplas O, Malo JL, Dugas M, Cartier A, Desjardins A, Levesque J, et al. Hypersensitivity pneumonitis-like reaction among workers exposed to diphenylmethane [correction to piphenylmethane] diisocyanate (MDI). Am Rev Respir Dis 1993;147(2):338-46.
32. Baur X. Hypersensitivity pneumonitis (extrinsic allergic alveolitis) induced by isocyanates. J Allergy Clin Immunol 1995;95(5 Pt 1):1004-10.
33. Lacasse Y, Cormier Y. Hypersensitivity pneumonitis. Orphanet J Rare Dis 2006;1:25.
34. Lacasse Y, Selman M, Costabel U, Dalphin JC, Ando M, Morell F, et al. Clinical diagnosis of hypersensitivity pneumonitis. Am J Respir Crit Care Med 2003;168(8):952-8.
35. Hughson GW, Aitken RJ. Determination of dermal exposures during mixing, spraying and wiping activities. Ann Occup Hyg 2004;48(3):245-55.
36. Delgado P, Porcel J, Abril I, Torres N, Teran A, Zugasti A. Potential dermal exposure during the painting process in car body repair shops. Ann Occup Hyg 2004;48(3):229-36.
37. Liu Y, Sparer J, Woskie SR, Cullen MR, Chung JS, Holm CT, et al. Qualitative assessment of isocyanate skin exposure in auto body shops: a pilot study. Am J Ind Med 2000;37(3):265-74.
38. Bello D, Sparer J, Redlich CA, Ibrahim K, Stowe M, Liu Y. Slow curing of aliphatic polyisocyanate paints in the automotive refinishing: a potential source for skin exposure. J Occup Environ Hyg 2007;4(6):406-411.

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Summary

In this thesis the association between isocyanates and respiratory health effects and specific sensitisation was studied. Exposure to isocyanates, a group of compounds characterized by reactive N=C=O groups, is among the most frequently identified causes of occupational asthma (OA) in industrialized countries. Diisocyanates, containing two NCO groups, are polymerizing agents used in polyurethane (PU) products, such as lacquers, kits, and (insulation) foams. In a large proportion of product formulations many diisocyanate monomers have been replaced by their oligomers, with lower vapor pressure, to reduce inhalation exposure. OA is regarded as the most predominant health effect resulting from isocyanate exposure. Besides OA a range of other respiratory health effects have been linked to isocyanates. Yet, epidemiological studies in isocyanate exposed populations are scarce and many aspects of the relation between isocyanate exposure and respiratory health effects are still not clear. This may be attributed to the complexity of isocyanate exposure assessment and uncertainties regarding the choice of a relevant exposure proxy. Also, as mentioned, respiratory health endpoints, with potentially overlapping symptoms and effects, are diverse, and valid immunological markers of specific sensitization are lacking.

Spray painters comprise a large population at risk, with potentially high isocyanate exposure because many lacquers contain hexamethylene diisocyanate (HDI). In several countries, a high occupational asthma incidence has been reported in individuals exposed in this industry. The primary objective of this thesis was to establish exposure-response relationships between isocyanate exposure and respiratory health end-points and specific sensitization in spray painters. Aims of this thesis were: 1) To identify relevant isocyanate compounds, exposure sources, exposure routes and potential determinants of exposure. With this information exposure control measures can be identified and personal exposure estimates for exposure-response modeling can be established, 2) To study the prevalence of respiratory health effects and their association with exposure, and 3) To study the prevalence of specific sensitization and its association with exposure as well as health effects.

Isocyanate inhalation exposure was assessed in car body repair shops and industrial painting companies. Personal task-based samples (n=566) were taken using impingers with di-n-butylamine (DBA) dissolved in toluene, to immediately capture and stabilize reactive isocyanates. Samples were analyzed by liquid chromatography mass spectrometry (LC-MS) for isocyanate monomers, oligomers and products of thermal degradation. 20 compounds could be detected out of a total of 23. HDI oligomers were dominant in frequency of occurrence and showed the highest exposure levels. This indicates that paint is the most important source and major contributor of isocyanate exposure in this industry. Thermal degradation of isocyanates, for instance as a result of welding

activities, or the use of other isocyanate containing products like kits or foams did not seem to be a major source of exposure. Exploratory factor analysis showed that the presence of HDI oligomers and HDI monomer was highly correlated. Exposure to the sum of HDI-based compounds was highest during spray painting. Exposure variability during PU spray painting was large (from < limit of detection to 2643 $\mu g/m^3$ NCO). Variability over time was considerably higher than differences between workers. Lower level exposure was found during other paint related tasks and even tasks without direct exposure to paint. Exposure patterns were very similar in the car body repair shops and industrial painting companies.
Because of regular contact with lacquers dermal exposure may occur. The relative contribution of dermal exposure may be significant because of the widespread use of respiratory protection during spray painting. No validated methods were available for quantitative dermal exposure assessment. Therefore an ad hoc method was developed, making use of nitrile rubber gloves for sampling. Immediately after sampling, gloves were extracted in DBA in toluene and analyzed for HDI and its oligomers by LC/MS. Parallel to inhalation sampling 95 personal task-based samples were taken. Dermal exposure occurred during tasks that involve direct handling of paint. In car body repair shops exposure to the skin was significantly less likely to be found when gloves were used (odds ratio (OR) 0.2, 95% confidence interval (CI) 0.1-0.6) and more likely to be found when inhalation exposure was higher (OR 1.3, 95%CI 1.0-1.8 for a 10-fold increase in inhalation exposure).
Estimation of actual exposure is complicated because exposure may occur through inhalation and dermal exposure. In addition the use of personal protection equipment is widespread. To gain more insight in actual internal dose received by workers in this industry hexamethylene diamine (HDA, the corresponding amine of HDI) was assessed in urine samples collected during 24 hours by 55 workers (291 samples). HDA in urine could be demonstrated in 36% and 10% of car body repair shop workers and industrial painting company workers, respectively. In car body repair shops, the frequency of detectable HDA was significantly elevated at the end of the working day (OR (95% CI): 2.1 (1.1-4.2) for 3-6 PM vs. 0-8 AM). Surprisingly, HDA was detectable in only ~25% of the spray painters in both industries but also in a considerable percentage of non spray-painters in car body repair shops. These results imply a different exposure pattern as observed by the exposure assessment described earlier. However, use of HDA as a marker of total HDI and HDI oligomer exposure has several limitations, which complicate its interpretation. Therefore these results were not further used in the epidemiological survey. Nevertheless, the presence of HDA in non spray-painters in car body repair shops may point towards considerable bystander exposure.

Relations were studied between isocyanate exposure, sensitization and respiratory symptoms in 581 workers in the spray painting industry. Results from task-based personal inhalation exposure assessment were combined with task activity information to estimate personal exposure for exposure-response

analyses. Respiratory symptoms were more prevalent in exposed than among non exposed office workers. Accordingly, log-linear exposure-response associations were found for asthma-like symptoms (wheezing or chest tightness), COPD-like symptoms (chronic cough or phlegm or shortness of breath) and work-related chest tightness (prevalence ratios (PRs) for an interquartile range (IQR) increase in exposure of 1.2, 1.3 and 2.0 respectively, $p \leq 0.05$).
Specific IgE and IgG to HDI were assessed in serum by ImmunoCAP and enzyme immunoassays (EIA) with HDI-human serum albumin (HSA) conjugates prepared with HDI in the liquid (HDI_L) or vapor phase (HDI_V), or with HDI-oligomers (N100, N3300). The prevalence of specific IgE sensitization was low (up to 4.2% in spray painters). Nevertheless, IgE to N100-HSA was associated with exposure and work-related chest tightness. The low prevalence of specific IgE suggests that at most, specific IgE plays a role in a minority of individuals with symptoms. The prevalence of specific IgG was higher (2-50%) and strongly associated with exposure. IgG was only weakly associated with symptoms, suggesting that it is merely a marker of exposure.
Bronchial hyperresponsiveness (BHR), baseline spirometry and exhaled NO (eNO) were investigated in a subset of the population (n=229). BHR, assessed by methacholine challenge, was more prevalent among exposed than among office workers and a positive association was found with estimated exposure (PR (95% CI) IQR: 1.8 (1.1-3.0)). Although sensitized (specific IgE and IgG) workers seemed more often hyperresponsive, this was statistically significant for IgG positives as assessed by ImmunoCAP assay only (PR (95% CI): 3.1 (1.1-8.2)). Exposure related obstructive lung function changes independent of BHR were also found (FEV1, FEV1/FVC and flow parameters associated with exposure, $p<0.05$). This indicates that besides asthma more chronic respiratory effects may result from isocyanate exposure. No differences in eNO levels were observed between exposure categories. Although eNO was not associated with exposure, workers with IgG to the oligomeric HDI conjugates had somewhat elevated levels of eNO.

Although several features of isocyanate asthma point towards an immunological mechanism, specific IgE is generally found in a small proportion of asthma cases. There has been considerable debate regarding the validity of serological assays. Therefore various immunoassays based on HDI-HSA conjugates prepared with HDI in the liquid (HDI_L) or vapor phase (HDI_V), or with HDI-oligomers (N100, N3300) and the commercial ImmunoCAP assay were used in this study. Concordance and correlation between most assays were moderate to high, and inhibition assays revealed cross-reactivity between the various HDI-conjugates. However, results of EIAs with N100-HSA showed the strongest associations with both exposure levels and with health end-points and seemed to provide the best combination of sensitivity and specificity in the spray painting industry with mainly exposure to HDI oligomers. Therefore, when assessing isocyanate specific sensitization the isocyanate compounds that the population is exposed to should be taken into account.

The results of this study do not suggest that specific IgE plays a major role in the development of isocyanate related asthma-like effects in this population. It has been hypothesized that isocyanate asthma may be mediated by non-IgE mediated mechanisms. Therefore in a subset of the population (n=101) the use of a new cellular diagnostic test was explored. This assays is based on the production of the cytokine monocyte chemotactic protein-1 (MCP-1) by peripheral blood mononuclear cells (PBMC) stimulated with isocyanate conjugates. Crude data did not suggest that HDI-induced MCP-1 release differed between the three exposure categories or between individuals with and without asthma-like symptoms, BHR or specific antibodies. However, high background levels and large variability found may have limited the detection of subtle effects on isocyanate-induced MCP-1 production.

To summarize, in this thesis clear associations between isocyanate exposure and specific sensitization (IgE and IgG), respiratory symptoms and BHR and lung function parameters were demonstrated. Because this is the first study in which such associations were assessed, interpretation of the associations should be done with care. Exposure to isocyanates is complex and several sources of exposure misclassification may exist. The exposure-response estimates may be biased. For instance, because the use of filtering devices and dermal exposure were not taken into account when estimating exposures. Despite a possible bias, exposure-response associations were found for various health end-points. Different phenotypical subgroups with respect to BHR, FEV1/FVC<70%, antibodies, and increased eNO were observed. These may reflect different health end-points, underlying mechanisms, and disease stages. These results indicate that isocyanate oligomers can induce respiratory health effects at levels commonly found in this industry. This stresses the importance of regulation and control of oligomer exposure.

Nederlandse samenvatting

In dit proefschrift is het verband tussen blootstelling aan isocyanaten en effecten op de luchtwegen onderzocht in spuiters. In geïndustrialiseerde landen is blootstelling aan isocyanaten een van de meest voorkomende oorzaken van beroepsastma. Isocyanaten zijn een groep verbindingen die worden gekenmerkt door reactieve N=C=O-groepen. Di-isocyanaten bevatten 2 NCO groepen en worden gebruikt als polymeriserende verbindingen in polyurethaan producten zoals lakken, (isolatie)schuimen en kitten. Om blootstelling via de lucht te verlagen zijn veel di-isocyanaat monomeren in (tussen) producten vervangen door hun oligomeren met lagere dampspanning. Naast beroepsastma, dat gezien wordt als belangrijkste gezondheidseffect, kunnen isocyanaten ook andere luchtwegaandoeningen veroorzaken. Veel aspecten van de relatie tussen isocyanaatblootstelling en gezondheidseffecten zijn nog onduidelijk doordat weinig grootschalig epidemiologisch onderzoek is gedaan. Dit komt mede doordat het meten van blootstelling aan isocyanaten complex is. Daarnaast is het bepalen van te bestuderen gezondheidseindpunten moeilijk door de verschillende gezondheidseffecten met mogelijk overlappende symptomen en het ontbreken van valide immunologische markers voor specifieke allergische sensibilisatie.

Verfspuiters zijn een grote groep werknemers met mogelijk hoge blootstelling aan isocyanaten doordat veel lakken hexamethyleen di-isocyanaat (HDI) bevatten. In landen met een goede registratie van beroepsziekten is bekend dat beroepsastma onder verfspuiters vaak voorkomt. Doordat in Nederland geen goede registratie bestaat kan inzicht in het vóórkomen van astma als gevolg van isocyanaten alleen worden verkregen door surveys uit te voeren.
Het belangrijkste doel van dit proefschrift was om het verband te onderzoeken tussen isocyanaatblootstelling, luchtwegeffecten en specifieke sensibilisatie in spuiters. Deeldoelstellingen waren: 1) Bepalen van relevante isocyanaatverbindingen, bronnen van blootstelling, blootstellingsroutes en mogelijke determinanten van blootstelling. Hierdoor kunnen beheersmaatregelen worden bepaald en kan persoonlijke blootstelling voor onderzoek naar blootstellings-respons relaties worden geschat, 2) Onderzoeken van het vóórkomen van luchtwegeffecten en de relatie met blootstelling, 3) Onderzoeken van het vóórkomen van specifieke sensibilisatie en de relatie met blootstelling en gezondheidseffecten.

In het beschreven onderzoek is blootstelling via de luchtwegen in autoschadeherstel bedrijven en industriële spuiterijen in kaart gebracht. Hiervoor zijn persoonlijke metingen uitgevoerd met behulp van impingers (wasflessen) tijdens taken zoals spuiten, aanmaken van lak, etc. In de monsters zijn 23 verschillende isocyanaten bepaald, waaronder isocyanaat monomeren, oligomeren en producten die kunnen worden gevormd na afbraak door verhitting. Van 23 geanalyseerde verbindingen zijn er 20 gedetecteerd. HDI

oligomeren kwamen het meest voor en ook waren de niveaus hiervan het hoogst. Dit wijst erop dat in deze industrie lakken de belangrijkste bron van isocyanaatblootstelling zijn. Het vóórkomen van HDI monomeer en de verschillende HDI oligomeren was sterk gecorreleerd. Om de verschillende isocyanaten te kunnen optellen is de concentratie uitgedrukt in eenheden NCO/m^3. De hoogste niveaus van de som van alle HDI-verbindingen kwam voor tijdens het spuiten. Er was echter veel variatie in blootstellingsniveaus tijdens het spuiten (van onder de detectielimiet tot 2643 µg/m^3 NCO). Variatie over tijd bij eenzelfde persoon was groot in vergelijking met verschillen tussen werknemers. Bij andere taken waarbij met lakken gewerkt werd, maar ook bij taken waarbij niet met lakken werd gewerkt, was de blootstelling lager. Blootstellingspatronen en niveaus in de autoschadeherstel en industriële spuiterijen waren goed vergelijkbaar.

Tijdens het werken met lakken kan huidblootstelling vóórkomen door huidcontact. De relatieve bijdrage van huidblootstelling aan de totale blootsteling is mogelijk groot door het wijdverspreide gebruik van maskers tijdens spuiten. Omdat voor het meten van huidblootstelling geen gevalideerde methoden bestonden is een ad hoc methode ontwikkeld die gebruik maakt van nitril rubberen handschoenen voor monstername. 95 handschoenmonsters zijn parallel aan luchtmetingen genomen tijdens verschillende taken. Huidblootstelling kwam voor tijdens alle taken waarbij met lakken gewerkt werd. In autoschadeherstelbedrijven kwam huidblootstelling significant minder vaak voor wanneer beschermende handschoenen werden gebruikt (odds ratio (OR): 0,2, 95% betrouwbaarheidsinterval (bthi): 0,1-0,6) en vaker voor wanneer inhalatoire blootstelling hoger was (OR: 1,3, 95% bthi: 1,0-1,8 voor een 10-voudige toename in inhalatoire blootstelling).

Het schatten van de werkelijke blootstelling van een werknemer is complex doordat zowel blootstelling via de huid als de lucht voor kan komen. Daarnaast worden persoonlijke bescherming zoals maskers of handschoenen vaak gebruikt. Om meer inzicht te krijgen in de werkelijke blootstelling is een afbraakproduct van HDI (HDA: hexamethyleen diamine) bepaald in de urine van werknemers. Hiertoe is urine verzameld van 55 werknemers gedurende 24 uur (291 urine monsters). HDA kon worden aangetoond in 36% van de werknemers van autoschadeherstel bedrijven en 10% van werknemers van industriële spuiterijen. In autoschadeherstelbedrijven kwam HDA significant vaker voor aan het eind van de werkdag (OR (95% bthi): 2,1 (1,1-4,2) voor 15.00-18.00 uur vs. 0-8.00 uur). Het is verrassend dat HDA in slechts ~25% van de spuiters in beide industrieën voorkwam en ook in een behoorlijk deel van de niet-spuiters in de autoschadeherstel. Deze resultaten wijzen op een ander blootstellingspatroon dan de resultaten van de eerder beschreven blootstellingsmetingen. Omdat veel onduidelijkheden bestaan rond de validiteit van het gebruik van HDA als marker van blootstelling aan HDI oligomeren zijn deze resultaten niet gebruikt in de epidemiologische analyses. Desondanks wijst het vóórkomen van HDA in niet-

spuiters erop dat mensen die niet direct met isocyanaten werken maar wel in de directe omgeving van spuiters werken mogelijk kunnen worden blootgesteld.

Het verband tussen isocyanaatblootstelling en luchtwegklachten is bestudeerd in 581 werknemers in de spuitbranche. Voor alle werknemers in de studie is persoonlijke blootstelling geschat op basis van de inhalatoire luchtblootstellingsmetingen en individuele tijdsbesteding op het werk. Astma-achtige klachten (piepen op de borst of benauwdheid), COPD-achtige klachten (chronisch hoesten of slijm ophoesten, of kortademigheid) en werkgerelateerde benauwdheid kwamen significant vaker voor in werknemers met hogere blootstelling (prevalentie ratio's (PRs) voor een interkwartiel range (IQR, het verschil tussen 25 en 75 percentiel van de blootstelling) toename in blootstelling van respectievelijk 1,2, 1,3 en 2,0, $p \leq 0,05$).
In een deel van de populatie (229 werknemers) zijn bronchiale hyperreactiviteit (BHR), longfunctie en NO in uitademingslucht (eNO) onderzocht. BHR is een objectieve methode om luchtwegvariabiliteit vast te stellen en een belangrijk kenmerk van astma. BHR kwam vaker voor bij werknemers met een hogere blootstelling (PR (95% bthi) IQR: 1,8 (1,1-3,0)). Daarnaast waren het FEV1, en de FEV1/FVC ratio en volumestroom parameters geassocieerd met blootstelling. Deze verbanden waren onafhankelijk van BHR. Dit suggereert dat naast astma mogelijk meer chronische effecten op de luchtwegen het gevolg kunnen zijn van isocyanaatblootstelling. eNO is vaak verhoogd tijdens allergische reacties die worden gemediëerd door IgE zoals na blootstelling aan huisstofmijten of proefdierallergenen. In deze studie zijn geen verschillen gevonden in eNO niveaus tussen de verschillende categorieën blootgestelden. Desondanks was eNO verhoogd in bepaalde groepen werknemers met antilichamen tegen isocyanaten.

De mechanismen achter het ontstaan van isocyanaatastma zijn onduidelijk. Ondanks dat verschillende kenmerken van isocyanaatastma wijzen op immunologische mechanismen (onder andere snelle sterke longfunctiedaling bij provocatie en herstel zoals bij vroege en late reacties), worden IgE antilichamen over het algemeen maar in een klein gedeelte van de astma gevallen gevonden. Er bestaat veel controverse over de validiteit van serologische tests. Daarom zijn in deze studie meerdere tests gebruikt om antilichamen te bepalen in de hoop hiermee meer werknemers met antilichamen te detecteren. Specifieke IgE en IgG antilichamen tegen HDI in serum zijn bepaald met behulp van ImmunoCAP en enzym immunoassays (EIA) met verschillende HDI-humaan serum albumine (HSA) conjugaten. Deze conjugaten zijn gemaakt met HDI in vloeistof- (HDI_L) of in gasfase (HDI_v), of met HDI-oligomeren (N100 of N3300). Specifieke IgE antilichamen kwamen zeer weinig voor (tot 4.2% in spuiters) en spelen waarschijnlijk op zijn hoogst in een klein deel van werknemers een rol bij de ontwikkeling van klachten. Desondanks kwam IgE tegen N100-HSA vaker voor bij werknemers met hoge blootstelling en bij werknemers met werkgerelateerde benauwdheid. Specifieke IgG antilichamen kwamen vaker voor (2-50%) en waren duidelijk verhoogd in werknemers met hoge blootstelling.

Specifieke IgG antilichamen kwamen niet vaker voor bij werknemers met klachten en leken daarom vooral een marker van blootstelling.
Over het algemeen waren de resultaten verkregen met de verschillende assays vergelijkbaar. Inhibitie-experimenten lieten zien dat de verschillende HDI-conjugaten kruisreacties vertoonden. Er waren echter ook verschillen in reactiviteit en associaties met blootstelling en gezondheidseffecten. EIAs met HDI-oligomeer conjugaten (N100-HSA) leken in deze populatie van spuiters, die vooral was blootgesteld aan oligomeren van HDI, de beste combinatie van sensitiviteit en specificiteit te geven. Dit wijst erop dat bij het bepalen van specifieke sensibilisatie rekening gehouden moet worden met aan welke isocyanaten een populatie wordt blootgesteld.
De resultaten wijzen er niet op dat specifieke IgE antilichamen in deze populatie een belangrijke rol spelen in de ontwikkeling van astma-achtige effecten. Eerder is gesuggereerd dat isocyanaatastma het gevolg is van niet-IgE-gemedieerde immunologische mechanismen. In een deel van de populatie (n=101) is daarom het een nieuwe cellulaire test gebruikt. Deze test is gebaseerd op de productie van het cytokine monocyte chemotactic protein-1 (MCP-1) door perifere bloed mononucleaire cellen die gestimuleerd zijn met isocyanaatconjugaten. De eerste resultaten lieten echter geen verband zien tussen MCP-1 afgifte na toevoeging van HDI en de mate van blootstelling, de aan- of afwezigheid van astma-achtige klachten, BHR of specifieke sensibilisatie.

Samenvattend wordt in dit proefschrift een duidelijk verband aangetoond tussen isocyanaatblootstelling en luchtwegklachten, BHR en longfunctie. Omdat dit de eerste studie is waarin deze relaties zijn bekeken moeten de resultaten met enige voorzichtigheid worden geïnterpreteerd. Blootstelling aan isocyanaten is een complex fenomeen en bij de epidemiologische analyse is alleen rekening gehouden met blootstelling via de lucht. Ondanks een mogelijke bias door misclassificatie van blootstelling zijn blootstellings-respons relaties gevonden.
Verschillende fenotypische subgroepen konden worden gedefinieerd op basis van verschillende combinaties van gezondheidsparameters (klachten, BHR, FEV1/FVC<70%, sensibilisatie en verhoogd eNO). Deze zijn mogelijk het gevolg van verschillende luchtwegaandoeningen en onderliggende verschillen in pathogenese. Daarnaast kan ook het stadium waarin de ziekte zich bevindt een rol spelen. Specifieke IgE sensibilisatie leek geen belangrijke rol te spelen bij de ontwikkeling van gezondheidseffecten en onderliggende mechanisme(n) blijven onduidelijk.
De resultaten duiden erop dat isocyanaat oligomeren effecten op de luchtwegen kunnen veroorzaken bij blootstellingsniveaus die gewoonlijk gevonden worden in deze industrie. De sterke associatie met BHR is verontrustend. Dit benadrukt het belang van regulatie en beheersing van oligomeerblootstelling.

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Curriculum vitae

Anjoeka Pronk werd geboren op 10 september 1977 te Oudenbosch. In 1995 behaalde ze haar VWO diploma aan het Thomas More College in Oudenbosch. Van 1996 to 2002 studeerde ze Milieuhygiene aan de Wageningen Universiteit. Ze deed afstudeervakken bij de vakgroep Toxicologie in Wageningen en het Institute for Risk Assessment Sciences (IRAS), Universiteit Utrecht en liep stage bij Landcare Research in Nieuw Zeeland. Haar studie werd in 2002 afgerond met het behalen van haar diploma met als specialisatie Milieu, Arbeid en Gezondheid. Daarna begon ze bij TNO Business Unit Food and Chemical Risk Analysis aan het promotieonderzoek beschreven in dit proefschrift. Dit werd uitgevoerd in samenwerking met het IRAS. Vanaf Augustus 2007 werkt ze als postdocteral fellow bij het bij de Occupational and Environmental Epidemiology Branch van het National Cancer Institute in Washington, Verenigde Staten.