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2017

Document Version:
Publisher's PDF, also known as Version of record

[Link to publication](#)

Citation for published version (APA):
Hamada, H. (2017). *Experimental and Clinical Studies on Contact Allergy to Diphenylmethane-4,4′-diisocyanate and Related Substances*. [Doctoral Thesis (compilation), Faculty of Medicine]. Lund University: Faculty of Medicine.

Total number of authors:
1

Experimental and Clinical Studies on Contact Allergy to Diphenylmethane-4,4′-diisocyanate and Related Substances

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Organization	Document name
LUND UNIVERSITY Dept. of Occupational and Environmental Dermatology Skåne University Hospital Malmö, Sweden	DOCTORAL DISSERTATION
	Date of issue: September 1, 2017
Author(s) Haneen Hamada	Sponsoring organization

Title and subtitle Experimental and Clinical Studies on Contact Allergy to Diphenylmethane-4,4′-diisocyanate and Related Substances

Abstract

The exposure to isocyanates is more of an occupational problem than a consumer problem. The main route of exposure is assumed to be through inhalation. Isocyanates that have high vapor pressure, such as TDI, can be airborne at room temperature. Other isocyanates such as MDI are volatile only when heated. Industrial processes that can cause exposure to isocyanates are, for example, spraying or heating. Inhalation of isocyanates can cause disorders of the respiratory tract. Isocyanates are well known to cause direct toxic and irritant effects, such as irritation of the nose, throat, and upper airways and also eye and skin irritation. In animal and human studies, it has been found that isocyanates cause skin sensitization and sensitization of the lung. The sensitization mechanism and metabolism of isocyanates is not fully understood. Concurrent reactions are often seen between different isocyanates and their different corresponding amines. It is not always evident whether the concurrent reactions are due to co-exposure or possible cross-reactivity. Patch test preparations of 4,4′-MDI have been shown to contain only fractions of what is stated. Concentrations of 4,4′-MDI in test preparations decrease rapidly.

The aim of the first two papers included in this thesis was to study the dermal uptake and metabolism of isocyanates by dermal provocation of volunteers with different doses of 4,4′-MDI. This was done by the tape-stripping technique and studies on plasma and urine over time. The aim of papers three, four, and five was to investigate the sensitizing capacities and cross-reactivity patterns of some common isocyanates and the corresponding amines in order to provide a better understanding of isocyanate contact allergy. In order to do so, the guinea pig maximization test (GPMT) was used. The aim of paper six was to investigate the cause behind the rapid decrease in 4,4′-MDI in patch test preparations.

The main findings were as follows. (i) The distribution after dermal uptake of diphenylmethane-4,4′-diisocyanate (4,4′-MDI) appears to be a slower process than what is seen for airway uptake. Instead, our results indicated that the distribution of 4,4′-MDI in the skin and the subsequent elimination is a slow process, and the proportion absorbed into the skin was approximately half of the amount applied. The main amount absorbed reacts with cell components or forms polyurea and is probably released as diphenylmethane-4,4′-diamine (4,4′-MDA) by spontaneous or enzymatic hydrolysis over weeks or months, distributed systemically, and finally eliminated. A proportion of reacted 4,4′-MDI is probably eliminated from the skin upon cellular renewal. Patch testing with freshly made preparations of 2% 4,4′-MDI might lead to active sensitization. Thus, a concentration of 0.5% in pet., which has been recommended by the European Society for Contact Dermatitis (ESCD) based on our results, should be used (Papers I and II). (ii) 4,4′-MDI, 4,4′-MDA, dicyclohexylmethane-4,4′-diisocyanate (4,4′-DMDI), dicyclohexylmethane-4,4′-diamine (4,4′-DMDA), and p-phenylene diamine (PPD) are strong sensitizers among our group of sensitizers. 4,4′-MDI sensitized animals cross-react to 4,4′-MDA and to 4,4′-DMDI and animals sensitized to 4,4′-MDA cross-reacted to 4,4′-DMDA. PPD-sensitized animals showed cross-reactivity to 4,4′-MDA and there was an indication of cross-reactivity to 4,4′-DMDA. 4,4′-MDI-sensitized animals did not show cross-reactivity to PPD, so PPD cannot be used as a marker of 4,4′-MDI allergy. (iii) 4,4′-MDI reacts with water, protein, or other components found in Freund's complete adjuvant used in the GPMT. Aged, pure 4,4′-MDI is instable, even when stored in the freezer. The outcome of 4,4′-MDI sensitization in the GPMT might be affected by the instability of the pure substance and also its reaction with Freund's complete adjuvant. Induction substances should be prepared in close connection with the intradermal injection (Papers III, IV, and V). (iv) Evaporation and reaction with water can be excluded as significant factors for the instability of the 4,4′-MDI patch test preparations. Most data indicate that trimerization—and perhaps also dimerization—is the main factor influencing this instability. The 4,4′-MDI trimer might be a weak allergen due to the higher molecular weight making it more difficult to penetrate through the skin. Since many patients have been patch tested with aged 4,4′-MDI patch test preparations (where the patients do not react), we can conclude that if the trimer is present, it does not cross-react with 4,4′-MDI (Paper VI).

Key words: 4,4′-MDI, diphenylmethane-4,4′-diisocyanate, 4,4′-MDA, PPD, 2,4-TDI, 4,4′-DMDI, 4,4′-DMDA, Contact allergy, dermal uptake, cross-reactivity, GPMT, Trimer, Freund´s complete adjuvant.

Classification system and/or index terms (if any)

Supplementary bibliographical information	Language English
ISSN and key title 1652-8220 Lund University, Faculty of Medicine Doctoral Dissertation Series 2017:110	ISBN 978-91-7619-492-8

Recipient's notes	Number of pages 172	Price
	Security classification	

Signature _Haneen Hamada_   Date _170731_

Experimental and Clinical Studies on Contact Allergy to Diphenylmethane-4,4′-diisocyanate and Related Substances

Haneen Hamada

To Mom, Dad, Mustafa, Farah, Christoffer, and Noah

Contents

The thesis at a glance

Paper I

Dermal uptake study with diphenylmethane-4,4′-diisocyanate led to active sensitization

Objectives:

To investigate the dermal uptake of diphenylmethane-4,4′-diisocyanate (4,4′-MDI) and to explore the indications that the commonly used patch test concentration 2% in petrolatum can cause active sensitization.

Methods:

Using liquid chromatography-mass spectrometry, we performed chemical analysis of the 4,4′-MDI preparation used in the application and of the amount of 4,4′-MDI not absorbed in the skin. The volunteers were tested with serial dilutions of 4,4′-MDI and the potentially cross-reacting substances 4,4′-diaminodiphenylmethane (4,4′-MDA), *p*-phenylenediamine (PPD) and dicyclohexylmethane-4,4′-diisocyanate (4,4′-DMDI).

Results:

Patch test results indicated that the volunteers were actively sensitized to 4,4′-MDI following the dermal uptake study since they had positive reactions to 4,4′-MDA, which is considered to be a marker of MDI allergy. No positive reactions to PPD or DMDI were seen. Chemical investigation confirmed that the correct concentration had been used for the dermal uptake study, and showed that about 70% of the 4,4′-MDI applied was not absorbed.

Conclusions:

This dermal uptake study with 4,4′-MDI in 2.0% petrolatum with an occlusion time of 8 hours induced active sensitization to 4,4′-MDI and subsequent contact allergy to 4,4′-MDA.

Paper II

Assessment of dermal uptake of diphenylmethane-4,4′-diisocyanate using tape stripping and biological monitoring

Objective:

To investigate the dermal uptake of diphenylmethane-4,4′-diisocyanate (4,4′-MDI).

Methods:

Four volunteers were dermally exposed to 10, 25, 49, or 50 mg 4,4′-MDI, respectively, for 8 hours. The exposed areas were tape-stripped. Urine and blood levels were biologically monitored for 48 hours. Tape strips, plasma, and urine were analyzed by liquid chromatography-mass spectrometry.

Results:

Between 35% and 70% of the dose of 4,4′-MDI applied was absorbed by the skin. Very low proportions of the dose applied were found in the tape-stripping. The concentration of 4,4′-MDA in plasma and urine was low, but it peaked in urine 10–14 hours after exposure and in plasma 8–32 hours after exposure.

Conclusions:

4,4′-MDI is readily absorbed by the human skin. Only small proportions of 4,4′-MDI as such remained in the superficial layers of the skin. The amounts found in blood and urine were only small fractions of the total doses applied, indicating that very small amounts of 4,4′-MDI penetrate the skin and reach the blood stream. This indicates that 4,4′-MDI polymerizes or reacts with cell constituents in the upper layers of the skin. The dermal uptake and distribution of 4,4′-MDI is much slower than what is seen with airway uptake. Our data strongly suggest that formation of 4,4′-MDA from 4,4′-MDI by reacting with water in the skin can only occur to a very limited extent.

Paper III

Sensitization and cross-reactivity patterns of contact allergy to diisocyanates and corresponding amines. Investigation of diphenylmethane-4,4′-diisocyanate, diphenylmethane-4,4′-diamine, dicyclohexylmethane-4,4′-diisocyanate, and dicylohexylmethane-4,4′-diamine

Objectives:

To investigate the sensitizing potential and the cross-reactivity pattern of diphenylmethane-4,4′-diisocyanate (4,4′-MDI), diphenylmethane-4,4′-diamine (4,4′-MDA), dicyclohexylmethane-4,4′-diisocyanate (4,4′-DMDI), and dicyclohexylmethane-4,4′-diamine (4,4′-DMDA).

Methods:

We used the guinea pig maximization test (GPMT).

Results:

The GPMT showed sensitizing capacities for all the substances investigated: 4,4′-MDI ($p < 0.001$), 4,4′-MDA ($p < 0.001$), 4,4′-DMDI ($p < 0.001$), and 4,4′-DMDA ($p < 0.001$). 4,4′-MDI-sensitized animals showed cross-reactivity to 4,4′-MDA ($p < 0.001$) and 4,4′-DMDI ($p < 0.05$). 4,4′-MDA-sensitized animals showed cross-reactivity to 4,4′-DMDA ($p = 0.0084$).

Conclusion:

All the substances investigated were found to be strong sensitizers. Animals sensitized to 4,4′-MDI showed cross-reactivity to 4,4′-MDA and 4,4′-DMDI, thus supporting previous findings in the literature. Animals sensitized to the aromatic amine 4,4′-MDA showed cross-reactivity the aliphatic amine 4,4′-DMDA.

Paper IV

Sensitizing capacities and cross-reactivity patterns of some diisocyanates and amines using the guinea pig maximization test. Can *p*-phenylenediamine be used as a marker for diisocyanate contact allergy?

Objectives:

To investigate the sensitizing capacities of toluene-2,4-diisocyanate (2,4-TDI) and PPD and the cross-reactivity of diphenylmethane-4,4′-diamine (4,4′-MDA), 2,4-TDI, dicyclohexylmethane-4,4′-diamine (4,4′-DMDA), dicyclohexylmethane-4,4′-diisocyanate (4,4′-DMDI), diphenylmethane-4,4′- diisocyanate (4,4′-MDI), and *p*-phenylenediamine (PPD).

Methods:

We used the guinea pig maximization test (GPMT).

Results:

PPD was found to be a strong sensitizer ($p < 0.001$). Animals sensitized to PPD showed cross-reactivity to 4,4′-MDA ($p < 0.001$), and there was some indication of cross-reactivity to 4,4′-DMDA ($p = 0.069$). The cross-reactivity did not go in both directions, i.e. animals sensitized to 4,4′-MDA and 4,4′-DMDA did not show cross-reactivity to PPD. Animals sensitized to 2,4-TDI developed toxic reactions at the induction site. Based on the 16 animals sensitized to 2,4-TDI, there was some indication of cross-reactivity to PPD ($p = 0.20$).

Conclusion:

PPD was found to be a strong sensitizer. However, it cannot be used as a marker for isocyanate contact allergy. On the other hand, positive reactions to 4,4′-MDA could indicate a PPD allergy. The intradermal induction concentration of 2,4-TDI (0.70%w/v) can induce strong local reactions in guinea pigs and should be reduced.

Paper V

Factors affecting the concentration of diphenylmethane-4,4′-diisocyanate in Freund's complete adjuvant. Can they affect the outcome of the guinea pig maximization test?

Objectives:

To investigate the stability of 4,4′-MDI and its possible reaction with components in Freund's complete adjuvant (FCA).

Methods:

The same preparations as used in the GPMT were prepared, i.e. 1.0% w/v 4,4′-MDI in FCA/paraffin oil (40/60 v/v), and stored under different conditions for 48 hours. The content of 4,4′-MDI in FCA/paraffin oil mixtures and also in samples of pure substance was monitored by chemical analysis using gel permeation chromatography (GPC).

Results:

The 4,4′-MDI content in the pure substance can decrease substantially within a few months. A rapid decrease in 4,4′-MDI concentration was also seen in 4,4′-MDI/FCA preparations stored in the refrigerator and at room temperature.

Conclusion:

The outcome of 4,4′-MDI sensitization in the GPMT might be affected by the instability of the pure substance as well as its reaction with FCA. Major effects can be expected if these two factors interact. Thus, fresh 4,4′-MDI should be used and preparations of induction substances should be prepared in close connection with the intradermal injection.

Paper VI

The mysterious instability of diphenylmethane-4,4′-diisocyanate (4,4′-MDI) in petrolatum patch test preparations for diagnoses of contact allergy

Objective:

To investigate why the concentration of 4,4′-MDI decreases in patch test preparations intended for detection of 4,4′-MDI contact allergy.

Method:

We performed chemical analysis of 4,4′-MDI preparations using gel permeation chromatography (GPC). We also performed synthesis of 4,4′-MDI trimer and analysis using Fourier transform infrared (FTIR) and nuclear magnetic resonance (NMR).

Results:

Addition of water to the 4,4′-MDI preparations did not cause significant loss of 4,4′-MDI. Hence, the degradation was not caused by polymerization due to its reaction with moisture in the petrolatum or in the air. Instead, the findings supported the hypothesis that trimerization is the cause of degradation. The characteristics of the synthesized trimer of 4,4′-MDI matched previously published data using FTIR. The synthesized trimer matched trimer extracted from the aged 4,4′-MDI preparations. NMR analysis of the synthesized trimer showed high amounts of impurities but indicated the presence of 4,4′-MDI trimer.

Conclusion:

Reaction of 4,4′-MDI with water and evaporation can be excluded as reasons for the instability of 4,4′-MDI in patch test preparations. There are strong indications that the cause of instability is due to the spontaneous formation of 4,4′-MDI trimer. Since many patients tested with aged preparations do not react, this indicates that the possible trimer does not cross-react with 4,4′-MDI and that it is probably a weaker allergen than 4,4′-MDI .

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List of publications

I. **Dermal uptake study with 4,4′-diphenylmethane diisocyanate led to active sensitization**
    Hamada H, Isaksson M, Bruze M, Engfeldt M, Liljelind I, Axelsson S, Jönsson B, Tinnerberg H, Zimerson E
    Contact Dermatitis 2013:66:101-105

II. **Assessment of dermal uptake of diphenylmethane-4,4′-diisocyanate using tape stripping and biological monitoring**
    Hamada H, Liljelind I, Bruze M, Engfeldt M, Isaksson M, Jönsson B, Tinnerberg H, Lindh C, Axelsson S, Zimerson E
    Accepted in The European Journal of Dermatology

III. **Sensitization and cross-reactivity patterns of contact allergy to diisocyanates and corresponding amines. Investigation of diphenylmethane-4,4′-diisocyanate, diphenylmethane-4,4′-diamine, dicyclohexylmethane-4,4′-diisocyanate, and dicylohexylmethane-4,4′-diamine**
    Hamada H, Bruze M, Zimerson E, Isaksson M, Engfeldt M
    Contact Dermatitis. 2017 May 29. doi: 10.1111/cod.12809. [Epub ahead of print] PMID: 28555927

IV. **Sensitizing capacities and cross-reactivity patterns of some diisocyanates and amines using the guinea pig maximization test. Can *p*-phenylenediamine be used as a marker for diisocyanate contact allergy?**
    Hamada H, Bruze M, Zimerson E, Isaksson M, Engfeldt M
    Submitted

V. **Factors affecting the concentration of diphenylmethane-4,4′-diisocyanate in Freund's complete adjuvant. Can they affect the outcome of the guinea pig maximization test?**
    Hamada H, Bruze M, Zimerson E, Isaksson M, Engfeldt M
    Journal of Clinical & Experimental Dermatology Research 2017:8:402

VI. **The mystical instability of diphenylmethane-4,4′-diisocyanate (4,4′-MDI) in petrolatum patch test preparations used for diagnoses of contact allergy**
    Hamada H, Engfeldt M, Bruze M, Isaksson M, Zimerson E
    In manuscript

Abbreviations

ACD	Allergic contact dermatitis
CLP	Classification, labeling, and packaging
D	Day
Da	Dalton
DBA	Di-n-butylamine
4,4′-DMDA	Diamino-4,4′-dicyclohexylmethane
4,4′-DMDI	Dicyclohexylmethane-4,4′-diisocyanate
DMSO	Dimethyl sulfoxide
DPRA	Direct peptide-reactive assay
ESCD	European society of contact dermatitis
FCA	Freund's complete adjuvant
FTIR	Fourier transform infrared spectroscopy
GC-MS	Gas chromatography–mass spectrometry
GHS	Globally harmonized system
GPC	Gel permeation chromatography
GPMT	Guinea pig maximization test
HDI	Hexamethylene diisocyanate
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum coherence
HRIPT	Human repeated insult patch test
ICD	Irritant contact dermatitis
ICDRG	International contact dermatitis research group
IPDI	Isophorone diisocyanate
LLNA	Local lymph node assay
4,4′-MDA	Diphenylmethane-4,4′-diamine, 4,4-methylenedianiniline
2,2′-MDI	Diphenylmethane-2,2′-diisocyanate
2,4′-MDI	Diphenylmethane-2,4′-diisocyanate
4,4′-MDI	Diphenylmethane-4,4′-diisocyanate
MEST	Mouse ear-swelling test
NMR	Nuclear magnetic resonance
OECD	Organization for economic cooperation and development
$P_{o/w}$	Partition-coefficient $_{octanol/water}$
pet.	Petrolatum
PPD	*p*-Phenylenediamine
PUR	Polyurethane
RADS	Reactive airway dysfunction syndrome
2,4-TDA	2,4-Toluene diamine
2,4-TDI	2,4-Toluene diisocyanate

1 Introduction

1.1 Contact allergy and allergic contact dermatitis

"Delayed contact hypersensitivity" and "type IV allergy" are synonyms of contact allergy. Contact allergy is a reaction that can develop after the skin has been exposed to sensitizing substances. These substances are called contact allergens. Clinically, contact allergy shows as allergic contact dermatitis (ACD). The ACD appears when a person is exposed to a dose of the allergen that exceeds that person's threshold (1). More than 4,000 substances are known to cause contact allergy (2).

A contact allergen must first penetrate the skin barrier and then react with proteins to form an antigen. The requirements for penetration of the skin are high lipophilicity ($logP_{o/w} > 1$) (3) and low molecular weight (< 1000 Da) (4). Such contact allergens are also called haptens or incomplete antigens, as they are too small to act as antigens. Many macromolecules such as proteins contain nucleophilic groups that are rich in electrons and can form covalent bonds with electrophiles, which are positively charged. Almost all haptens are electrophilic and can therefore react with proteins, but not all of them; some need to undergo metabolic processes to gain electrophilic properties while others gain these properties by, for example, auto-oxidation (1, 5).

There are two phases in contact allergy. The sensitization phase is when the hapten binds to the protein(s) of the skin and forms antigen. The antigens are then taken up by antigen-presenting cells called Langerhans cells and are transported to the nearby lymph nodes. In the lymph node, the Langerhans cells are presented to the uncommitted T-cells, which become activated. Activated T-cells release cytokines, which lead to proliferation and differentiation of the T-cells to make them specific memory cells of the hapten. The sensitization phase may take 4 days to several weeks to develop. The second phase is the elicitation phase, which occurs when the individual is re-exposed to the hapten. Here, the Langerhans cells present the antigen to the hapten-specific memory T-cells. The memory T-cells become activated and start to proliferate, and a cascade of inflammatory events develops in the area of skin that has been exposed. The inflammation then causes an eczematous reaction, which develops 18–48 hours after exposure to the allergen (1). Some substances have a longer elicitation phase—up to 2–3 weeks (6-9).

Contact allergy is a lifelong condition. If the sensitized individual avoids contact with the allergen or to chemically related substances, he/she will not develop ACD.

1.2 Patch testing

Patch testing is the method used to diagnose contact allergy. It was first established by Jadassohn more than 100 years ago. The method was described by Bloch in 1929 (10). Patients with a history of dermatitis can be patch tested and re-exposed to the suspected allergen under controlled conditions (Figure 1). The patch testing method is continuously being developed and standardized with regard to allergens, vehicles, concentrations, doses, and scoring (11-15).

Patch testing is performed by evenly distributing allergens in suitable vehicles and concentrations in test chambers, which are then applied to the back of the patient for 48 hours. When reading the patch test reactions, they are scored according to the International Contact Dermatitis Research Group (ICDRG) criteria: (+), doubtful reaction; +, weak positive reaction; ++, strong positive reaction; and +++, extreme positive reaction (15). Readings of the patch test reactions on day (D) 3/4 and D7 are recommended, as they have been shown to be most accurate (16).

Late patch test reactions are reactions that appear at the site of the patch test later than D7 (15). Possible explanations for late patch test reactions are a low test concentration, that the patient has low reactivity, or slow penetration of the allergen. Late reactions can also be a sign of active sensitization.

Active sensitization is an unfavorable effect of patch testing, which is generally defined as being when a previously negative patch test has a flare-up reaction appearing after 10–20 days and where a positive reaction on D3 is seen on retesting (14). Some sensitized patients can react to lower concentrations of the allergen after D7 (6, 7), so it is recommended that the patient should be patch tested with serial dilutions of the allergen when there is a suspicion of active sensitization (13).

False-positive reactions are (by definition) caused by irritation and have a similar morphology to a contact allergic reaction (15). In order to investigate if the substance causes false-positive reactions it is recommended that the substance is tested in serial dilutions. Patch testing in controls can give further evidence of the nature of the reaction (14).

False-negative reactions are defined as failing to induce a positive patch test reaction in a contact allergic patient (15). It is important to test with an adequate dose, a stable substance in the vehicle of choice, and an evenly distributed substance in order to minimize the occurrence of false-negative reactions. If the substance is

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tested in an unsuitable vehicle or test chamber, it can sometimes change into a non-sensitizing substance (17, 18).
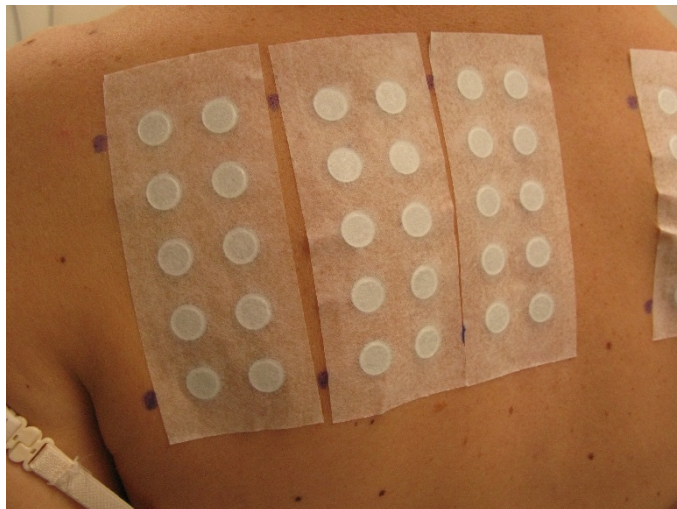
Figure 1.
Applied patch test.

1.3 Isocyanates

Isocyanates are chemical compounds that are reactive and contain $-N = C = O$ groups. They can be monoisocyanates, diisocyanates, or polyisocyanates depending on how many –NCO groups there are in the molecule. Isocyanates are either aromatic or aliphatic. Due to their high reactivity, they react with compounds such as water, alcohols, amines, and thiols. Diisocyanates and polyisocyanates are used in the plastic industry in the production of polyurethanes (PURs). PUR is produced by reacting the specific isocyanate with polyols. Polyols are either hydroxyl-terminated polyethers or polyesters (19). The number of reactive hydroxyl groups per polyol molecule can determine the mechanical properties of the polymer. In the production of polyurethanes, additives are used to control the reactions or to obtain the desired properties in the finished product. These additives can be catalysts (amines), pigments, flame retardants, and blowing agents that produce polyurethane foams. A wide range of products can be obtained, such as flexible and rigid foams, coatings, adhesives, elastomers, sealants, and lacquers. The general reactions in the production of PUR are shown in Figure 2.
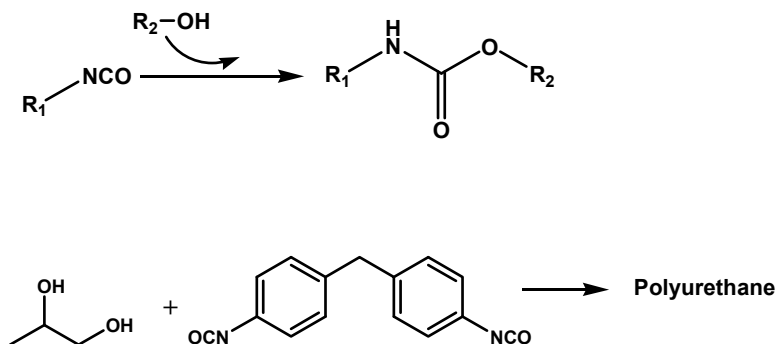
Figure 2.
General reaction of isocyanates. Upper reaction: combination of an isocyanate with alcohol to form a urethane. Lower reaction: propylene glycol reacts with diphenylmethane-4,4′-diisocyanate to form polyurethane.

The diisocyanates that are used in the production of PURs are generally aromatic, of low molecular weight, volatile, and are highly reactive. The main isocyanates produced are toluene diisocyanate (TDI) and diphenylmethane diisocyanate (MDI) (20). MDI usually replaces the use of TDI due to the volatility of the latter, which constitutes a respiratory health hazard. MDI and TDI are aromatic diisocyanates and are used in the production of for example foams, adhesives, coatings, and elastomers (21). Aliphatic isocyanates are more expensive to produce than MDI and TDI. Examples of aliphatic isocyanates are isophorone diisocyanate (IPDI) and hexamethylene diisocyanate (HDI) (22). The global annual production of isocyanates is expected to reach 12×10^6 tons in 2020 (23).

To alter the reactivity of the isocyanate groups or to minimize the vapor emission of isocyanates in solution, they are often used in a prepolymerized form. In order to polymerize isocyanates, di- or polyfunctional alcohols in small quantities are mixed together with an excess of isocyanates. This process replaces the volatile monomers with isocyanates of higher molecular weight (20). Aromatic isocyanates are more reactive than aliphatic isocyanates. This is because of the structure of an aromatic isocyanate. The aromatic benzene ring is electron-drawing, which results in drawing of the negative charge of the nitrogen―making the carbon positively charged and therefore reactive. This is why most of the reactions take place across the $C = N$ bond (24).

In industry, technical-grade isocyanates are used. Technical-grade means that it is a product that contains a mixture of isomers of isocyanates. The isomers can be monomers or oligomers i.e. diisocyanates or isocyanates with several –NCO groups. The oligomers are also called polyisocyanates.

In the literature describing contact allergy to isocyanates, it is not uncommon that only a general abbreviation such as MDI or TDI is given for the isocyanate and it is

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not specified whether the patient has come in contact with technical-grade products—and in that case, what different isomers and oligomers he/she has been exposed to. However, only one isomer of each isocyanate is present in the commercial patch test preparations that patients are tested with (Table 1).

Table 1.
Commercial isocyanate and amine patch test preparations from Chemotechnique Diagnostics

Isophorone diisocyanate	1.0% in pet.
Toluene-2,4- diisocyanate	2.0% in pet.
Isophorone diamine	0.1% in pet.
Hexamethylene diisocyanate	0.1% in pet.
Diamino-4,4′-dicyclohexylmethane	0.5% in pet.
Dicyclohexylmethane-4,4′-diisocyanate	1.0% in pet.
Diphenylmethane-4,4′-diamine	0.5% in pet.
Diphenylmethane-4,4′-diisocyanate	0.5% in pet.
Polymeric diphenylmethane disisocyanate	2.0% in pet.

From here on, when a general abbreviation (MDI, TDI, IPDI, or HDI) is used in this thesis, it denotes all isomers of the diisocyanates, respectively.

1.3.1 Exposure to isocyanates

Exposure to isocyanates is more of an occupational problem than a consumer problem. It is estimated that 280,000 workers are exposed to isocyanates in the United States alone (25). However, exposure to isocyanates in domestic settings has also been reported (24, 26-30). The main route of exposure is assumed to be by inhalation. Isocyanates that have high vapor pressure, such as TDI, can be airborne at room temperature. Other isocyanates such as MDI are only volatile when heated (31). Thermal degradation in PUR products when heated to temperatures over 150°C leads to dissociation of PURs into a mixture of isocyanates, amines, and aminoisocyanates (32). Industrial processes that can cause exposure to isocyanates are, for example, spraying or heating. Aircraft painters and auto repair shop workers can be exposed to high doses and have a high risk of developing respiratory diseases (33, 34).

1.3.2 Respiratory and carcinogenic effects

Inhalation of isocyanates can cause disorders of the respiratory tract. Isocyanates are well known to cause direct toxic and irritant effects, such as irritation of the nose, throat, and upper airways—as well as eye and skin irritation (35, 36). Their effects on the respiratory tract have been extensively described (21, 37, 38). Exposure to high concentrations of isocyanates have been reported to cause reactive airway dysfunction syndrome (RADS) and intestinal obstruction (39, 40). An association of isocyanates with occupational rhinitis and asthma has recently been

described (41). Acute exposure to high levels of isocyanates can cause respiratory sensitization, but low levels of isocyanate exposure can also have adverse health effects on the upper airways, intestinal tract, and skin (42). The mechanism behind isocyanate-induced asthma is yet not fully understood. Animal and human studies have been performed and have found both immunological and non-immunological mechanisms (38, 43). There is evidence from animal studies that isocyanates cause skin sensitization and sensitization of the lung (44-46). This was also described later in humans (47-50).

Based on animal studies, TDI and MDI have been proposed to be carcinogenic. However, only TDI is classified as being carcinogenic in humans (51, 52). On the other hand, the amines diphenylmethane diamine (MDA) and 2,4-toluene diamine (2,4-TDA), corresponding to the above-mentioned isocyanates, have been shown to be carcinogenic in animals (53, 54). In Sweden, a special licence, issued by the authorities, is needed in order to be allowed to use or even store MDA and 2,4-TDA (55).

1.3.3 Contact allergy to isocyanates

In the commercial isocyanate patch test series, only specific isomers are included. However, the technical grade of each isocyanate contains a mixture of isomers of the isocyanate in question as well as larger oligomers and their respective isomers. Since the isocyanate group is extremely reactive and readily reacts with skin proteins, this—in theory—makes isocyanates strong contact allergens. Skin sensitization to isocyanates has not been investigated as thoroughly as sensitization of the respiratory tract. Thus, there have been few reports of contact allergy to isocyanates. Perhaps the strict regulations regarding isocyanates to prevent respiratory disorders could be a contributory factor that can reduce isocyanate skin contamination (56). Due the reactivity of isocyanates, it has been suggested that they react before penetrating the skin, i.e. with water or proteins on the surface of the skin, making them less allergenic and therefore not sensitizing (57). Other plausible explanations have been suggested for the low rate of contact allergy cases, such as insufficient diagnosis by patch testing due to inaccurate concentrations of patch test allergens or not reading the test after D7 (9, 12, 58-60).

In those cases that have been described in the literature, there are often concurrent reactions between different isocyanates and their different corresponding amines. It is not always evident whether the concurrent reactions are due to co-exposure or possible cross-reactivity. Allergic reactions to the aromatic isocyanate MDI and the aliphatic isocyanate DMDI have been reported. DMDI has been found to be a strong dermal sensitizer (61-66). Simultaneous reactions to 4,4′-DMDI and the corresponding amine 4,4′-DMDA, and to the aromatic amine 4,4′-MDA have been reported (62). Patients sensitized to 4,4′-DMDI were also found to have positive

reactions to 1,6-HDI and IPDI. These patients had had no previous contact with the aforementioned isocyanates (62). A similar pattern has been described by Militello et al. in two patients who were exposed to MDI and DMDI, but who also showed positive reactions to TDI, IPDI, and HDI (28).

Cross-reactivity has been studied by Thorne et al. using the mouse ear-swelling test (MEST), and showed cross-reactivity between MDI, TDI, HDI, and DMDI (67). The capacity of sensitization of the isocyanates was also investigated and was found to be the following (in declining order): HDI > DMDI > MDI > TDI (67).

The sensitizing capacity and cross-reactivity of a selection of isocyanates will be discussed further in the context of papers III and IV. 2,4-TDI has been shown to be a strong sensitizer when investigated with the local lymph node assay (LLNA) (68), and has also been found to cause active sensitization in humans following patch testing with 1% 2,4-TDI in pet. (69)

In the literature, concurrent reactions have also been seen between MDI and MDA (25, 28, 58, 62, 70-75). In workers exposed to MDI, positive patch test reactions to MDA and negative reactions to MDI have been described in many reports (25, 58, 62, 70, 76-78). This pattern may be explained by the instability of the MDI patch test preparations. The patch test preparations that have been used have not been stable, and might therefore have failed to diagnose contact allergy to MDI. MDA has been proposed to be a marker for MDI allergy, since MDA is formed once MDI reacts with water. However, the conversion of 4,4′-MDI into 4,4′-MDA by reaction with water is probably not the explanation for the concomitant reactions. Instead, a suggested explanation is that several reactions occur, leading to MDI conjugates and urea reacting with skin constituents, which then hydrolyze into 4,4′-MDA. These reaction events take longer to process, which might explain the late MDI reactions (i.e. after D7) (9).

Isocyanates have also been reported to cause other skin disorders, namely irritant contact dermatitis and urticaria (79-81).

1.3.4 Sensitization and cross-reactivity

The sensitizing capacity of a chemical can be determined using animal testing. There are many different animal testing methods such as the Buhler test, the guinea pig maximization test (GPMT), the mouse ear-swelling test (MEST), and the local lymph node assay (LLNA). *In vitro* assays such as direct peptide-reactive assay (DPRA) can also be used. Human testing—such as human repeated insult patch testing (HRIPT) — exists but it is not part of any official test guideline, since it is considered to be unethical (82). The GPMT is a standardized method that is used for determination of limits and cross-reactivity between chemicals.

The term cross-reactivity refers to when an individual initially sensitized to one chemically defined substance (A) reacts to a second chemically defined substance (B) that he or she has not been in previous contact with. The first compound is the primary sensitizer and the other is the secondary sensitizer (83). Cross-reactivity can occur because A and B are structurally similar, or because A is metabolized to a compound that is similar to B and vice versa, or because A and B are both metabolized into similar compounds (84). Cross-reactivity does not have to go in both directions; thus, if A is a primary sensitizer giving rise to a cross-reaction to the secondary sensitizer B, this does not automatically mean that a primary sensitization to B also gives rise to a cross-reaction to A (85).

2 Aims

- To study of the dermal uptake and metabolism of isocyanates by dermal provocation (papers I and II).

- To investigate the sensitizing capacities and cross-reactivity patterns of some common isocyanates and the corresponding amines in order to provide a better understanding of isocyanate contact allergy (papers III, IV, and V).

- To investigate the cause behind the rapid decrease in the concentration of 4,4′-MDI in patch test preparations (paper VI).

3 Materials and methods

3.1 Chemical structures of the isocyanates and amines investigated

Table 2.
All the substances investigated are listed together with some common synonyms, their CAS number, their classification according to the CLP regulation[§§], their log $P_{o/w}$, and the purity of each investigated substance as stated by the manufacturer

Name[§]	Synonyms	CAS no.	Structure	Harmonized classification[§§] — Class and category code	log $P_{o/w}$	MW	Swedish work environment authority limits	IARC classification
Diphenylmethane-4,4'-diisocyanate	4,4'-MDI; 4,4'-Diisocyanatodiphenylmethane; 4,4'-Methylenebis(phenyl isocyanate); 4,4'-Methylenediphenyl diisocyanate	101-68-8		Skin Sens. 1 Resp. Sens. 1 Carc. 2	5.22	250	0.03 mg/m³	Group 3 Not classifiable as to its carcinogenicity to humans
Diphenylmethane-4,4'-diamine	4,4'-MDA; 4,4'-Methylenedianiline; 4,4'-Dimethylenediamine; 4,4'-Diaminodiphenyl methane.	101-77-9		Carc. 1B Muta. 2 Skin Sens. 1	1.59	198	Carcenogenic. Special approval required.	Group 2B Possibly carcinogenic to humans
Dicyclohexylmethane-4,4'-diisocyanate	4,4'-DMDI; 4,4'-HMDI; Methylene bis(4-cyclohexylisocyanate); 4,4'-Methylenedicyclohexyl diisocyanate; Hydrogenated MDI.	5124-30-1		Resp. Sens. 1 Skin Sens. 1	6.11	262	2 mg/m³	Not classified
Dicyclohexylmethane-4,4'-diamine	4,4'-DMDA; 4,4'-HMDA; 4,4'-Diaminodicyclohexylmethane; 4,4'-Methylenebis(cyclohexylamine),	1761-71-3		Skin Sens. 1	3.26	210	-	Not classified
p-Phenylenediamine	PPD; 1,4-diaminobenzene; benzene-1,4-diamine; para-phenylenediamine.	106-50-3		Skin Sens. 1	0.43	108	-	Group 3 Not classifiable as to its carcinogenicity to humans
Toluene-2,4-diisocyanate	2,4-TDI; 4-methyl-m-phenylene diisocyanate; 2,4-diisocyanato-1-methylbenzene.	584-84-9		Skin Sens. 1 Resp. Sens. 1 Carc. 2	3.74	174	0.014 mg/m³	Group 2B Possibly carcinogenic to humans

[§] Name as used in this thesis; [§§] Classification as found in Annex VI of Regulation (EC) No. 1272/2008 on the classification, labeling, and packaging of substances and mixtures (CLP Regulation). International Agency for Research on Cancer (IARC).

3.2 Studies I and II

3.2.1 Subjects

In study I, we included two volunteers (healthy women aged 19 and 35 years; volunteers 1 and 2) with no previous history of contact allergy to isocyanates or any respiratory symptoms that could be related to isocyanates. The original exposures and the investigation of the suspected sensitization were carried out at the Department of Occupational and Environmental Dermatology, Malmö. This study was performed in 2010 (86). In study II, a 47-year-old woman and a 54-year-old man (volunteers 3 and 4) were dermally exposed in 2012. Volunteers 3 and 4 were diagnosed as having contact allergy to 4,4′-MDI before the second part of the study.

3.2.2 Dermal provocation

The cutaneous exposure doses, areas, and amounts of 4,4′-MDI preparations in 2% petrolatum are presented in Table 3. 4,4′-MDI in 2% petrolatum was applied to filter papers, which were placed on the outer aspect of the upper arms of each volunteer. The filter paper was covered with aluminium foil and then secured with Scanpor tape. The exposure time was 8 hours (Figure 3). The surface concentration of 4,4′-MDI was approximately 0.8 mg/cm^2 for all volunteers. Different doses were obtained by altering the area of exposure, i.e. the size of the filter paper used for application. Volunteers 3 and 4 were exposed by applying duplicate 31 cm^2 filter papers, one on each arm.

Table 3.
Provocation concentrations of 4,4′-MDI, exposure areas, and amounts of 4,4′-MDI in 2% petrolatum (pet.)

Volunteer		Exposure dose of 4,4′-MDI in pet., mg	Exposure area, cm^2	Amount of preparation (2% 4,4′-MDI in pet.), mg
1		10	12.5	500
2		25	31	1,250
3		49	62	2,400
4		50	62	2,500

Figure 3.
Dermal exposure. Diphenylmethane-4,4′-diisocyanate in petrolatum was applied on filter paper, which was covered with aluminium foil and later secured with Scanpor tape.

The removed filter paper with its aluminium foil backing and the contaminated compresses from the exposed volunteers were placed in glass flasks, one for each volunteer, containing di-n-butylamine (DBA) in methylene chloride and toluene.

3.2.3 Tape-stripping

Tape-stripping was performed in study II. After removal of the filter paper and after any excess petrolatum preparation had been wiped off, 10 cm^2 of the exposed skin area was tape-stripped using a set of 20 tape strips (2.5 × 4 cm) (Figure 4). This was done on both arms of volunteers 3 and 4, 1 set on the right arm and 1 set on the left. Tape-stripping was not performed on volunteers 1 and 2. The tape-strip technique has been described in detail elsewhere (87). The set of tape strips from the right arm was intended for measurement of 4,4′-MDI levels. Each of these tape strips was placed in a glass test tube containing DBA in methylene chloride and toluene.

Figure 4.
Tape-stripping of the area exposed to diphenylmethane-4,4′-diisocyanate.

3.2.4 Patch test concentrations

In study I, patch testing was performed on volunteers 1 and 2 due to suspicion of sensitization. They were patch tested with petrolatum preparations of 4,4′-MDI, 4,4′-MDA, PPD, and 4,4′-DMDI. The patch test concentrations of the dilution series of the substances were made equimolar. The dilution series were based on the factor √10. The patch test concentrations are given in Table 4. 20 mg of each preparation was applied on Finn Chambers (8 mm; Allerderm, Phoenix, AZ, USA) on Scanpor (Norgesplaster A/S, Vennesla, Norway). The tests were applied to the upper part of the back for 48 hours and then discarded. The tests were read by a dermatologist on D3 or D4 and also on D7 and D10.

Table 4.
Equimolar patch test concentrations of dilution series in petrolatum for patients 1 and 2

4,4′-MDI % (w/w)	4,4′-MDA % (w/w)	PPD % (w/w)	4,4′-DMDI % (w/w)
0.20	0.16	0.091	0.17
0.063	0.050	*	*
0.020	0.016	*	*
*	0.0050	*	*
*	0.0020	*	*

*Not tested.

3.2.5 Urine and plasma samples

All 4 volunteers had plasma and urine samples taken just before the dermal exposure, and full urine collections were done over 48 hours from the start of the exposure. The volunteers were issued with urine flasks to be labeled with the time and date of each collection. Five plasma samples were drawn from each volunteer and sent (together with the urine samples) to the Department of Occupational and Environmental Medicine in Lund for chemical analysis.

3.2.6 Chemical investigation, workup procedure and sampling

In study I, the provocation materials were removed and all excess petrolatum was wiped off using compresses soaked in acetone. The materials removed from the volunteers' arms were then placed in an impinger flask containing DBA in methylene chloride and toluene. Samples of the 4,4′-MDI preparations that were used in the dermal provocation were also treated in the same reagent solutions in impinger flasks. To each impinger sample, an aliquot of 4,4′-MDI-D$_9$DBA was added as internal standard. The samples of petrolatum preparations and removed materials were analyzed using high-performance liquid chromatography-mass spectrometry (HPLC-MS). A detailed description of the HPLC-MS method can be found in paper I. Chemical analysis was performed at the Department of Occupational and Environmental Medicine, Örebro University Hospital, Örebro.

Analysis of 4,4'-MDI in excess petrolatum preparation and tape strips in study II

The samples of 4,4′-MDI recovered from the skin and the tape-strip sets on the right arms of volunteers 3 and 4 were analyzed using liquid chromatography-mass spectrometry (LC-MS). 4,4′-MDI was derivatized and measured in the form of its stable reaction product with DBA. The method was identical to the method used in paper I.

Analysis of urine and plasma samples for systemically distributed 4,4'-MDI – Studies I and II

The dermal uptake of 4,4′-MDI was determined by measurement of 4,4′-MDA in hydrolyzed urine and plasma. The 4,4′-MDA levels in the hydrolyzed urine and plasma samples were analyzed according to Sennbro et al. (88). Briefly, the biological samples were hydrolyzed in sodium hydroxide in order to release the 4,4′-MDI-corresponding amine 4,4′-MDA, which was extracted with toluene. After derivatization with pentafluoropropionic acid anhydride, the derivatives were quantified by gas chromatography and mass spectrometry (GCMS). This method detects 4,4′-MDI that has reacted with the cell components, and also 4,4′-MDA if present in the sample. The chemical analysis was performed at the Department of Occupational and Environmental Medicine, Skåne University Hospital, Lund.

3.3 Studies III, IV, and V

3.3.1 Guinea pig maximization test

In studies III and V, the GPMT was performed according to the original description (89). However, in order to standardize the test and objectify the evaluation of the patch test reactions, some modifications in accordance with those proposed by Bruze in 1985 were used (90). These modifications included statistical calculations, blind readings, and use of a positive control group (90, 91).

In the method proposed by Bruze, all animals are given the same degree of inflammation by treating them all with the same dose of sodium lauryl sulfate (SLS). The test substance is then tested with the highest possible concentration that does not elicit irritant reactions. Before sensitization and cross-reactivity patterns can be assessed, the topical irritancy thresholds must therefore be determined, in order to ensure that the test concentrations chosen do not give rise to irritant reactions. This was performed by applying different concentrations of each of the investigated substances intended for induction, as a closed patch test for 2 days, on both the neck and the flank of one side of 4 animals. All animals were pretreated with Freund's complete adjuvant (FCA). In order to maximize the number of test concentrations that could be evaluated, the animals were tested first on one side of the body and then on the other side. Concentrations that did not cause irritation were chosen for topical induction and elicitation.

The investigation of sensitizing capacity and cross-reactivity of the selected suspected allergens (Tables 7 and 8 in section 4.2) was performed by inducing the guinea pigs with the suspected allergen. For each sensitization series, 24 test animals, 12 controls, and 6 positive controls were used (Figure 5). The induction and challenge procedure is described in detail in papers III and IV.

3.3.2 Analysis of 4,4′-MDI in FCA/paraffin oil

Gel permeation chromatography (GPC)

Analysis of 4,4′-MDI was carried out using gel permeation chromatography, which is a type of size exclusion chromatography (SEC). The identity of each analyte is determined by a specific peak, which is identified by a certain retention time and its UV spectrum. The detector was set at a wavelength of 238 nm for the detection of 4,4′-MDI. A detailed description of the method can be found in paper V.

In paper V, chemical analyses were performed in order to determine (1) the effect that the age of the 4,4′-MDI might impose on the sensitization rate in the GMPT, and (2) whether the reaction between 4,4′-MDI and FCA might affect the GMPT outcome.

Two preparations of 4,4′-MDI in a vehicle consisting of FCA and paraffin oil were prepared. One was placed in a refrigerator and one was stored at room temperature. 4,4′-MDI was first dissolved in FCA and then diluted further in paraffin oil to the intended concentration, i.e. 1.0% (w/v) 4,4′-MDI in FCA/paraffin oil. Samples were taken from each emulsion at 0, 2, 4, 6, 24, and 48 hours. Double samples were dissolved in dichloromethane and analyzed in triplicate with GPC.

Batches of 4,4′-MDI

Three different batches of 4,4′-MDI were analyzed to determine the concentration of 4,4′-MDI found in each package. One package of 4,4′-MDI was new and was used as reference substance. The second was 4.5 months old, and the third was 3 years and 8 months old. All batches of 4,4′-MDI had been stored in the freezer. Samples of 4,4′-MDI flakes were mixed with dichloromethane to prepare a solution with an intended concentration of 1.0% (w/v). The solutions were filtered when required. The samples were diluted 100 times and analyzed with GPC.

3.3.3 Statistics

The proportion of positive animals in the test group was compared with the proportion of positive animals in the control group. In the animals challenged with the induction substance on both the cranial and caudal patches (12 test animals and 6 negative control animals) (Figure 5), only one of the patches―chosen in advance―was included.

Statistical significance for the sensitizing capacity and cross-reactivity was calculated with Fisher's exact test (one-sided). When significant values ($p < 0.05$) were obtained with Fisher's exact test, the compound was considered to be a sensitizer or to show cross-reactivity to other compounds based upon set criteria ($p < 0.001$, strong; $p < 0.01$, moderate; $p < 0.05$, weak).

Induction of Irritancy

The neck is shaved. 0.2 ml of 10% SLS in DAE 433 is spread over a 2×4 cm area of all 42 animals to induce irritation and enhance sensitization.
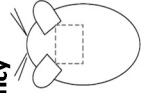
The elastic compression band is removed.

The elastic compression band is removed.

The test sites are read.

D0   D6 D7 D8 D9   D21   D22 D23

Induction

Challenge

Intradermal sensitization

An area of 2×4 cm is shaved on the neck of the animal. Every animal is injected at 2×3 places on the shaved area. Injection volume 0.1 ml. The following solutions are injected:
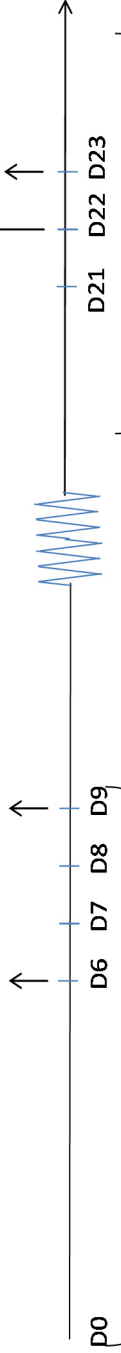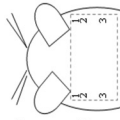
Test animals (24 animals)
1. FCA/H2O 1:1
2. Test substance/propylene glycol or test substance/liquid paraffin
3. Test substance/FCA/propylene glycol or test substance/FCA/liquid paraffin

Negative controls (12 animals)
1. FCA/H2O 1:1
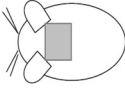2. Propylene glycol
3. FCA/propylene glycol

Positive controls (6 animals)
1. FCA/H2O 1:1
2. 2-MP/propylene glycol
3. 2-MP/FCA/propylene glycol

Epidermal sensitization

An area of 2×4 cm is shaved on the neck of the animal

Test animals (24 animals)
0.2 ml of the induction substance in appropriate concentration in ethanol or acetone is applied over a 2×4 cm area.

Negative controls (12 animals)
0.2 ml of the vehicle is applied over a 2×4 cm area.

Positive control (6 animals)
0.2 ml of 25% 2-MP in ethanol is applied over a 2×4 cm area.

Challenge I

Challenge II

Both flanks are shaved.

Cross-reacting substances are tested randomized according to a latin square table

Test animals (24 animals)
30 µl of up to 6 substances can be tested

Negative controls (12 animals)
30 µl of up to 6 substances can be tested

Positive control (6 animals)
Are not tested on this side.

Test animals (24 animals)
12 animals
30 µl test substance
30 µl test substance
6 animals + 6 animals
30 µl test substance   ● + ○
30 µl vehicle   ○ + ●

Negative controls (12 animals)
6 animals
30 µl test substance
30 µl test substance
3 animals + 3 animals
30 µl test substance   ● + ○
30 µl vehicle   ○ + ●

Positive control (6 animals)
2 animals
30 µl 2-MP
30 µl 2-MP
2 animals + 2 animals
30 µl 2-MP   ● + ○
30 µl vehicle   ○ + ●

Figure 5. General scheme of the sensitization and induction process. D, day; FCA, Freund's complete adjuvant; 2-MP, 2-methylol phenol; DAE, dimethyl acetamide/acetone/ethanol; H2O, water; SLS, sodium lauryl sulfate.

3.4 Study VI

3.4.1 Chemical investigation of 4,4′-MDI in petrolatum

Gel permeation chromatography (GPC)

The chemical analysis of the 4,4′-MDI preparations was the same as used in study V (section 3.3.2), except that the flow rate was 2 ml/min.

Nuclear magnetic resonance (NMR) spectroscopy

To determine the identity of substances present in the 4,4′-MDI petrolatum preparations, proton and carbon nuclear magnetic resonance spectra were recorded using nuclear magnetic resonance (NMR) spectroscopy. This is an analysis technique that investigates the magnetic properties of atomic nuclei based on chemical and physical properties, such as structure and the individual functional groups. The analyses were performed at the Institution of Biomedical Sciences, Malmö University, Malmö.

Fourier transform infrared (FTIR) spectroscopy

For analysis of the synthesized trimer, FTIR spectroscopy was used. FTIR is a technique that obtains infrared spectra of solids, liquids, or gases. Different organic functional groups produce different spectra.

Sample preparation

From a syringe containing 4,4′-MDI test preparation at 0.5% in petrolatum, 4 g was accurately weighed and mixed with water. After mixing, the mixed petrolatum preparation was transferred to a new syringe. Likewise, from another syringe containing 4,4′-MDI test preparation at 0.5% in petrolatum, 4 g was accurately weighed and mixed with water and acetone. Another mixture with ethanol was made in a manner similar to that for the other mixtures. After mixing, the mixed petrolatum preparation was transferred to a new syringe.

For GPC analysis of the concentration of MDI in the test syringes with added water, about 20 mg test preparation was dissolved in 5 ml dichloromethane. As an internal standard for the GPC analysis, acetone was added. The test preparations were analyzed in duplicate before addition of water, then 15, 30, 45, and 60 minutes after addition of water, and thereafter duplicate samples were taken on a daily basis―over 17 days for the 4,4′-MDI water and acetone mixture and over 25 days for the 4,4′-MDI and water mixture. The 4,4′-MDI and ethanol mixture was followed for 60 minutes due to the rapid reaction.

Dimer/trimer synthesis

A synthetic method was developed at our department. This synthesis was done by mixing 2% 4,4′-MDI in acetone with trimethylamine, which served as catalyst. The product crystallized within 24 hours. The solution was then vacuum-filtered, washed with acetone, and dried under vacuum. The crystals were later transferred to a test tube with a screw cap. The test tube was filled with nitrogen and stored in a −20°C freezer. From here on, this substance is referred to as reaction product T1. Another synthesis was done according to the original description by Zhang et al., which is referred to as T2 from hereon (92). In the synthesis of T2, toluene was used as a solvent and 2,4,6-tris(dimethylaminomethyl)-phenol was used as a catalyst. This product was filtered and treated in the same way as T1.

Extraction of old MDI syringes

Three preparations of 4,4′-MDI in petrolatum of different age (Table 5) were dissolved in heptane and then extracted with DMSO using a separation funnel. The DMSO phases were washed several times with heptane until clear solutions were obtained. The filtered DMSO phases were then analyzed.

Table 5.
Aged 4,4′-MDI preparations

Preparation	Amount of petrolatum	Age at analysis
0.5% MDI (Chemotechnique)	4.2 g	2.0 years
2.0% MDI (in-house)	2.1 g	6.6 years
2.0% MDI (in-house)	7.6 g	2.0 years

3.5 Ethics

Studies I and II were approved by the Research Ethics Committee of Umeå University, Sweden. The volunteers gave written informed consent to participate in the study.

Studies III and V were approved by the Lund Ethical Committee on Animal Experimentation, Lund, Sweden.

4 Results

4.1 Studies I and II

Study I

Of the total amount of 4,4′-MDI applied during the dermal uptake study, 7.0 (70%) and 17.5 mg (70%) were recovered, i.e. 10.0 mg and 25.0 mg respectively. Seven to 10 days after this study, an itchy eczema appeared corresponding to the area of application on both volunteers. The concentration of 4,4′-MDI in petrolatum preparations was verified and was found to be 1.9% (w/w). Results from patch testing with 4,4′-MDI, 4,4′-MDA, PPD, and 4,4′-DMDI are given in Table 6.

Table 6.

Patch test results to diphenylmethane-4,4'-diisocyanate (4,4'-MDI), diphenylmethane-4,4'-diamine (4,4′-MDA), p-phenylenediamine (PPD), and dicyclohexylmethane-4,4′-diisocyanate (4,4′-DMDI) at a dilution series in petrolatum in the 2 volunteers with reading on D3 (or D4), D7, and D10

Substance, % (w/w)	Volunteer 1			Volunteer 2		
	D3/4	D7	D10	D3/4	D7	D10
4,4′-MDI						
0.20	-	-	-	-	++	-
0.063	-	-	-	-	++	-
0.020	-	-	-	-	-	-
4,4′-MDA						
0.16	++	++	++	+++	++	+
0.05	++	++	+	++	++	+
0.016	-	-	+	-	+	+
0.005	-	-	-	-	-	-
0.002	-	-	-	-	-	-
PPD						
0.091	-	-	-	-	-	-
4,4′-DMDI						
0.17	-	-	-	-	-	-

Study II

The amounts of unreacted and unabsorbed 4,4′-MDI in the excess 4,4′-MDI petrolatum preparation recovered was 7.0 and 17.5 mg for volunteers 1 and 2, respectively, which represents 70% of the dose applied to each volunteer. During transportation for analysis, one sample got crushed and the solvent wiped off the marking of the other 3 samples, leaving them unidentified. Three out of the 4 samples from volunteers 3 and 4 contained 5.2, 9.0, and 11.3 mg of 4,4′-MDI. By considering all possible combinations of these unmarked samples, the average value of unabsorbed 4,4′-MDI in the three samples analyzed was calculated to be 35% ± 4% of the dose applied.

In hydrolyzed plasma, the concentration of 4,4′-MDA did not rise from the baseline concentration for volunteer 1, but had a maximum concentration (after approximately 8 hours of exposure) of 0.18 ng/ml, 0.22 ng/ml, and 0.20 ng/ml for volunteers 2, 3, and 4, respectively. In hydrolyzed urine, the concentration of 4,4′-MDA did not rise for volunteer 1 but had a maximum concentration (after 10–14 hours of exposure) of 1.4 ng/ml, 2.0 ng/ml, and 4.3 ng/ml for volunteers 2, 3, and 4, respectively. The results are shown in Figures 6 and 7.

The total amount of 4,4′-MDI found in the skin layers removed with tape strips was 21.14 μg and 6.64 μg for volunteers 3 and 4, respectively. Data for each tape-strip layer are given in Figure 8.

Figure 6. Diphenylmethane-4,4'-diamine concentrations in plasma. The dermal exposure doses were 10 mg (V1), 25 mg (V2), 49 mg (V3), and 50 mg (V4) diphenylmethane-4,4′-diisocyanate. V, volunteer.

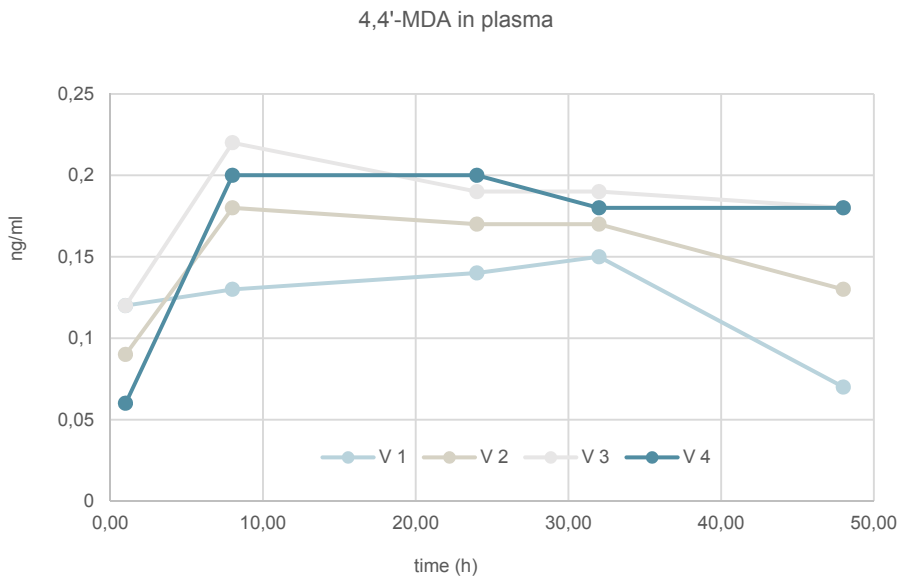**Figure 7.** Diphenylmethane-4,4'-diamine concentrations in urine. The dermal exposure doses were 10 mg (V1), 25 mg (V2), 49 mg (V3), and 50 mg (V4) diphenylmethane-4,4′-diisocyanate. V, volunteer.

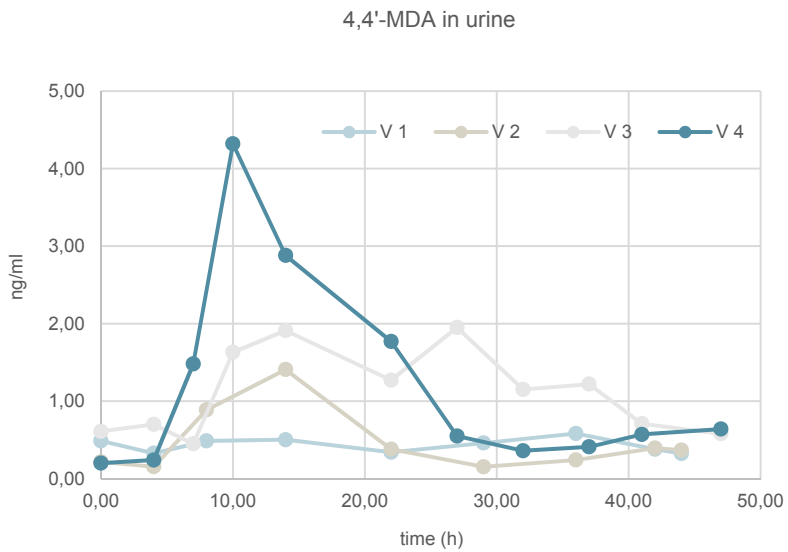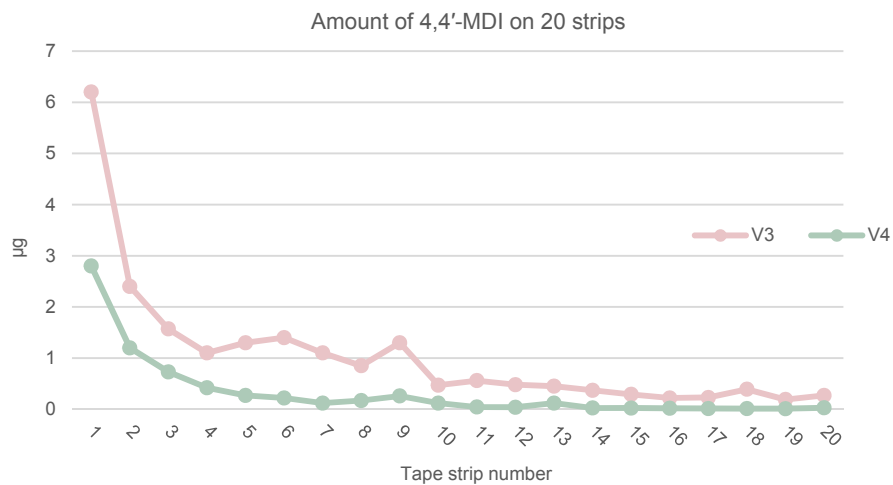**Figure 8.** Amounts of diphenylmethane-4,4′-diisocyanate found on tape strips. The dermal exposure doses for the tape-stripped areas (10 cm^2) were 8 mg diphenylmethane-4,4′-diisocyanate for V3 and V4. V, volunteer.

4.2 Studies III and IV

Eight different sensitization series were performed on different occasions with the same method during a period stretching from May 2013 to September 2015. The results regarding sensitization and cross-reactivity patterns for each of these series are given in Tables 7 and 8. In all sensitization series, at least 4 of the positive control animals showed positive reactions, indicating that the method was functioning well without negative influences due to, for example, sick animals or adjuvant with impaired effect.

Sensitizing capacity
4,4′-MDI was used as induction substance on three different occasions. On the first and last occasions, it was found to be a sensitizer ($p<0.001$ and $p=0.024$, respectively) but on the second occasion the induction failed ($p = 0.19$) (Table 7). 4,4′-MDA, 4,4′-DMDI, and 4,4′-DMDA were found to be potent sensitizers ($p < 0.001$, $p < 0.001$, and $p < 0.001$, respectively).

PPD was found to be a strong sensitizer ($p < 0.001$). The sensitizing capacity of 2,4-TDI ($p = 0.22$) was based on 16 animals, since 8 animals had to be sacrificed due to oozing wounds at the intradermal injection site (Table 8). Their capacity to dry and form crusts deviated from what was considered acceptable in the ethical approval.

Table 7.

Summary of the sensitization and cross-reactivity rates of diphenylmethane-4,4'-diisocyanate, dicyclohexylmethane-4,4'-diisocyanate, and their corresponding amines diamino-4,4'-diphenylmethane and diamino-4,4'-dicyclohexylmethane using the guinea pig maximization test

	Sensitization series A	Sensitization series B		Sensitization series C	Sensitization series D	Sensitization series E	Sensitization series F
Induction Sensitizing substance Intradermal Epidermal	Diphenylmethane-4,4'-diisocyanate (4,4'-MDI) 1.0% p.o (40 mM) 1.0% ac (40 mM)	Diamino-4,4'-diphenylmethane (4,4'-MDA) 0.79 p.g (40 mM) 0.79% EtOH (40 mM)		Dicyclohexylmethane-4,4'-diisocyanate (4,4'-DMDI) 1.0% p.o (40 mM) 1.0% ac (40 mM)	Diamino-4,4'-dicyclohexylmethane (4,4'-DMDA) 0.84 p.g (40 mM) 0.84% EtOH (40 mM)	Diphenylmethane-4,4'-diisocyanate (4,4'-MDI) 1.0% p.o (40 mM) 1.0% ac (40 mM)	Diphenylmethane-4,4'-diisocyanate (4,4'-MDI) 1.0% p.o (40 mM) 1.0% ac (40 mM)
Challenge I concentration	1.0% ac (40 mM) C = 1/12 T = 18/24 V = 0/12 Pos = 4/6 **p < 0.001**	0.79% EtOH (40 mM) C = 0/12 T = 22/24 V = 3/12 Pos = 4/5 **p < 0.001**		0.63% ac (24 mM) C = 0/12 T = 21/24 V = 0/12 Pos = 5/6 **p < 0.001**	0.84% EtOH (40 mM) C = 1/12 T = 18/24 V = 3/12 Pos = 5/6 **p < 0.001**	1.0% ac (40 mM) C = 3/12 T = 2/24 V = 0/12 Pos = 6/6 **p = 0.19**	1.0% ac (40 mM) C = 0/12 T = 8/24 V = 1/12 Pos = 4/6 **p = 0.024**
Challenge II 4,4'-MDI	0.6% ac (24 mM) C = 1/12 T = 7/24 **p = 0.16**	1.0% ac (40 mM) C = 3/12 T = 3/24 **p = 0.31**		1.0% ac (40 mM) C = 0/12 T = 1/24 **p = 0.67**	1.0% ac (40 mM) C = 0/12 T = 3/24 **p = 0.28**	1.0% ac (40 mM) C = 2/12 T = 5/24 **p = 0.57**	1.0% ac (40 mM) C = 0/12 T = 9/24 **P = 0.0139**
4,4'-MDA	0.48% EtOH (24 mM) C = 2/12 T = 13/24 V = 0/12 **P = 0.034**	NT		0.79% EtOH (40 mM) C = 1/12 T = 6/24 V = 0/12 **p = 0.23**	0.79% EtOH (40 mM) C = 4/12 T = 13/24 **p = 0.21**	0.79% EtOH (40 mM) C = 4/12 T = 13/24 **p = 0.21**	0.79% EtOH (40 mM) C = 2/12 T = 21/24 **p < 0.001**
4,4'-DMDI	0.63% ac (24 mM) C = 0/12 T = 5/24 **p = 0.11**	0.63% Ac (24 mM) C = 4/12 T = 8/24 **p = 0.65**	1.0% Ac (40 mM) C = 3/12 T = 12/24 **p = 0.14**	1.0% ac (40 mM) C = 02/12 T = 23/24 **p < 0.001**	1.0% ac (40 mM) C = 3/12 T = 11/24 **p = 0.20**	1.0% ac (40 mM) C = 5/12 T = 20/24 **p = 0.016**	1.0% ac (40 mM) C = 4/12 T = 13/24 **p = 0.21**
4,4'-DMDA	0.50% EtOH (24 mM) C = 0/12 T = 2/24 **p = 0.44**	0.84% EtOH (40 mM) C = 1/12 T = 13/24 **P = 0.0084**		0.84% EtOH (40 mM) C = 1/12 T = 2/24 **p = 0.75**	0.84% EtOH (40 mM) C = 2/12 T = 16/24 **p = 0.0058**	0.84% EtOH (40 mM) C = 1/12 T = 1/24 **p = 0.56**	0.84% EtOH (40 mM) C = 2/12 T = 3/24 **p = 0.55**

p.o, liquid paraffin; p.g, propylene glycol; EtOH, ethanol; ac, acetone; C, control animals (12 in total); T, test animals (24 in total); V, reactions to the vehicle in test animals (12 in total); pos, positive control animals (6 in total).

Table 8.
Summary of the sensitization and cross-reactivity rates of diphenylmethane-4,4'-diisocyanate, dicyclohexylmethane-4,4'-diisocyanate, and their corresponding amines diamino-4,4'-diphenylmethane and diamino-4,4'-dicyclohexylmethane using the guinea pig maximization test

	Sensitization series G	Sensitization series H	Sensitization series B	Sensitization series C	Sensitization series D	Sensitization series F
Induction Intradermal concentration Epidermal concentration	p-phenylenediamine (PPD) 0.43% p.g 0.43% EtOH	Toluene-2,4-diisocyanate (2,4-TDI) 0.70% p.o 0.70% ac	Diamino-4,4' diphenylmethane (4,4'-MDA) 0.79 p.g 0.79% EtOH	Dicyclohexylmethane-4,4'-diisocyanate (4,4'-DMDI) 1.0% p.o 1.0% ac	Dicyclohexylmethane 4,4'-diamine (4,4'-DMDA) 0.84 p.g 0.84% EtOH	Diphenylmethane-4,4'-diisocyanate (4,4'-MDI) 1.0% p.o 1.0% ac
Challenge I concentration	1.0% ac C = 1/12 T = 20/24 V = 0/12 Pos = 5/6 **p < 0.001**	1.0% ac C = 2/12 T = 6/16 V = 1/4 Pos = 5/6 **p = 0.22**	0.79% EtOH (40 mM) C = 0/12 T = 22/24 V = 3/12 Pos = 4/5 **p < 0.001****	0.63% ac (24 mM) C = 0/12 T = 21/24 V = 0/12 Pos = 5/6 **p < 0.001****	0.84% EtOH (40 mM) C = 1/12 T = 18/24 V = 3/12 Pos = 5/6 **p < 0.001****	**1.0% ac (40 mM)** **C = 0/12** **T = 8/24** **V = 1/12** **Pos = 4/6** **P = 0.024****
Challenge II PPD	0.43% EtOH C = 1/12 T = 18/24 **p < 0.001**	0.43% EtOH C = 3/12 T = 1/16 **p = 0.20**	0.43% EtOH C = 1/12 T = 2/24 **p > 0.3**	0.43% EtOH C = 1/12 T = 0/24 **p > 0.3**	0.43% EtOH C = 1/12 T = 0/24 **p > 0.3**	0.43% EtOH C = 1/12 T = 1/24 **p > 0.3**
2,4-TDI	0.70% ac C = 0/12 T = 1/24 **p > 0.3**	0.70% ac C = 2/12 T = 6/16 **p = 0.22**	0.70% ac C = 0/12 T = 3/24 **p = 0.28**	0.70% ac C = 4/12 T = 1/24 **p > 0.3**	0.70% ac C = 1/12 T = 0/24 **p > 0.3**	0.70% ac C = 1/12 T = 2/24 **p > 0.3**
4,4'-MDI	1.0% ac C = 2/12 T = 2/24 **p > 0.3**	1.0% ac C = 1/12 T = 2/16 **p > 0.3**	**	**	**	**
4,4'-MDA	0.79% EtOH C = 3/12 T = 21/24 **p < 0.001**	0.79% EtOH C = 5/12 T = 6/16 **p > 0.3**	**	**	**	**
4,4'-DMDI	1.0% ac C = 0/12 T = 2/24 **p > 0.3**	1.0% ac C = 3/12 T = 8/16 **p = 0.17**	**	**	**	**
4,4'-DMDA	0.84% EtOH C = 0/12 T = 6/24 **p = 0.070**	0.84% EtOH C = 3/12 T = 6/16 **p > 0.3**	**	**	**	**

p.o, liquid paraffin; p.g, propylene glycol; EtOH, ethanol; ac, acetone; C, control animals (12 in total); T, test animals (24 in total); V, reactions to the vehicle in test animals (12 in total); pos, positive control animals (6 in total).
** Presented in Table 7.

Cross-reactivity

The cross-reaction pattern between the substances investigated, when tested in equimolar concentrations, are presented in Figure 9. Animals primarily sensitized to 4,4′-MDI showed cross-reactivity to the secondary sensitizers 4,4′-DMDI and 4,4′-MDA ($p=0.016$ and $p<0.001$, respectively) whereas animals primarily sensitized to 4,4′-MDA showed cross-reactivity to 4,4′-DMDA ($p=0.0084$).

Animals sensitized to PPD showed cross-reactivity to 4,4′-MDA ($p<0.001$) and there was an indication of cross-reactivity to 4,4′-DMDA ($p=0.069$). Since 8 animals in the 2,4-TDI induction series (series H, Table 8) were sacrificed, no statistically significant cross-reactivity patterns could be found. However, based on the 16 animals sensitized to 2,4-TDI, there was some indication of cross-reactivity to PPD ($p=0.20$).

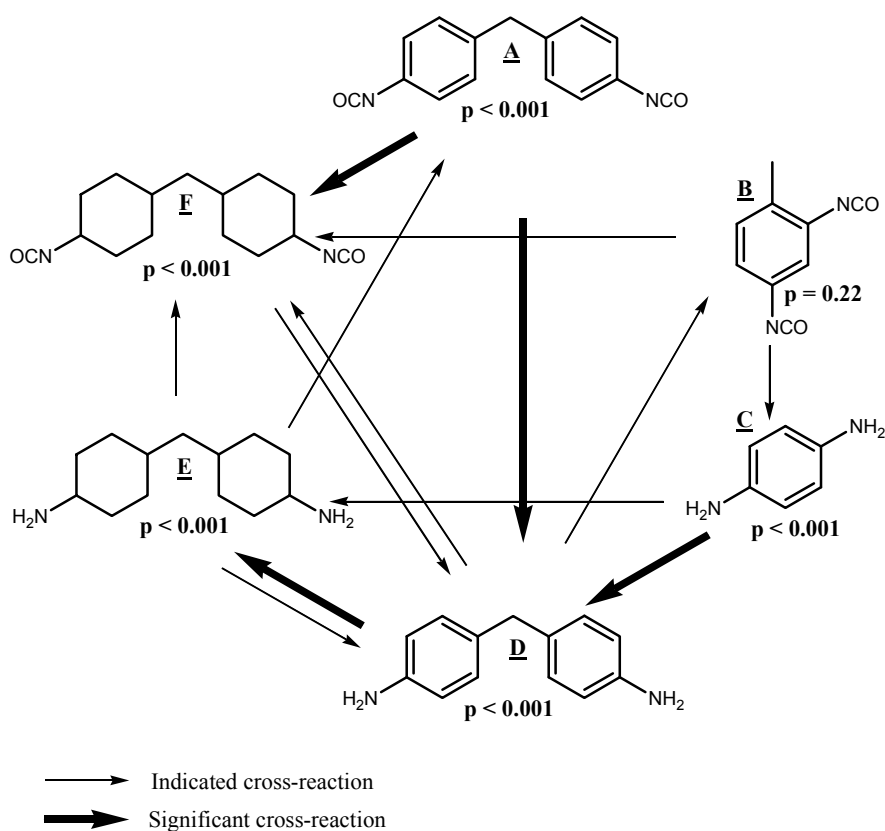**Figure 9.**
Summary of cross-reaction patterns in studies III and IV.
A, diphenylmethane-4,4′-diisocyanate; B, toluene-2,4-diisocyanate; C, *p*-phenylenediamine; D, diphenylmethane-4,4′-diamine; E, dicylohexylmethane-4,4′-diamine; F, dicyclohexylmethane-4,4′-diisocyanate. Some indication of cross-reaction was assumed if $0.05 < p \le 0.3$. Significant cross-reaction was assumed if $p < 0.05$.

4.3 Study V

In order to investigate the influence of FCA on the stability of 4,4′-MDI, duplicate samples were withdrawn continuously over 48 hours from preparations stored at room temperature and in the refrigerator, and analyzed. The mean concentration of 4,4′-MDI for the duplicate samples was plotted against time. A decline in the concentration of 4,4′-MDI over the 48 hours was seen in preparations stored at room temperature and at 8°C (Figures 10 and 11). As expected, the decrease was more rapid at room temperature, as the concentration of 4,4′-MDI had declined to 0.33% (w/v) after 48 hours while the corresponding concentration in the refrigerator-stored preparation after 48 hours was 0.67% (w/v).
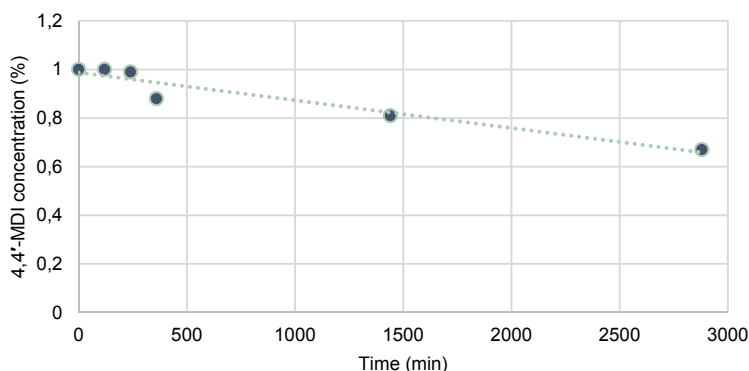

Figure 10. Diphenylmethane-4,4′-diisocyanate in Freund's complete adjuvant/paraffin oil stored at 8°C.
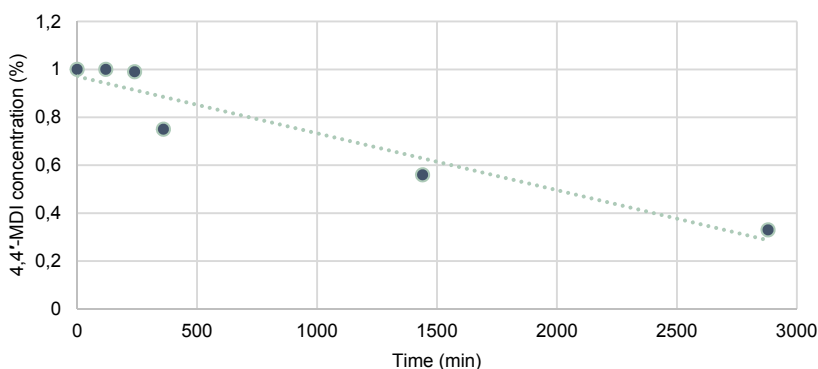
Figure 11. Diphenylmethane-4,4′-diisocyanate in Freund's complete adjuvant/paraffin oil stored at room temperature (25°C).

Compared to a fresh and newly opened batch of 4,4-MDI, which was used as reference substance, we found that the 4.5-month-old batch contained 57% (w/w) 4,4′-MDI and the 3.7-year-old batch contained 42% (w/w) 4,4′-MDI (Figure 12).
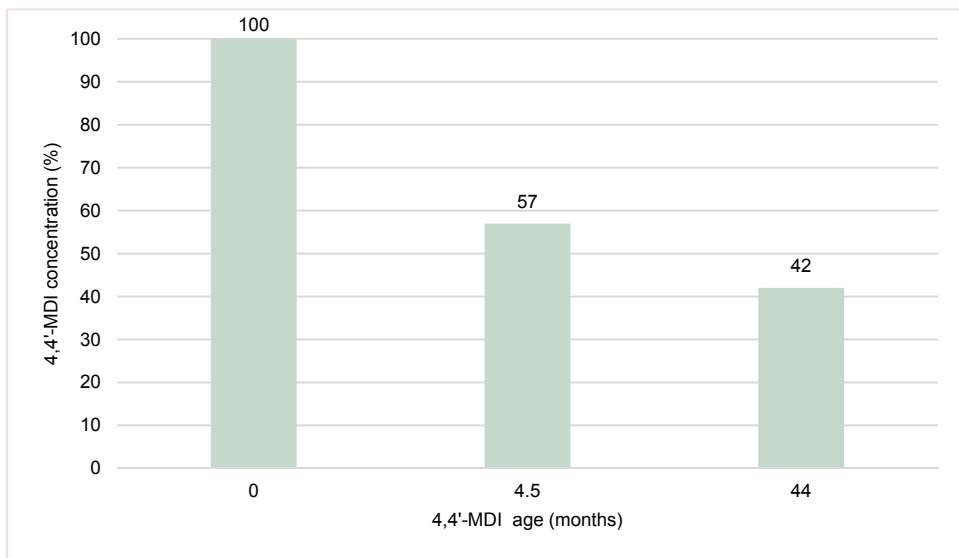
Figure 12. Concentrations of 4,4'-MDI in different batches.

4.4 Study VI

Results of GPC analysis of petrolatum mixtures

The results of the reaction of 4,4′-MDI in different petrolatum mixtures are presented in Figures 13, 14, and 15. The mixtures were as follows: 0.5% 4,4′-MDI and water in pet., 0.5% 4,4′-MDI and water + acetone in pet., and 0.5% 4,4′-MDI and ethanol in pet.

Figure 13. The concentration of diphenylmethane-4,4′-diisocyanate in petrolatum mixed with 3 µl water per g, followed for 25 days.

Figure 14. The concentration of diphenylmethane-4,4′-diisocyanate in petrolatum mixed with 3 µl water and 6 µl acetone per g, followed for 17 days.

0.5% 4,4′-MDI and ethanol in petrolatum
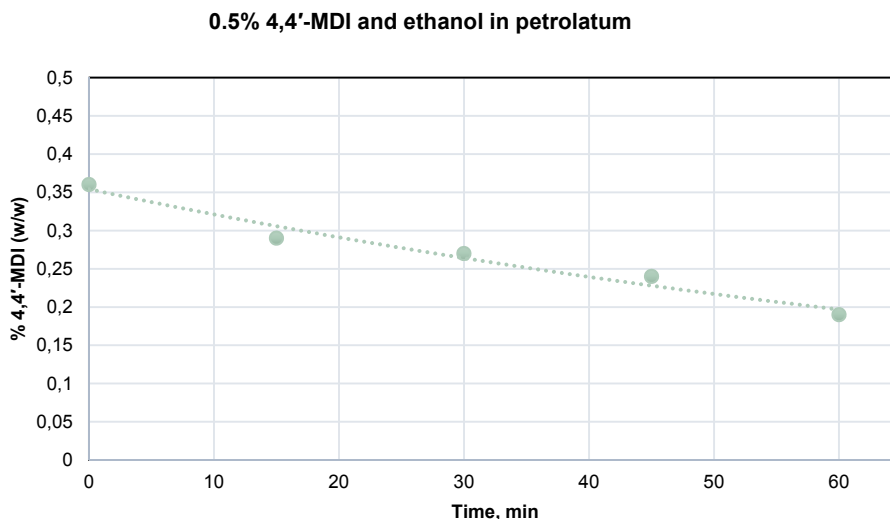
Figure 15. The concentration of diphenylmethane-4,4′-diisocyanate in petrolatum mixed with 9 µl ethanol per g, followed for 60 minutes.

Results of dimer/trimer synthesis

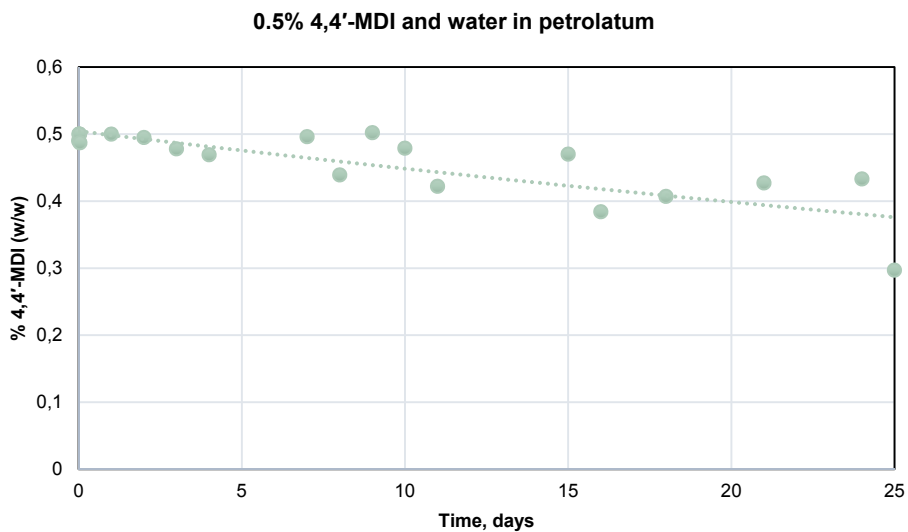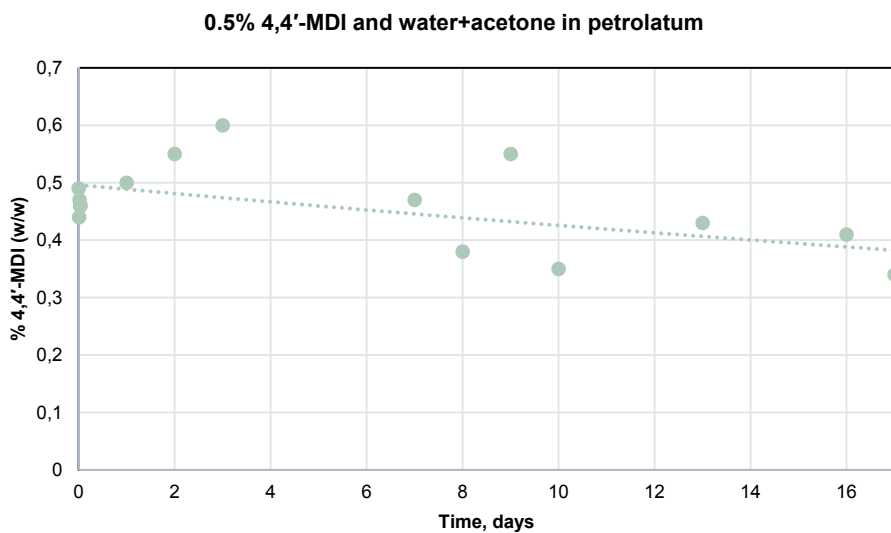The synthesized trimer matched the trimer described in the original publication by Jing Zhang et al. (92). Using FTIR spectroscopy, the trimer could be distinguished by three characteristic peaks at 1,406, 1,699 and 2,248 cm^{-1}, which were also described earlier (92) (Figure 16). The peak at 2,248 cm^{-1} is characteristic of aromatic rings, and the peaks at 1,699 and 1,406 cm^{-1} are characteristic of carbonyl and amide groups and can come from the isocyanurate ring (Figure 17). A peak at 2,340 cm^{-1} is characteristic of asymmetric stretch of the isocyanate group (93). An absorption at this wavelength can be seen in the FTIR spectrum of 4,4′-MDI. This peak could be seen in the spectrum of substance T2, but the isocyanate peak would be expected to be smaller, and could be present as a broadening of the base of the dominant aromatic peak at 2,248 cm^{-1}.

In general, the FTIR spectrum of synthesized trimer (T2) matched the previously published spectrum (92). The spectrum of the synthesized trimer (T1) showed the same characteristic peaks but contained interfering impurities.

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Figure 16. Identification of FTIR spectroscopy peaks of MDI trimer and diphenylmethane-4,4′-diisocyanate.
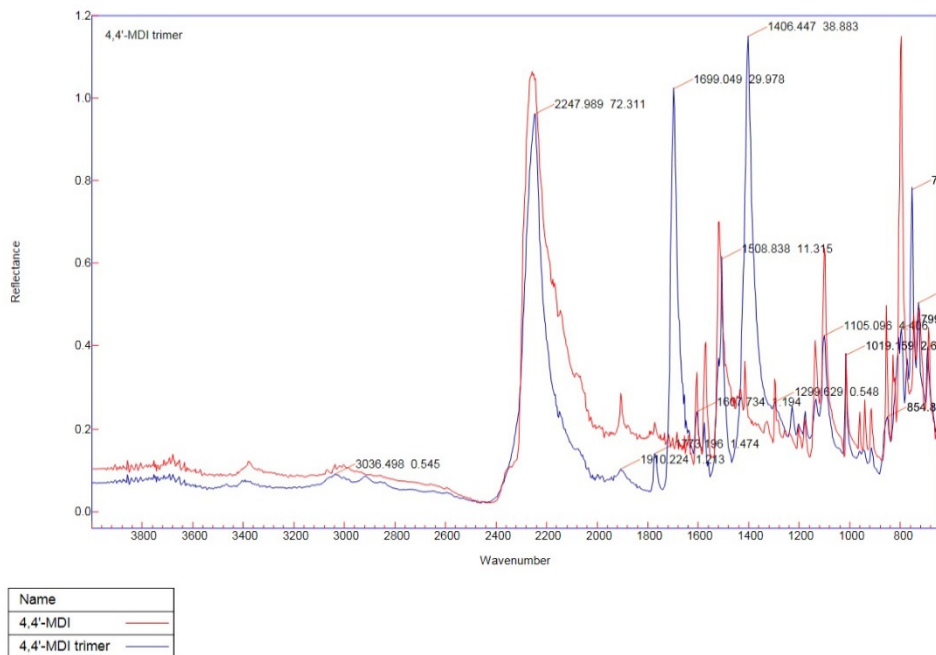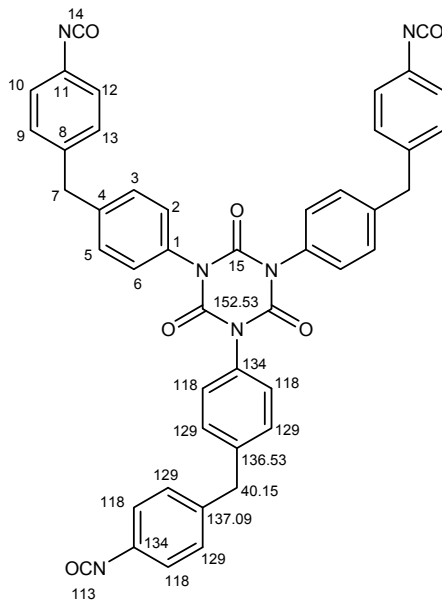

Figure 17. Chemical structure of 4,4′-MDI trimer with ^{13}C shifts shown (lower part of the molecule). 1–15 denote the carbon numbers (Table 9).

Results of GPC analysis of aged 4,4'-MDI preparations

The different preparations of aged 4,4′-MDI (Table 5) were analyzed using GPC. The chromatograms were compared with the chromatograms of a reference sample of 4,4′-MDI and the synthesized trimer (T2). The results are shown in Figure 18. In the overlays of the chromatograms from the extract of the 2-year-old 2% 4,4′-MDI preparation and the chromatogram of the synthesized 4,4′-MDI trimer (T2), a similar peak appeared at a retention time of 21 minutes. The peak for the 4,4′-MDI reference had a retention time of 22 minutes.
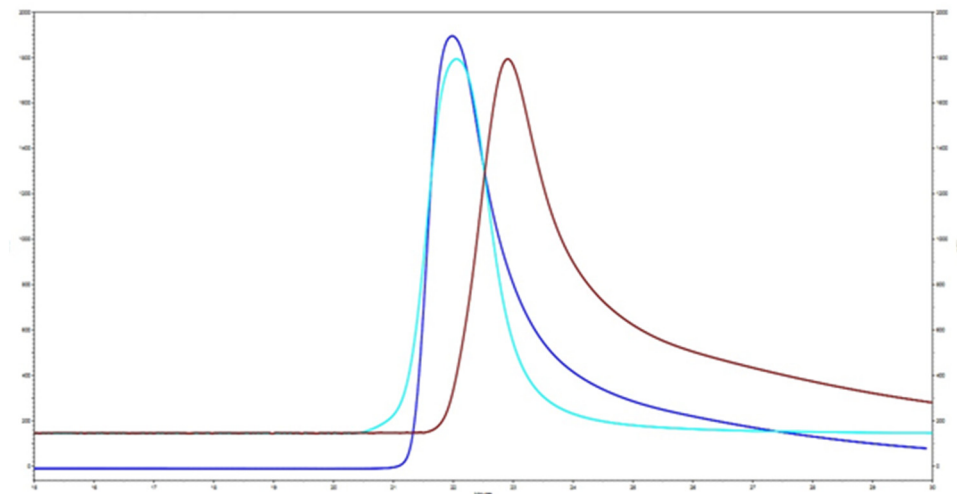
Figure 18. Synthesized 4,4′-MDI trimer (T2) (tourquoise) and substance extracted from 2-year-old 2% 4,4′-MDI preparation in petrolatum (blue) compared to a fresh reference 4,4′-MDI preparation (brown).

The UV-spectra at 21.3 min of an extract of the aged 4,4′-MDI preparation and of the solution of 4,4′-MDI trimer (T2) are presented in Figure 19. Since DMSO was used as solvent for the chromatography, we had no UV spectrum for wavelengths under 260 nm, which is the UV cutoff for DMSO. The UV spectrum for synthesized 4,4′-MDI trimer (green) had a somewhat narrower peak (with the maximum at 270 nm) than the extracted product, which indicated the presence of other substances that might broaden the peak and shift its maximum by several nm.
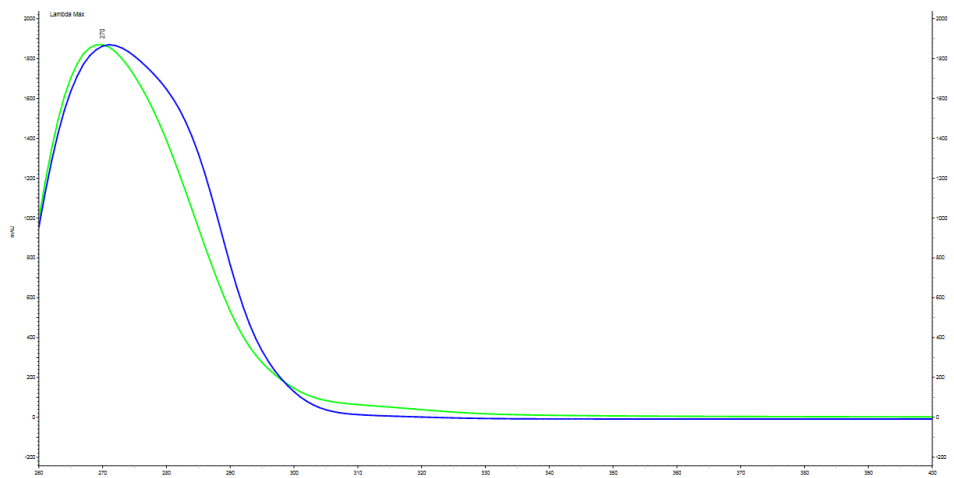
Figure 19. UV-spectra of material extracted from a 2-year-old preparation of 2% 4,4′-MDI in petrolatum (blue) and of synthesized 4,4′-MDI trimer (T2) (green).

Nuclear magnetic resonance (NMR) spectrometry

NMR spectroscopy of both T1 and T2 was performed and evaluated. The ^{1}H- and ^{13}C-NMR results have been compared to estimated data. In the ^{1}H-NMR of T2, the aromatic multipletes could not be resolved due to residues of solvent and impurities. It was difficult to separate toluene from this product during workup. Some peaks characteristic of aromatic amines indicated that around 15% of the isocyanate groups had been hydrolyzed. Peaks appeared in T2 that deviated from the main signal at δ 3.80 for the methylene group (CH_2 groups between the aromatic rings) (Figure 17), indicating up to 50% other substances. However, the integral of aromatic protons compensated for residual toluene, and impurities gave the expected number of protons compared to the protons indicated by the main methylene group peak (Table 9). The ^{1}H-NMR of T1 showed methylene group protons and aromatic protons in relative numbers that matched what could be expected in the 4,4′-MDI trimer. Other impurities appeared in this spectrum compared to the T2 spectrum.

For ^{13}C, the estimated signals were assigned to the most probable signals from the T1 substance as well as the heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond correlation (HMBC) spectra. Multiple close peaks at δ 118, 129, and 134 could not be resolved and were therefore given rough numbers. The assigned ^{13}C data (Table 10) are shown in Figure 17.

It was not possible to obtain help with professional interpretation of the NMR data produced, and this limited the extraction of more information from the spectra.

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Table 9.
^{1}H-NMR of synthesized trimers and estimated shifts for the proposed trimer structure

Trimer			Estimated		HMQC	HMBC
Shift (ppm)	Type	Integral	Shift (ppm)	Integral	BOUND to C (shift, ppm)	Coupling to C (shift, ppm)
3.80	s	6H	3.81	6H (s)	40.15	134.36 + 129
7.0–7.2	m broad	24H	7.04 and 7.1*	6H (d) 12H (dod)	118	136.53 + 137.09
7.3–7.4	m broad		7.52	6H (d)	129	118 + 133.21

* This was taken as an uncertain estimation, as the program treated the -NCO group as an unknown substituent.
s, singlet; m, multiplet; d, doublet; dod, doublet of doublet; HMQC, heteronuclear multiple quantum coherence ;
HMBC, heteronuclear multiple bond correlation; H, hydrogen.

Table 10.
^{13}C-NMR of synthesized trimer and estimated shifts for the proposed trimer structure.

Trimer shift (ppm)	Estimated shifts (ppm)	Carbon no. in Figure 17
40.15	45.8	7
113.97	125.7	14
118	122.1	2, 6
118	125.6	10, 12
129	128.4	3, 15
129	129.3	9, 13
134	130.2	1
134	131.1	11
136.53	137.1	4
137.09	138.7	8
152.53	151.1	15

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5 Discussion

5.1 Studies I and II

It has been shown previously that occupational airway exposure to 4,4′-MDI can be traced and monitored by analyzing 4,4′-MDA as a biomarker for this exposure in hydrolyzed plasma and urine from humans and animals (94, 95). 4,4′-MDA in hydrolyzed blood and urine samples has been identified as a metabolite of 4,4′-MDI from airway exposure in humans (96, 97). This has been used to trace the absorbed amount of 4,4′-MDI in blood and urine samples (88). In our studies, we used similar methods to detect dermal uptake of 4,4′-MDI by analysis of 4,4′-MDA in hydrolyzed blood and urine samples. Tape-stripping of the exposed skin areas was also used to detect 4,4′-MDI remaining in the superficial skin layers.

Studies I and II involved dermal exposure of 4 volunteers to 2.0% 4,4′-MDI in pet., with a surface concentration corresponding to approximately 0.8 mg/cm^2. The amount applied in the study corresponded to the one recommended at that time, namely 20 mg (11) as 2.0% MDI in petrolatum. However, volunteers 1 and 2 exhibited eczematous dermatitis on the exact area of application 7–10 days after the application. Positive reactions that are seen 7–10 days after application are usually considered to be a sign of active sensitization. However, it has previously been shown that such late positive reactions to isocyanates may also appear in already sensitized individuals (9). In order to determine whether or not the late reactions were due to active sensitization, we performed retesting of 4,4′-MDI in serial dilutions and also testing with potentially cross-reacting substances such as 4,4′-MDA, PPD, and 4,4′-DMDI.

Since the volunteers showed such strong reactions at the site of the provocation, they were not tested with the highest concentration of 4,4′-MDI, i.e. 2.0%, and thus no re-test was performed with the same concentration as the one used at the suspected active sensitization. Only one of the volunteers reacted to the diluted preparation of 4,4′-MDI on D7. In our experience at the department in Malmö, patch testing with 4,4′-MDI can be difficult since there could be significant individual variation in test response from one test occasion to another, and we have seen a case in which the same patient showed early reaction, late reaction, and no reaction at all to 4,4′-MDI when tested on different occasions. Based solely upon the testing with

diluted preparations of 4,4′-MDI, the results did not fulfill the criteria described in section 1.2 for establishing that active sensitization had occurred in both volunteers, since one did not react to 4,4′-MDI. Still, we consider it certain that both volunteers had been sensitized through the dermal uptake, since they both reacted to 4,4′-MDA. Concurrent allergic reactions to 4,4′-MDA and 4,4′-MDI have been described in an earlier report (9).

The concentration and dose per unit area (2.0% and 0.8 mg/cm^2, respectively) that we used in these studies have been established and used in patch testing over many years without reported active sensitization. We therefore believed that using this dose per unit area was safe to use, yet two volunteers were sensitized. A regular patch test is performed using a commercial 4,4′-MDI preparation. In our study we also patch tested the volunteers with fresh 4,4′-MDI preparation made at our department. In a previous study from our department (59), it was found that commercial patch test preparations of 4,4′-MDI contained only small fractions of the concentrations intended. It is therefore likely that most patients who were patch tested with commercial 4,4′-MDI preparations were patch tested with concentrations that were too low to even elicit a positive reaction. In our provocation study, we may have unintentionally exposed the volunteers to a much higher concentration and dose per unit area than patients who were routinely patch tested earlier. It is possible that the concentration used and the dose per unit area used induced a certain amount of irritation that could have promoted sensitization.

It has been considered that the dose per unit area is the most important factor for sensitization, and more important than the total area exposed. A study performed on humans found that irritation and concentration were important factors for sensitization but the authors reported that a "notable finding is that the area size is not a very influential factor." When the surface area decreased from 0.8 cm^2 to 0.08 cm^2 a difference in sensitization rate was found. However, a relatively high concentration, sensitizing almost the whole group exposed at 0.8 cm^2, was used on these surfaces and therefore it is difficult to compare the results from these two areas with the rest of the presented data since different doses and exposure areas were used (98). However, in 1966 Kligman found that the sensitization rate increased significantly in the area range 0.36–14.0 cm^2 when keeping the dose / area unit constant. In areas larger than 14.0 cm^2, the sensitization rate did not increase (99). This means that our provocation areas (12.5 and 31 cm^2) possibly could increase the risk of sensitization when compared to the patch test area, i.e. 0.50 cm^2.

Our conclusion was that the sensitization could have been due to provocation with a higher concentration and dose per unit area than had been used in practice earlier for patch testing. Our provocation areas were in the area range where sensitization is significantly increased. Another factor for the sensitization was the possible irritation that may have been induced by the fresh preparations of 4,4′-MDI.

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Based on the results above, the European Society for Contact Dermatitis (ESCD) recommended reduction of the recommended patch test concentration of 4,4′-MDI to 0.50% (w/w) in Europe (100).

PPD was tested due to the suspicion that one of the volunteers was allergic to it. PPD could have been the primary sensitizer, cross-reacting with 4,4′-MDA. The volunteers were patch tested with PPD and had no reactions to it. Since DMDI had been reported to cross-react with 4,4′-MDI (62), the volunteers were also patch tested with this substance but no concurrent reactions were evident in the present study (Table 4). The cross-reactivity of these diisocyanates and corresponding amines, and of PPD, was explored further in papers III and IV.

The physical properties, log $P_{o/w}$ and the molecular weight, of 4,4′-MDI are favorable for skin penetration. Our data showed that a substantial proportion―approximately 50% of the dose of 4,4′-MDI―had been absorbed by the skin. Although the crushing of one sample flask during transportation resulting in removal of the identity of the other sampling flasks from volunteers 3 and 4, we believe that we made a good estimation of the amounts absorbed. At clinical patch testing, many allergens are tested in petrolatum preparations and similar proportions of the substances being tested are absorbed by the skin (101). These results are well in line with the physical properties of 4,4′-MDI, and indicate that 4,4′-MDI readily penetrates the skin (102) and can cause active sensitization when tested as 2.0% in pet. and when using fresh preparations (86).

The amounts of 4,4′-MDA detected in hydrolyzed blood and urine of the volunteers were very low, and only represented a very small fraction of the dose of 4,4′-MDI applied. We estimate, based on the assumption of a plasma volume of approximately 5 l, that the sum of the amounts in plasma and urine was in the range of 0.01–0.2% of the 4,4′-MDI dose used for volunteers 2, 3, and 4. For volunteer 1, no such calculations could be done due to the blood and urine concentrations being below the detection limits. The non-absorbed amounts of 4,4′-MDI cannot explain this result. Events more complicated than just diffusion-based penetration take place in 4,4′-MDI exposed skin, which is the predominant skin penetration mechanism for many other non-polar substances. The most plausible explanation is that 4,4′-MDI reacts and binds chemically to skin constituents. This explanation is supported by earlier findings suggesting that 4,4′-MDI undergoes specific reactions to the site of first contact and forms adducts, conjugates, and/or polyureas (103).

Biological monitoring of exposure to airborne 4,4′-MDI has been done in workers exposed to airborne 4,4′-MDI through evaporation from an industrial MDI product (104). The study showed that 8 hours of exposure to a median air concentration of 0.2 μg/m^3 4,4′-MDI resulted in 4,4′-MDA concentrations with medians of 2 ng/ml in hydrolyzed urine and 0.7 ng/ml in hydrolyzed plasma. Approximately 10 hours after the volunteers had started the dermal application of 4,4′-MDI in the present

study, we found that the highest concentrations of 4,4′-MDA were 4.32 ng/ml in hydrolyzed urine and 0.22 ng/ml in hydrolyzed plasma (Figures 6 and 7). Thus, 8 hours of skin exposure to 25–50 mg 4,4′-MDI gave similar blood and urine values of 4,4′-MDA as air exposure to 2 µg over 8 hours, based on an inhaled air volume of 10 m^3 per day. This indicates that the distribution and elimination after dermal uptake of 4,4′-MDI are much slower processes compared to the similar events in airway uptake.

Analysis of the tape strips showed that only 0.1–0.3% of the total applied dose of 4,4′-MDI could be retrieved from volunteers 3 and 4, showing that accumulation of unreacted 4,4′-MDI in the upper layers of the stratum corneum is very low. However, following increased applied dermal doses ranging from 25 to 50 mg, an increase in urinary metabolites could be seen. The peak in urinary metabolites (4.32 ng/ml) appeared after 10–14 hours and declined to approximately starting values 48 hours after the start of the exposure. This indicates that 4,4′-MDI probably reacts with cell constituents and stays in the upper layers of the skin. A proportion of the reacted 4,4′-MDI is probably eliminated from the skin upon cellular renewal while the remaining amount of bound 4,4′-MDI is hydrolyzed and absorbed as 4,4′-MDA which then can be distributed to plasma and urine. The absorption and elimination of 4,4′-MDI is a slow process, and it can probably take weeks or even months to eliminate it from the body.

It has been suggested that 4,4′-MDI reacts with water in the skin to form 4,4′-MDA, which then penetrates the skin. If this suggestion was true, it could explain the low amounts of 4,4′-MDI found in the tape strips. In human studies, 4,4′-MDA has been shown to penetrate the skin easily (105, 106), which should result in high 4,4′-MDA levels in the urine. However, we found only traces of 4,4′-MDA, indicating that only a very small fraction of 4,4′-MDI could have reacted with water and formed 4,4′-MDA; this mechanism is therefore most likely not responsible for the instability of 4,4-MDI test preparations (107). This was investigated further in paper VI.

Patch testing in individuals who were allergic to 4,4′-MDI has shown that positive test reactions to 4,4′-MDI in already sensitized individuals may appear after 2–3 weeks whereas a reaction from contact allergy usually appears after 1–3 days (9). The authors hypothesized that there are unusually late reactions from the binding of 4,4′-MDI to cell constituents, with a subsequent slow release of 4,4′-MDA as hydrolysis of the conjugates that are formed occurs. The low recovery of 4,4′-MDI in study II supported our suspicion that a large proportion of the skin-absorbed 4,4′-MDI rapidly reacted with cell constituents in the upper layers of the skin. The late positive patch test reactions would then be caused by 4,4′-MDA allergy. Polymerization in the upper skin layers might also be a contributory mechanism. The very low levels of 4,4′-MDA in hydrolyzed plasma and urine samples would

indicate that only a small fraction of 4,4′-MDI penetrates the skin in an unaltered form.

Efforts were made to verify the amount of 4,4′-MDI that reacted with cell components in the tape strips by hydrolysis and conversion to 4,4′-MDA. The analysis at another laboratory failed, and it was impossible to re-analyze the samples.

5.2 Studies III and IV

5.2.1 Sensitizing capacity in GPMT

In studies III and V, we wanted to investigate the sensitizing capacity and cross-reactivity of 4,4′-MDI, 4,4′-DMDI, and 2,4-TDI, and of the amines 4,4′-MDA, PPD, and 4,4′-DMDA. We used the GPMT method to investigate the allergenic properties of these chemicals.

In order to elicit allergic contact dermatitis, a chemical must have the physiochemical characteristics necessary to penetrate the stratum corneum. Once in the viable epidermis, it must be able to form reaction products with proteins for the elicitation of an immune response. Thus, contact allergens are either protein-reactive in themselves or are metabolized in the skin into protein-reactive species (108). Isocyanates are (in theory) potent contact allergens, since they possess electrophilic carbons that can be readily attacked by nucleophilic atoms present on macromolecules in the skin. However, it has been proposed that their reactivity is so high that they might polymerize before they penetrate the skin (65). Amines are lipophilic, and penetrate the skin quite readily. However, in order to react with proteins in the skin, the amines must be metabolized.

In the literature, there have been some animal studies investigating the sensitizing capacity of 4,4′-MDI. In 1976, the sensitizing capacity of 4,4′-MDI was studied with GPMT, and it was concluded that the proportion of test animals that reacted to 4,4′-MDI (10% in pet.) showed that it was a strong allergen. In that study, they used higher concentrations than in paper III, with intradermal injections of 5.0% 4,4′-MDI in olive oil and epicutanous sensitization with 25.0% 4,4′-MDI in petrolatum (109). In paper III, we had difficulties in sensitizing with 4,4′-MDI. It was used as induction substance on three separate occasions. On the first occasion, it was found to be a strong sensitizer―with 18 out of 24 test animals reacting to 1% 4,4′-MDI in acetone ($p < 0.001$). However, in this first sensitization series there was a suspicion that 4,4′-DMDI might cause irritant reactions if patch tested at a concentration equimolar to 1.0% 4,4′-MDI. Thus, the concentrations of 4,4′-MDI at challenge I and challenge II were not the same. The concentration at challenge II was lower―on

account of being able to patch test in a concentration equimolar to 4,4′-DMDI. As expected, a lower proportion of test animals were positive in challenge II, where they were patch tested with a lower concentration of 4,4′-MDI than in challenge I (18 of 24 animals were positive in challenge I as opposed to 7 of 24 in challenge II). In the second sensitization series, two concentrations of 4,4′-DMDI were investigated and it was concluded that a concentration of 1.0% did not cause irritant reactions. Hence, a new series was performed in order to induce with 4,4′-MDI and perform challenge II with concentrations equimolar to those in challenge I. This time, the induction failed and only 2 out of 24 test animals were sensitized. Since the positive controls reacted, there were no obvious methodological reasons for the failure. 4,4′-MDI was used as an induction substance a third time. This time, 8 out of 24 test animals reacted ($p < 0.05$), making it a weak allergen based on the criteria set.

In paper III, we showed that 4,4′-MDA, 4,4′-DMDI, and 4,4′-DMDA were strong sensitizers among our group of sensitizers. This was supported by previous clinical observations (61, 62, 65, 66). 4,4′-MDA is known to sensitize patients when tested at 0.5% in petrolatum (110, 111).

In paper IV, 8 of the test animals induced with 2,4-TDI were sacrificed due to oozing wounds at the site of the intradermal injection. Their capacity to dry and form crusts was considered to deviate too much from what was allowed in the ethical approval. Thus, 2,4-TDI was not considered to be a sensitizer based upon our set criteria, when using Fisher's exact test on the test results from the remaining 16 animals. However, if the sacrificed animals had been positive, the results of 2,4-TDI would have been statistically significant and would have indicated that 2,4-TDI is a moderate skin sensitizer.

PPD is an ingredient of hair dyes, and is considered to be a potent contact sensitizer. It is also associated with 4,4′-MDA allergy (110) and is usually used to detect hair dye allergy (68, 112, 113). In paper IV, we found that PPD was a strong sensitizer in our tests. This result is an accordance with other studies, in which PPD has been shown to be a strong sensitizer in both LLNA and GMPT (114-116). According to the GHS and the CLP regulation, PPD can be classified as a strong skin sensitizer.

5.2.2 Cross-reactivity in GPMT

Animals primarily sensitized to 4,4′-MDI show statistically significant cross-reactivity to 4,4′-MDA. This indicates that individuals who are sensitized to 4,4′-MDI may show simultaneous reaction to 4,4′-MDA.

In paper III, we also found that animals sensitized to 4,4′-MDI showed cross-reactivity to 4,4′-DMDI. However, when 4,4′-DMDI was the primary sensitizer, no cross-reactivity to 4,4′-MDI was found. To our knowledge, there have been no reports in the literature describing concurrent reactions between the two isocyanates.

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Instead, concurrent reactions between 4,4′-MDA and 4,4′-DMDI have been described (61, 62, 65). The lack of concurrent reactions between the two isocyanates possibly stems from the fact that commercially available patch test preparations of 4,4′-MDI have a high risk of false-negative reactions (59). Finally, it was found that animals sensitized to 4,4′-MDA also showed cross-reactivity to 4,4′-DMDA. Concurrent reactions between 4,4′-MDA, 4,4′-DMDA, and 4,4′-DMDI have been described in 2 patients who worked for a medical company, where a lacquer based on 4,4′-DMDI was used (62).

In 2012, Engfeldt et al. published results from consecutive patch testing with 4,4′-MDA and 4,4′-MDI in Belgium and Sweden (110). It was concluded that positive reactions to 4,4′-MDA were associated with contact allergy to PPD. A study published by Liippo et al. showed that one-third of their 4,4′-MDA-positive patch test patients also reacted to PPD (117). In 2002, Uter et al. presented clinical patch test data indicating that "para-amino" compounds could cross-react with each other. Patients who were positive against PPD were also positive against 4,4′-MDA and other para-amino compounds that are similar in structure (118). Cross-reactivity has also been reported between PPD and azo-dyes (119). We have seen that guinea pigs sensitized to PPD show cross-reactivity to 4,4′-MDA. It is possible that a positive reaction to 4,4′-MDA may indicate sensitization to PPD. This cross-reactivity could be an explanation for the concurrent reactions in humans that have been observed earlier (110). PPD is one of the most common contact allergens in the baseline series. The cross-reactivity between 4,4′-MDA and PPD should be taken into consideration if 4,4′-MDA is used as a sign of 4,4′-MDI allergy. It should be considered that a positive reaction to 4,4′-MDA could also be a sign of hair dyeing habits, and not only of isocyanate exposure. Since no cross-reactivity could be found between 4,4′-MDI and PPD, one can assume that individuals with hair dye allergy can work with isocyanates. Accordingly, there is nothing in the results from this study to suggest that 4,4′-MDI-sensitized individuals have a higher risk of developing eczema when dying their hair. However, we cannot draw any conclusions on whether 2,4-TDI-sensitized individuals are more likely to develop eczema from hair dyes containing PPD, since the result could not be fully evaluated. However, it should be noted that some indication of cross-reactivity to PPD was seen in animals sensitized with 2,4-TDI. It is possible that significant results would have been seen if it had been possible to read the tests in all the animals.

There was some indication of cross-reactivity to 4,4′-DMDA in animals sensitized to PPD. This and cross-reactivity to 4,4′-DMDA in animals sensitized to 4,4′-MDA―and also an indication of cross-reactivity in the reverse situation―highlights the need for further studies to investigate the cross-reactivity patterns between the amines investigated and other structurally related substances such as 2,4-TDA and azo-dyes.

Our substances were tested at equimolar amounts in order to be able to compare the sensitizing capacities between these substances. GPMT is a method that is defined by maximization, which means sensitizing animals with the highest non-irritating concentration of a substance regardless of the equimolarity. If our substances had been tested with the highest non-irritating concentration in the sensitization and challenge, we would probably have seen more cross-reactions and the indication of a cross-reaction could have turned into a statistically significant cross-reaction.

5.3 Study V

In paper III, where the GPMT was used to investigate the sensitizing capacity and cross-reactivity of 4,4′-MDI, the sensitization gave different results when performed on our test substances on three different occasions—namely, strong sensitizer, non-sensitizer, and weak sensitizer (102). The sensitization procedure was investigated for errors, but no obvious reasons for the different results were found. The positive controls reacted as expected. However, it was suggested that one plausible explanation could be a variation in 4,4′-MDI content in the preparations used in the intradermal sensitization. Therefore, we analyzed the preparations containing 4,4′-MDI and those that had been used in the intradermal induction at the third and last sensitization trial with 4,4′-MDI. We found that the preparation containing 4,4′-MDI and paraffin oil was stable while the other, containing FCA, 4,4′-MDI, and paraffin oil, had declined in 4,4′-MDI concentration. We therefore investigated the stability of 4,4′-MDI when mixed with FCA. Mixtures of this kind were followed over time after storage in the refrigerator and at room temperature. Another factor that might have affected the outcome was the stability of the raw material, i.e. 4,4′-MDI. Thus, 4,4′-MDI of different ages was also investigated.

4,4′-MDI is a reactive substance, and it reacts readily with water, amino-, thiol-, and hydroxyl groups in other substances. There are proteins and water in FCA which are targets for 4,4′-MDI, due to its reactivity with amino- and hydroxyl groups. FCA contains water, oil, emulsifier, and killed *Mycobacterium* bacteria. At the start of the study, the 4,4′-MDI preparation was prepared according to the guidelines that had been used in the previous GMPT studies. These guidelines involved dissolving 4,4′-MDI in FCA and later mixing with the vehicle of choice (102, 120).

In all three sensitization trials with 4,4′-MDI, the induction emulsion with FCA was made one day prior to the intradermal sensitization and stored in a refrigerator. The emulsion was then taken out of the refrigerator after 24 hours, delivered to the animal testing laboratory, and stored at room temperature while the animals were being prepared for the induction phase. The results showed that the storage conditions play a vital role. When stored at room temperature, the concentration of

4,4′-MDI in the mixture had almost halved after 24 hours. At the end of the assay (48 hours), only 0.33% 4,4′-MDI (w/v) remained in the emulsion.

For the mixture stored in the refrigerator, the concentration also declined—but more slowly compared to storage at room temperature. After the end of the assay, the concentration of 4,4′-MDI stored in the refrigerator was 0.67% (w/v) (Figures 10 and 11). The decrease in 4,4′-MDI concentration could affect the sensitization process. As a conclusion from the results above, the approximate concentration of 4,4′-MDI in the intradermal FCA preparations that were used could be extrapolated from Figures 1 and 2 to be around 0.61% (w/v) as compared to the intended concentration i.e. 1.0% (w/v).

Analysis of 4,4′-MDI of different ages showed that pure 4,4′-MDI is a very reactive chemical. Despite being stored at −21°C, the concentration decreased substantially in just few months (Figure 12). If both factors interact—instability of the pure substance and the reactivity with FCA—this could have a major effect on the outcome of the method, since it might affect both the intradermal and the epidermal induction, and also the elicitation and detection of sensitization.

The use of FCA in the GPMT is a generally sensitive method for characterizing and identifying contact allergens by enhancing sensitization in the guinea pig, but it is important to point out that reactive chemicals may not be at the intended concentration in the preparations used. Based on our findings, the induction emulsion in FCA should be prepared in direct connection with the administration of the intradermal injection of the substance. Furthermore, it is most important to use fresh 4,4′-MDI to guarantee correct concentrations in both induction and elicitation. In similar assays for other aromatic diisocyanates or other highly reactive substances, it may be of interest to compare the intended concentration and the actual concentration injected.

5.4 Study VI

The 4,4′-MDI test preparation had been examined by our department earlier, and we found that test preparations contained small fractions of what was stated in the descriptions (59). We wanted to investigate what happens in the preparations, what is formed, and how. One explanation might be that the decrease in concentration is caused by contact with water. Water was mixed in excess with a petrolatum preparation of 4,4′-MDI and followed over time. The addition of water did not affect the rate of decrease, probably because it is almost impossible to disperse water in petrolatum. The mixed water resurfaced again after mixing. The rate of decrease

was slightly slower in this experiment than what was observed in an earlier investigation of the stability of pure 4,4′-MDI in petrolatum (107).

Acetone was also mixed with the 4,4′-MDI preparation. We tried to add a mixture of acetone and water. This did not affect the rate of decrease either. We did not succeed in distributing the water evenly enough to get it in close contact with the 4,4′-MDI. Our tries strongly suggested that water does not cause the observed disappearance of 4,4′-MDI in petrolatum preparations. Evaporation of 4,4′-MDI from petrolatum preparations was also excluded.

We also mixed ethanol with the preparation in petrolatum. Ethanol has a hydroxyl group that 4,4′-MDI can react with. The reaction was fast, and after 1 hour more than half the amount of 4,4′-MDI had reacted. This showed that 4,4′-MDI really can react inside the petrolatum preparation.

Isocyanates are reactive molecules, especially 4,4′-MDI, and they can react with each other. Spontaneous dimerization and trimerization can occur even at room temperature. The dimer has been described mainly as an intermediate in the formation of the more stable trimer (121). There are no published data on the 4,4′-MDI dimer and the 4,4′-MDI trimer. Searching of major databases revealed very little information on the 4,4′-MDI dimer, while the 4,4′-MDI trimer has hardly featured in the previous literature (122, 123).

We wanted to investigate whether trimerization is what happens in aging 4,4′-MDI petrolatum preparations, but 4,4′-MDI trimer could not be obtained commercially. At our laboratory, we developed a method for synthesis of 4,4′-MDI trimer using acetone, 4,4′-MDI, and the catalyst triethylamine. The synthesized 4,4′-MDI trimer was difficult to dissolve, and although we tested all the usual solvents we found only two that could dissolve the synthesized 4,4′-MDI trimer, dimethylacetamide and dimethylsulfoxide (DMSO). We found a published method for synthesis of the 4,4′-MDI trimer (92). The product from this synthesis was soluble in the same solvents. The only proof of identity that could be found was an FTIR spectrum (92).

The synthesized substance was almost insoluble, which was a huge obstacle in the analysis for identification of the structure using Gas chromatography–mass spectrometry (GC-MS)—due to the low volatility of DMSO. FTIR gave a spectrum that was similar to that published. NMR provided us with some information, but we only found limited evidence that the substance under investigation was 4,4′-MDI trimer. It was clear from the NMR data that the substance we were analyzing was not pure. However, we found NMR signals that were compatible with the proposed structure of the 4,4′-MDI trimer. Our suggested NMR shifts for the substance must be studied further.

We could conclude from our NMR analysis that further analysis and purification of the 4,4′-MDI trimer synthesized would be necessary. It was also important to find a

solvent that would be compatible with GC-MS. Mass spectrometry is crucial for identification of the 4,4′-MDI trimer.

GPC was used for analysis of the 4,4′-MDI trimer using the synthesized 4,4′-MDI trimer as reference substance. The different preparations from batches of 4,4′-MDI in petrolatum of different ages were extracted and analyzed using GPC. All the extracts of the different batches of aged petrolatum preparations showed a peak with the same retention time as the synthesized trimer. They also showed similar, though not identical, UV-spectra due to impurities.

The trimer of 4,4′-MDI has a molecular weight of 750, which makes it more difficult to diffuse and penetrate the skin. The 4,4′-MDI trimer still had reactive isocyanate groups. These groups can bind to proteins or to other skin constituents, and the substance could still be an allergen. The substance could also bind to the site of skin exposure in the same way as 4,4′-MDI appears to do (124), and this would prevent or slow down penetration. Because many patients have been patch tested with aged 4,4′-MDI patch test preparations and the results from these indicated that the patients did not react, we can conclude that if the trimer is present it does not cross-react with 4,4′-MDI and that it is probably a weaker allergen than 4,4′-MDI.

What we currently know is that we have been able to dissolve the synthesized 4,4′-MDI trimer only in DMSO or dimethylacetamide. The extractable substance(s) from the petrolatum preparations showed similar solubility properties. FTIR and NMR data suggested that the substance synthesized was mainly 4,4′-MDI trimer. It is also probable that this is the main substance found in aged test preparations. Furthermore, we can exclude the idea that evaporation or reaction with water would be significant factors in the instability of 4,4′-MDI patch test preparations. The data mostly suggest that trimerization—and perhaps also dimerization—is the main factor influencing this instability.

6 Summary and concluding remarks

From studies I and II, we have seen that dermal absorption of 4,4′-MDI occurs in human skin. Only small fractions of 4,4′-MDI remain as such in the superficial layers of the skin. The distribution after the dermal uptake of 4,4′-MDI appears to be a slower process than what is seen in airway uptake. Our results suggest that the distribution of 4,4′-MDI from the skin and the subsequent elimination is a slow process. The proportion of absorption was half of the amount applied The 4,4′-MDI absorbed reacts with cell constituents. This absorbed amount is probably released as 4,4′-MDA by spontaneous or enzymatic hydrolysis over a matter of weeks or months, systemically distributed, and finally eliminated. A proportion of reacted 4,4′-MDI is probably eliminated from the skin upon cellular renewal. Although a small proportion of 4,4′-MDI penetrates the skin in unchanged form, our studies have shown that the subsequent processes in the skin ultimately lead to an allergic reaction caused by exposure to 4,4-MDI. In fact, patch testing with freshly made preparations of 2% 4,4-MDI might lead to active sensitization. Thus, the concentration of 0.5% in pet., which is recommended by the ESCD based on our results, should be used instead

In studies III and IV, we found that 4,4′-MDI, 4,4′-MDA, 4,4′-DMDI, 4,4′-DMDA, and PPD were strong sensitizers among our group of sensitizers. In the evaluation of cross-reactivity, the clinical observations previously noting that 4,4′-MDA is an indicator for 4,4′-MDI were verified, as animals sensitized to 4,4′-MDI also reacted to 4,4′-MDA. Furthermore, animals sensitized to 4,4′-MDI cross-reacted with 4,4′-DMDI and animals sensitized to 4,4′-MDA cross-reacted with 4,4′-DMDA.

PPD-sensitized animals showed cross-reactivity to 4,4′-MDA and there was an indication of cross-reactivity to 4,4′-DMDA. 4,4′-MDI sensitized animals did not show cross-reactivity to PPD, so PPD cannot be used as a marker for 4,4′-MDI allergy. From the results of GPMT studies, it can be concluded that reactions to certain chemicals may not always indicate allergy to a specific substance, but rather a cross-reaction to a chemically related substance.

The intradermal induction concentration (1.0%) of 2,4-TDI can induce strong local reactions in guinea pigs, and should be chosen carefully. Animals sensitized to 2,4-TDI showed strong local reactions and had to be sacrificed.

FCA is used to enhance sensitization in animals. The allergens are mixed with FCA. It contains killed bacteria in an emulsion of water and oil. In study V, we found that 4,4′-MDI reacts with water, protein or other components of the mixture. We also found that aged pure 4,4′-MDI is instable even when stored in the freezer. The outcome of 4,4′-MDI sensitization in GPMT might be affected by the instability of the pure substance as well as its reaction with FCA. Major effects can be expected if these two factors interact. Hence, fresh 4,4′-MDI should be used and induction substances should be prepared in close connection with the intradermal injection. These factors might explain the variation in sensitization with 4,4′-MDI seen in study III.

Our results in study VI strongly indicated that water does not cause the previously reported disappearance of 4,4′-MDI in petrolatum patch test preparations. We can exclude the idea that evaporation or reaction with water would be significant factors for the instability of the 4,4′-MDI patch test preparations. Most of our data indicated that trimerization, and perhaps also dimerization, is the main factor influencing this instability.

The 4,4′-MDI trimer has reactive isocyanate groups. These groups can bind to proteins or to other skin constituents and cause allergy. However, the allergenicity might be weak due to the fact that as the molecular weight of a substance increases, it is more difficult for it to move through the skin. Because many patients have been patch tested with aged 4,4′-MDI preparations (in which they did not react), we can conclude that if the trimer is present it does not cross-react with 4,4′-MDI and that it is probably a weaker allergen than 4,4′-MDI.

7 Popular scientific summary in Swedish

Huden är kroppens största organ och är vårt skydd mot yttre faktorer. Vid kontakt med främmande ämnen kan kroppens immunförsvar lära sig känna igen dessa ämnen och vid förnyad kontakt med dessa ämnen kan det uppstå ett allergiskt kontakteksem. Kontaktallergi kan diagnostiseras genom att de misstänkta ämnena späds i lämplig vehikel, oftast vaselin. Dessa spädningar placeras i testkammare (lapptest) som sedan fästs på ryggen och tillåts sitta i två dagar. Vid dag 3/4 och 7 avläses testreaktionerna av en hudläkare.

I denna avhandling undersöker vi de kontaktallergiframkallande egenskaperna hos isocyanater. Isocyanater används i tillverkningen av polyuretan (PUR). PUR bildas när en isocyanat reagerar med en flervärd alkohol. PUR-produkter finns i många vanligt förekommande produkter i vår omgivning, exempelvis färg, lim, skumgummi och lack.

Isocyanater är kända för att ge upphov till luftvägssjukdomar såsom bronkit och astma. Hudexponering för isocyanater kan också orsaka kontaktallergi vilket är mindre uppmärksammat. För att diagnosticera kontaktallergi mot isocyanater, lapptestas patienter med en speciell isocyanatserie. I denna serie testas olika isocyanater i vaselin med en specifik koncentration. I denna serie finns också de motsvarande aminerna till de olika isocyanaterna. Dessa aminer bildas när isocyanaterna hydrolyseras.

I denna avhandling studerar vi i arbete I och II hudupptag och absorption av isocyanaten difenylmetan-4,4′-diisocyanat (4,4′-MDI) samt utsöndringen av denna via blod och urin. Detta gör vi genom en tejp-stripping metod samt med hjälp av vätskekromatografiska metoder.

Flera fallrapporter har beskrivit samtidiga reaktioner för olika isocyanater och deras motsvarande aminer. Därför undersöker vi i delarbetena III och IV hur sensibiliserande isocyanater är och hur de korsreagerar dels med varandra och dels även med sina motsvarande aminer. Detta gör vi med en *in vivo* metod som heter Guinea Pig Maximization Test (GPMT). Studien gör att vi kan få en klarare bild över reaktionsmekanismer och hur immunförsvaret reagerar på ämnen med liknande kemisk struktur.

Avhandlingen undersöker svårigheter som vi upptäckte vid djurförsöken. Frågeställningen var om ämnenas höga reaktivitet påverkar utfallet av försöken. Vi har även undersökt stabiliteten och åldrandet av 4,4′-MDI i de beredningar som används för att göra djuren allergiska.

I delarbete VI studerar vi instabiliteten av beredningar av 4,4′-MDI. Denna har tidigare påvisat brister i metoden för detektering av kontaktallergi mot isocyanater. Vi undersöker vad som händer i vaselinberedningarna och vad som bildas istället. Detta är en viktig del av avhandlingen då den belyser ett problem med diagnostiken och den låga frekvensen av kontaktallergi mot isocyanater och om detta är huvudorsaken till de låga frekvenserna.

Från studie I och II har vi sett att 4,4′-MDI absorberas bra av huden. Ämnet sprids och utsöndras långsamt från huden till övriga kroppen. Endast små fraktioner av 4,4′-MDI finns kvar som sådant i de ytliga hudlagren. Våra resultat tyder på att en stor andel av 4,4′-MDI reagerar snabbt med cellkomponenter och endast en liten del av 4,4′-MDI-dosen verkar penetrera huden i oförändrad form. Trots att 4,4′-MDI binder in till huden står det klart att 4,4′-MDI kan ta sig ner till de levande delarna och framkalla hudallergi. Hudupptaget behöver studeras mer ingående genom provokation av allergiska personer med 4,4′-MDI och följa halterna av 4,4′-MDI i plasma och urin under en längre period. Detta kan ge viktig information om hudupptaget och utsöndring.

Två försökspersoner exponerades för en beredning av 2.0% w/w 4,4′-MDI med en koncentration som vi vanligtvis lapptestar med. Trots detta ledde försöket till sensibilisering av försökspersonerna. Tidigare ansåg man att ytkoncentrationen är den avgörande faktorn sensibilisering oberoende av ytans storlek. I våra studier använde vi större exponeringsytor än vid en vanlig lapptestning med färsk beredning 4,4′-MDI i petrolatum. Det är samma koncentration som anges på kommersiella testberedningar. Sensibiliseringen skedde troligen pga större yta och att den färska beredningen faktiskt innehöll 2.0% w/w medan de kommersiella beredningarna innehåller mycket lägre halter. Ett resultat av våra studier är att ESCDs rekommendation för lapptestkoncentration sänkts till 0.50% w/w 4,4′-MDI i vaselin för att undvika sensibilisering.

I studie III, IV har vi jämfört styrkan på de kontaktallergena egenskaperna hos den grupp ämnen som testats. 4,4′-MDI, diamino-4,4′-difenylmetan (4,4′-MDA), dicyklohexylmetan-4,4′-diisocyanate (4,4′-DMDI), diamino-4,4′-dicyklohexyl-metan (4,4′-DMDA) och p-fenylendiamin (PPD) visades vara starka allergener. Undersökningen av 2,4-toluendiisocyanat (2,4-TDI) misslyckades delvis då testdjuren fick kraftiga reaktioner efter intradermala injektioner och därför fick avlivas. Sensibiliseringsgraden blev inte signifikant med de återstående testdjuren. Hade alla avlivade djur varit positiva så hade sensibiliseringen blivit statistiskt signifikant och då hade 2,4-TDI kunnat klassas som svagt allergen i jämförelse med

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de andra undersökta ämnena. 2,4-TDI har tidigare klassats som stark allergen när den har undersökts med local lymph node assay (LLNA).

Korsallergimönstret av de ovannämnda ämnena studerades också. Djur sensibiliserade med 4,4′-MDI korsreagerade mot 4,4′-MDA och 4,4′-DMDI. Djur sensibiliserade med 4,4′-MDA korsreagerade mot 4,4′-DMDA. Djur sensibiliserade mot 4,4′-MDA eller 4,4′-DMDI korsreagerade inte mot 4,4′-MDI. Inga av de ovan nämnda ämnena gav korsallergi mot PPD. PPD-sensibilisering gav upphov till korsallergi mot 4,4′-MDA. Eftersom GPMT är en välbeprövad metod kan vi dra slutsatsen att även människor reagerar på samma sätt. Positiva testreaktioner för 4,4′-MDA kan förklaras av antingen 4,4′-MDI i t ex. byggnadsindustrin eller sensibilisering för PPD vid t ex hårfärgning. Exponering för 4,4′-MDA är också en tänkbar orsak till sensibilisering men högst osannolik då det är ett ämne som är cancerframkallande och knappast förekommer i vår omgivning.

Vid sensibiliseringsförsöken av 4,4′-MDI har vi sett en variation i resultaten. Detta har vi studerat närmare i studie V. Vid närmare undersökning stod det klart att åldern på 4,4′-MDI kunde påverka kvaliteten. 4,4′-MDI blandas med Freund's complete adjuvant (FCA) som består av avdödade bakterier, vatten och olja. FCA används för att öka sensibiliseringen. Vårt försök visade att 4,4′-MDI reagerar med beståndsdelarna i FCA. Åldrad 4,4′-MDI som reagerar med beståndsdelarna i FCA kan ha en stor effekt på detektion av sensibilisering och kan förklara variationen i försöken.

I arbete VI undersökte vi instabiliteten av 4,4′-MDI i testberedningar som används för klinisk detektion av 4,4′-MDI–allergi. Våra resultat visar att varken flyktighet eller absorption av vatten orsakar polymeriseringen av 4,4′-MDI-molekylerna. Vi har försökt påvisa att 4,4′-MDI-trimeren existerar i åldrade 4,4′-MDI beredningar. Vi syntetiserade en 4,4′-MDI-trimer för att kunna jämföra det med kromatogramen av åldrade 4,4′-MDI beredningar. Syntesen gav inte en ren produkt som kunde identifieras med säkerhet. Den syntetiserade 4,4′-MDI trimeren är svårlöslig och har därför försvårat de kromatografiska analyserna och uppreningen. Instabiliteten av 4,4′-MDI-beredningarna beror troligen på trimerisering. Trimeren i sig har hög molekylvikt och är troligen inte ett starkt allergen. Många patienter har lapptestats med åldrande 4,4′-MDI preparationer där allergiska patienter inte visat några reaktioner. Därför kan vi dra slutsatsen att om trimeren finns korsreagerar den inte med 4,4′ -MDI och är troligen är ett svagare allergen än 4,4′-MDI.

Acknowledgments

To my family who believed in me and supported me with the never-ending unconditional love and have provided me with moral and emotional support in my life. When I count my blessings I count you twice, I could not have asked for a better family: because I owe it all to you.

Thank you!

Many thanks to:

Super(hero)visor and life-coach, *Erik Zimerson*. Co-supervisor *Malin Engfeldt* for the help, encouragement during my rollercoaster journey. Thank you *Malin*, you are the best! Co-supervisor *Marléne Isaksson* and group leader *Magnus Bruze* for their excellent ideas and supervision.

My eternal cheerleader and best friend, *Annarita Antelmi* for the encouragement through all the ups and downs.

My deepest gratitude to my former colleague and good friend *Lena Persson*. Thank you for all the help, support and love. I miss our chat moments by the AAS. Thank you for listening to my whining, moaning and agreeing with my disagreement!

My forever interested, encouraging and always enthusiastic in-laws: they were always keen to know what I was doing and how I was proceeding, although it is likely that they have never grasped what it was all about!

I would also like to thank all my friends for encouraging me to keep going.

With a special mention to my colleagues *Ewa Young, Lena Svensson, Kornelia Griekspoor Åsevik, Henrietta Passlov, Ann-Charlotte Thorsson, Christina Persson, Ola Bergendorff, Jakob Dahlin , Martin Mowitz, Linda Rosén, Linda Ljungberg and Ann-Kristin Björk*. It is fantastic to have the opportunity to work with you all. What a cracking place to work at!

And finally, last but by no means least, my deepest appreciation and love to my sweetheart husband and son who made this whole thing possible. Thank you for bearing with me. You guys rock!

Thanks for all your help and encouragement!

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