Dose-response relationships in contact allergy and studies on single and repeated exposures – perspectives for prevention

Louise Arup Fischer Neergaard

This PhD thesis is based on the following manuscripts:

I. Fischer LA, Johansen JD, Menné T. Nickel allergy: relationship between patch test and repeated open application test thresholds. *British Journal of Dermatology 2007*; 157, 723-729.

II. Fischer LA, Johansen JD, Menné T. Methyldibromo glutaronitrile allergy: relationship between patch test and repeated open application test thresholds. *British Journal of Dermatology 2008*; 159, 1138-1143.

III. Fischer LA, Menné T, Avnstorp C, Kasting GB, Johansen JD. Hydroxyisohexyl 3-cyclohexene carboxaldehyde allergy - relationship between patch test and repeated open application test thresholds. Accepted for publication in *British journal of Dermatology February 2009.*

IV. Fischer LA, Voelund A, Andersen KE, Menné T, Johansen JD. The dose-response relationship between the patch test and ROAT and the potential use for regulatory purposes. *Submitted to Contact Dermatitis March 2009.*

Supervisors
Torkil Menné, Professor, MD, DMSc
Department of Dermato-allergology
Gentofte Hospital
University of Copenhagen, Denmark

Jeanne Duus Johansen, Professor, MD, DMSc
National Allergy Research Centre
Department of Dermato-allergology
Gentofte Hospital
University of Copenhagen, Denmark

Abbreviations:

LLNA	Local Lymph Node Assay
ROAT	Repeated Open Application Test
MDBGN	Methyldibromo glutaronitrile
HICC	Hydroxyisohexyl 3-cyclohexene carboxaldehyde

Acknowledgements

This study was carried out at the National Allergy Research Centre, Department of Dermato-allergology, Gentofte Hospital in the period 2004-2009. Grants from Aage Bangs foundation and the Nickel Producers Environmental Research Association (NiPERA) supported the project.

This project could not have been carried out without the help from a number of people working at the Department of Dermato-allergology, Gentofte Hospital. I am very grateful to all who contributed to the work.

I would like to thank my two supervisors: Professor Jeanne Duus Johansen, for her daily support and invaluable guidance and Professor Torkil Menné, for his enthusiasm and creative support. I am grateful to both Jeanne and Torkil for their ability to provide an optimistic and inspirering working environment. Aage Vølund is gratefully acknowledged for his statistical assistance. Gerald B Kasting is acknowledged for his calculations in study III. Klaus Ejner Andersen is acknowledged for his assistance in study IV. Lone Holm Clausen, Ania Kayser, Hanne Knudsen and Mette Ramm, the Allergy Laboratory, are appreciated for their skilful assistance. I would like to thank Susanne Schweitz for her valuable daily assistance. I would like to thank Susanne Voltelen for her assistance in the research projects. I would like to thank all my colleagues (also the formerly employed) at the National Allergy Research Centre for supporting me with ideas, inspiring discussions, practical help, laughs, coffee-breaks and friendship.

I am very grateful to all the patients who took their time to participate in the studies, without them, the studies would not have been possible.

Last but not least, I want to thank my family and friends for their support and encouragement. I would especially like to thank you, Morten for your endless love and patience and my two boys August and Carl-Emil, for sharing your joy of life with me.

Louise Arup Fischer Neergaard

March 2009

Table of contents

1. Introduction

1.1 Contact allergy and allergic contact dermatitis

It is estimated that around 20% of the general population in the western world is sensitized to chemicals in the environment[1]. Allergic contact dermatitis may occur upon re-exposure and is dependent on individual threshold of response. It is a type IV immunological reaction which entails two phases: a sensitization phase and an elicitation phase. Sensitization is the induction phase, where, for the first time, the immunological system generates the memory of a specific allergen upon exposure. The elicitation phase, is the phase where the allergen specific T-cells are triggered by the renewed exposure, and the overall process results in a contact allergic skin reaction[2]. Typical symptoms are itching and erythema; formation of fissures and vesicles may occur as well as scaling of the skin. The location of the dermatitis may indicate the allergen causing the dermatitis, such as nickel eczema on the earlobe, foot dermatitis from chromium allergy or axillary dermatitis from fragrances, but patch testing is needed to confirm the diagnosis[3]. The location of allergic contact dermatitis is most frequently observed on the hands and face in women and on the hands in men[4]. It is a common disease that often requires treatment, sometimes sick leave and in the worst cases affects the ability to work[5]. Accordingly, it can have consequences not only for the individual, but also for society in e.g. increased national health expenses[6;7]. The most frequent contact allergens today are nickel followed by fragrances [8]. The hands are most often affected in occupational dermatitis[7]. Among women the most frequent occupational allergies are rubber additives and biocides due to wet occupations. Among men the most frequent allergies are chromium, due to leather; rubber, due to rubber gloves; and nickel due to exposure from the metalworking industry and work tools, in addition, epoxy is a common occupational allergen among men[9]. Allergic contact dermatitis is an environmentally driven disease, and as such it is preventable.

1.2 Prevention of allergic contact dermatitis

Allergens occur both in the home environment and at the workplace. The prevention strategy includes primary, secondary and tertiary prevention[10]. In primary prevention the focus is on minimizing the risk of induction of sensitization among workers and consumers. A risk assessment of the sensitization potential for new cosmetic ingredients may be performed before they are introduced on the market[11]; nevertheless, a considerable number of chemicals causes sensitizations and allergic contact dermatitis[12]. The quantitative risk assessment suggested by the cosmetic industry is based on animal studies, and the murine local lymph node assay (LLNA) is the method of choice today[13]. This method evaluates the relative sensitizing potential of different allergens, and is aimed at preventing sensitization, by estimating an "acceptable exposure level" of allergen[14;15]. The test chemical is applied on the ear of mice, and the dose needed to produce a three-fold increase

in the proliferative response in the local lymph node, the EC3 value, is used to classify the chemicals according to the sensitization potential[16;17]. To identify the sensitization hazard, tests on healthy individuals may be used[18], but such testing is controversial for ethical and efficacy reasons, since induction of contact allergy is life-long and failures in identifying potential human sensitizers have ocurred[12;19]. The "non-sensitizing area dose", derived from the EC3 value or human sensitization testing, is used to define the "acceptable non-sensitizing exposure level" by combining the EC3 value with different factors[14;15]. At the work place primary prevention also includes minimizing the contact between allergens and the skin. Nonetheless, some occupations have a high skin exposure to allergens, for instance, because of intense handling of tools/products containing allergens for example, nickel exposure among locksmiths[20] or epoxy-resin among windmill production workers[21]. Furthermore in some occupations such as hairdressers and health care workers, there is a very high frequency of wet work, which introduces an increased risk of developing hand eczema, and an increased exposure to allergens, because of the repeated exposures, which might result in allergic contact dermatitis[22]. Once eczema has developed, facilitation of skin penetration of allergens is expected, resulting in an increased risk of sensitization and elicitation. For this reason, primary prevention should also entail education on how to minimize the risk of eczema at the work place among employees in risk occupations, since this has proven effective[23;24]. In the quantitative risk assessment suggested by the cosmetic industry, extreme conditions, such as those encountered in some working environments, are not considered when defining the "Acceptable exposure level". As for secondary and tertiary prevention, the risk assessment procedure developed by the cosmetic industry is not able to predict the elicitation risk of chemicals; hence the "acceptable exposure level" does not protect those already sensitized. Secondary and tertiary prevention aims at reducing the risk of elicitation and the morbidity among those with eczema. Product labelling is one way of handling this issue[25]. But since a part of the sensitized population might not be diagnosed, and the main part of those who know they are sensitized find it difficult to read the labelling and identify the allergens to which they are allergic[26;27], this way of preventing allergic contact dermatitis may not be efficient.

Allergens have different thresholds below which no elicitation occurs. Experience from regulation of nickel shows that if safe levels of allergen exposure were determined based on elicitation data, allergic contact dermatitis could be prevented in a large part of those who are sensitized. Furthermore the use of such data in prevention would also result in primary prevention, because the number of incident cases of sensitizations would decrease[28-30]. A link between the patch test and the Repeated Open Application Test (ROAT) could be a valuable tool in preventive strategies. So, when product ingredients are already on the market, or allergens are present at the work place, the

evaluation of acceptable allergen exposure could be based on the elicitation potential of the allergens.

2. Experimental methods in allergic contact dermatitis

2.1 Patch test

The patch test was introduced by Josef Jadassohn in 1895, and Poul Bonnevie proposed a standard patch test series in 1938[31]. The patch test of today is primarily used as a diagnostic tool to diagnose different contact allergies. The European standard series consists of 28 patches[32], which are supplemented with additional testing according to specific exposures and occupations. The test is also used experimentally to establish elicitation thresholds in already sensitized individuals. This is done by patch testing a group of allergic persons with a dilution series of the allergen in question. These data can be used in elicitation risk assessment of allergens. The patch test is standardised and easy to perform. It is performed on the upper back, as this site has shown to be more reactive than the extremities[33]. The reading scale is also standardised according to the ICDRG criteria (International Contact Dermatitis Research Group)[34]. A method often used is the Finn chamber method and for diagnostic use the dose metric is percentage. Another method used is the True-test, and here the dose metric used is dose per area ($\mu g/cm^2$). For the experimental elicitation purpose, the reading scale is further developed, to recognize smaller differences in reactivity[35].

2.2 Repeated Open Application Test

The ROAT was standardised by Hannuksela[36]. It is a test which can be used for both diagnostic and experimental purposes. The test is used diagnostically if there is a suspicion of allergy, for example, to a certain cosmetic product, but no obvious relation between the patch test result and the product in question. The test is used experimentally to establish elicitation thresholds for different exposure situations for different allergens. The exposure conditions often vary in the ROAT[37], the originally published method was of a duration of 7 days, but evidence is given that longer exposure is reasonable, since some allergic reactions occur beyond the first week[38-41]. The outcome of the ROAT depends on exposure conditions such as exposure frequency, duration[42-44], location[40;45;46] (e.g. axillae, arm, neck, face), the size of the exposed area[47] and the vehicle[48]. A standard reading scale for the ROAT has been suggested[49]. The disadvantage of the ROAT is that it is time consuming compared with the patch test; however, its advantage over the patch test is that it mimics some real-life exposure situations, making the thresholds found in the ROAT often more relevant to real-life exposure, than the those found in the patch test. A link between these two test methods would therefore make it be possible to better estimate the real-life elicitation risk based on a patch test dilution series.

2.3 Dose-response in contact allergy

Many factors influence the sensitization and elicitation thresholds. Human and animal studies have established that the dose of allergen per unit area of skin - and not the total applied dose - is essential for the sensitization rate. As early as 1942 it was concluded that sensitization of guinea pigs with DNCB depended on the concentration of allergen and not on the size of the exposed area[50]. This has later been verified in both animals[51] and humans[52;53], though below a critical small area, fewer are sensitized[52;54]. In one of the pivotal studies using DNCB, it was shown that if the dose per unit area was kept constant and the area halved or doubled, approximately the same number of individuals were sensitized, whereas if the dose per unit area was increased and the area kept constant, the number of sensitizations was also increased[53]. The explanation of this could be that when the dose per area is kept constant, the same amount of allergen is available for the Langerhans cells, disregarding the size of the exposed area, whereas when the amount of allergen per area of skin is increased, more allergen is available per Langerhans cell, resulting in an increased stimulus of the immune system[55;56]. Even though the percentage is often used as the dose metric for experimental patch test and ROAT studies, when defining elicitation thresholds it is actually the dose per area which is changed in the dilution series, because the area and amount of applied dilution is kept constant, whereas the percentage is increased. For example, if the amount of applied substance was doubled and the percentage and area unchanged, increasing reaction would still be expected because the total amount of allergen had been doubled. Whether the dose per unit area is essential for the elicitation response has not been established, but it seems plausible that this is the case. Increasing the percentage of allergen, keeping the area and amount of applied substance the same, results in increasing elicitation reactions above the threshold[22;35;38;39;42-44;57-60]. In one study of nickel elicitation, it was shown that also the size of the exposed area, and thereby the total dose applied, had an influence on the elicitation response when this dose was close to the threshold[47]. Therefore, for induction the dose per area determines the response. For elicitations the dose per area also determines the response, but at some doses the elicitation reaction may also be influenced by the size of the exposed area. The relationship between the dose needed to sensitize and the dose needed to elicit an allergic reaction has not been established. In humans, it has been shown that when the sensitization dose of DNCB is increased, the elicitation dose is then decreased[61], similar results have been shown in mice[62]. Furthermore only 8% of healthy individuals are sensitized by a dose of 62.5 µg DNCB, whereas all sensitized individuals have an elicitation reaction to 12 µg DNCB or less, indicating that the dose needed to sensitize is higher than the dose needed to elicit an allergic reaction[61]. A review provide information that the relation between sensitizing doses and elicitation doses varies to a great extent from 1.9-7760, and that the sensitizing doses (derived from mice and healthy human beings) are higher than the elicitation

doses (in human beings)[63]. Several other factors influence the response to allergens, for example the individual reactivity. Individuals with multiple allergies are more easily sensitized with DNCB and shows a greater elicitation response compared with normal individuals, or individuals with nickel allergy[64], though no increased elicitation response in polysensitized individuals have also been found[65]. When individuals are sensitized to more than one allergen, they might react to a lower threshold when they are stimulated with both allergens simultaneously, e.g. nickel and cobalt allergy[66] or fragrance allergy[67]. In this thesis, it was decided to use the dose per area as the dose metric, firstly because this seems to be the most appropriate measure, given the aforementioned considerations, and secondly, because it facilitates a comparison between the elicitation threshold and the sensitization threshold in general.

3. Objectives

3.1 Overall objective

The aim of this thesis was to study the elicitation dose-response relationship and thresholds for allergens with different physico-chemical properties in patients with contact allergies to:

- examine if a relationship exists between the patch test and the ROAT results using both the dose per application of allergen and the total applied dose.
- examine if this relationship is independent of physico-chemical characteristics of the allergen.
- suggest a model for conversion of data from patch tests to ROATs.

The perspective of this thesis is to propose a method that can be used as part of preventive strategies.

The experimental studies were performed with three different allergens using identical methodology: a metal (nickel), a preservative (methyldibromo glutaronitrile, MDBGN) and a fragrance ingredient (hydroxyisohexyl 3-cyclohexene carboxaldehyde, HICC).

3.2 Nickel dose-response study (I)

The aim of this study was to investigate the elicitation dose-response of nickel with the patch test and the ROAT and compare the elicitation dose-response for the two test-methods in nickel allergic individuals.

3.3 MDBGN dose-response study (II)

The aim of this study was to investigate the elicitation dose-response of MDBGN with the patch test and the ROAT and compare the elicitation dose-response for the two test-methods in MDBGN allergic individuals.

3.4 HICC dose-response study (III)

The aim of this study was to investigate the elicitation dose-response of HICC with the patch test and the ROAT and compare the elicitation dose-response for the two test-methods in HICC allergic individuals. Further a theoretical estimation of the evaporation-loss was calculated.

3.5 Relation between the patch test and the ROAT (Study IV)

The aim of this study was to introduce a model to convert human patch test data into ROAT data, for non-volatile compounds using the results from Study I and II. Further the aim was to characterize the relationship between the patch test and the ROAT for volatile compounds using the results from Study III and data from a fourth study from the literature.

4. Material and Methods

4.1 Material and methods in general (I-III)

4.1.1 Test subjects

The inclusion criteria were a former positive standard patch test to nickel sulphate, MDBGN or HICC respectively, based on the ICDRG criteria[34] and age > 18. The exclusion criteria were active eczema, the use of systemic immunosuppressive therapy, UV-light on the test area within 3 weeks of the testing, pregnancy, breastfeeding, and not being capable of cooperating. All test subjects received a compensation, for each meeting, approved by the Local Ethical Committee.

4.1.2 Control subjects

The inclusion criteria were a negative patch test to nickel sulphate, MDBGN or HICC, and age > 18. The exclusion criteria were the same as for the test subjects. Subjects were recruited by advertisement and received the compensation, for each meeting as did the test subjects.

4.1.3 Study design

Test subjects were eczema patients with contact allergy to one of the chosen allergens. A control group was included for the ROAT. A serial dilution patch test and a ROAT were performed simultaneously on the same allergic individuals. The dose per application in the ROAT was present in the patch test. Furthermore the accumulated one-week dose, the accumulated two-week dose and the accumulated three-week dose from the ROAT were also present in the patch test (Table 1), except for nickel, where the accumulated 2-week dose was not present in the patch test. The doses were similar in all three studies and were based on previously published dose-response studies. The placement of the different patch test dilutions (Figure 1) and ROAT dilutions (Figure 2) was randomised and blinded for the investigator and the subjects. The randomisation code was broken after termination of each study. The study was performed according to the Helsinki II declaration and was approved by the Local Ethics Committee (The capital region of Denmark, KA04032).

Table 1
Study design for the dose-response studies with nickel, MDBGN and HICC. The dose per application and accumulated dose after 1 and 3 weeks in the ROAT. The doses in italic were also present in the patch test.

Area in the ROAT (the numbers were randomised for each test-subject)	Dose per application ($\mu g/cm^2$)	Number of applications after 1 week	Total accumulated dose after 1 week ($\mu g/cm^2$)	Number of applications after 3 weeks	Total accumulated dose after 3 week ($\mu g/cm^2$)
1	0(vehicle)	14	0	42	0
2	*0.0357*[a]	14	*0.5*	42	*1.5*
3	*0.357*[a]	14	*5*	42	*15*
4	*3.57*[a]	14	*50*	42	*150*
5	35.7[b]	14	500	42	*1500*[b]

[a] In the nickel study this dose was 0.035, 0.35 and 3.5 respectively
[b] These doses were present only in the HICC study

4.1.4 Patch test

A patch test dilution series with 19 dilutions and one vehicle was used for each study. 15µl was applied on a filter paper in a small Finn Chamber of 0.5 cm^2 (Smart Practice Finland OY, Finland) on Scanpore® (Actavis Norway AS, Norgesplaster). The patch test was occluded for 2 days and the reactions were read on Day 2, 3 or 4 and Day 7. The reading Day 3 or 4 was used for the statistical calculations. The reading scale for the patch test was based on the ICDRG reading scale, further developed by Hindsen and Bruze [35] , and modified so that weaker reactions were registered too. The scale was as follows: 0 = no reaction; 1= few papules with no erythema, no infiltration; 2 = faint erythema with no infiltration or papules; 3= faint erythema with few papules and no homogenous infiltration; 4=erythema, homogenous infiltration; 5= erythema, infiltration and a few papules; 6=erythema, infiltration and papules; 7=erythema, infiltration, papules and a few vesicles; 8=intensive erythema, infiltration and vesicles. The threshold concentration was defined as the weakest concentration giving a visible reaction (minimum score=1) on Day 3 or 4 in a continuous line of patch test reactions starting from the highest test concentration. The same three nurses applied and randomised the patch tests. The principle investigator (LAF) performed all the readings.

4.1.5 ROAT

Each test-subject was tested on 4 (Study I and II) or 4-5 (Study III) areas on the volar aspect of the forearms on areas of 9 cm^2. 20µl was applied twice daily using a fixed volume micropipette (Acura 815, 20 µl, Buch&Holm, Herlev, Denmark) and numbered bottles. Each number referred to a numbered area. The solutions were spread over the area with the tip of the pipette and left to dry by evaporation. To ensure test subjects understood how to use the pipette correctly, they received thoroughly manual instructions along with written instructions and were also given a telephone number on which they could contact study personnel 24 hours a day. They were instructed to apply the solution in the morning and evening and were requested not to use moisturizer, soap or perfume on or near the exposed areas. The reactions were read on all subjects routinely once a week and on the days of the patch test readings; readings were also performed if a reaction occurred between these days. With a score of 5 or above (based on the involved area, erythema, number of papules and number of vesicles), the exposure to that area was terminated, and if no reaction occurred, or with a score below 5, the exposure was terminated on Day 21. The same investigator (LAF) performed the readings by using a ROAT reading scale that has been developed [49]. The threshold concentration was defined as the weakest concentration which gave a visible reaction that remained at the end of week 3 if the exposure had not been terminated, or the weakest concentration giving a reaction with a score of 5 or above.

Figure 1
Example of the patch test from the nickel study. The placement of the different concentrations is randomised.

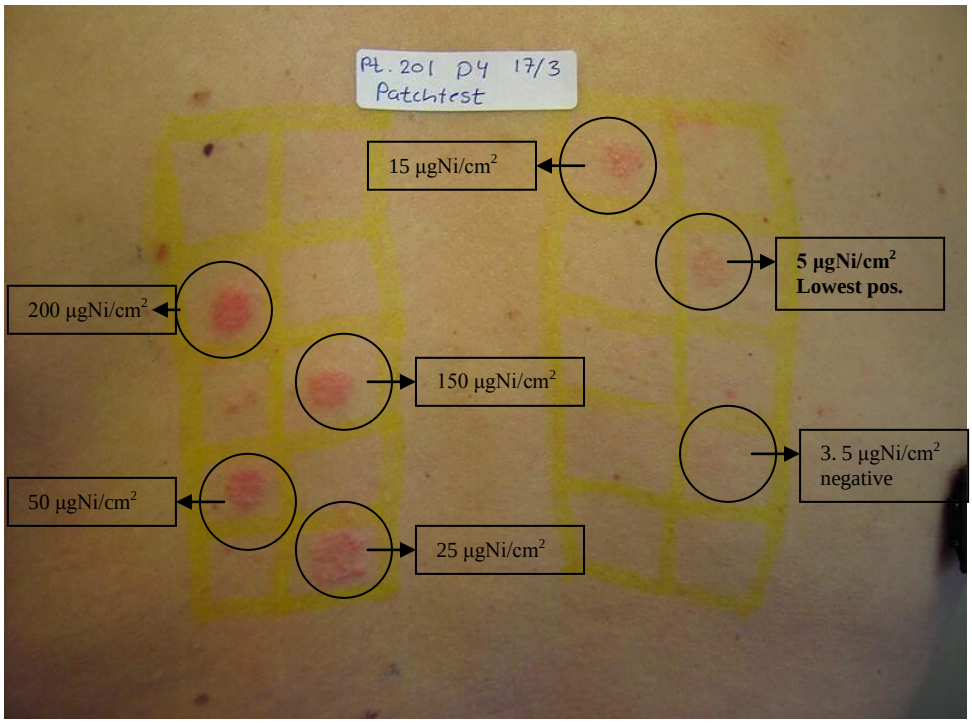
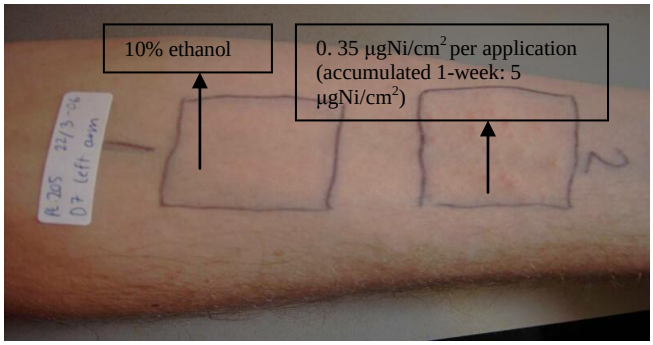
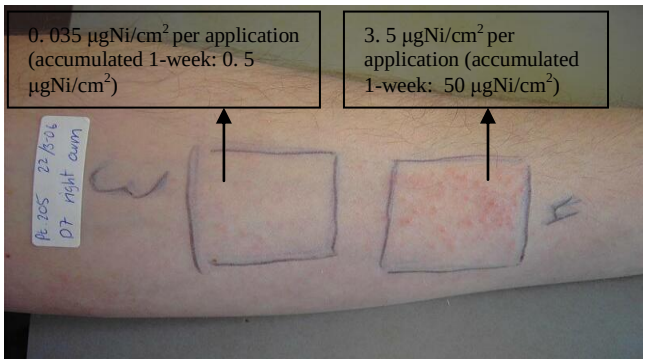


Figure 2

Example of the ROAT from the nickel study.





4.1.6 Statistics

A standard logistic regression analysis was used in each study to estimate the dose-response relation in the patch test (the logistic dose-response model has been used earlier to describe the dose-response relation in contact allergy[59;68;69]). The elicitation doses, in the patch test, that will elicit a reaction in 50%, 25%, 10%, 5% and 1% of allergic individuals respectively, the $ED_{xx,}$ was calculated and fitted dose-response curves were drawn. The 95% confidence interval for ED_{xx} was calculated by using Fieller´s method. McNemars test for paired binary observations was used to compare the elicitation response to the dose per application in the ROAT (μg allergen/cm^2/application) and to the corresponding dose in the patch test (μg allergen/cm^2). A fitted dose response curve for the patch test and the observed dose-response curve for the ROAT, expressed in dose per application, was calculated and drawn. In addition the elicitation reactions to the accumulated week-doses in the ROAT (accumulated μg allergen/cm^2) were compared with the elicitation reactions to the corresponding doses in the patch test (μg allergen/cm^2) also using McNemars test. A fitted dose response curve for the patch test and the observed ROAT responses were drawn. When the patch test and the ROAT dose-response reactions were compared, only the results from the subjects who participated in both tests were used.

4.2 Nickel dose-response study (I)

4.2.1 Test subjects (I)

20 patients from the Department of Dermato-allergology (former: Department of Dermatology), Gentofte hospital, Denmark with a former positive standard patch test to nickel sulphate, tested within the past 6 years, were enrolled in the study after oral and written consent. Subjects were aged 19-67 years, mean age: 44.6 years. (Women: 18, men: 2).

4.2.2 Control subjects (I)

18 control persons with no nickel allergy were enrolled in the study after oral and written consent. Subjects were aged 18-30 years, mean age: 23.3 years. (Women: 8, men: 10).

4.2.3 Study design and materials (I)

The Copenhagen County Hospital Pharmacy, Denmark manufactured the test solutions ($NiSO_46H_2O$ in 10% ethanol with 90% water and the control: 10% ethanol with 90% water).

4.2.4 Patch test (I)

Dilution series of $NiSO_4 6H_2O$ in 10% ethanol with 90% water (µg/ Ni cm^2): 200, 150, 50, 25, 15, 5, 3.5, 1.5, 0.78, 0.5, 0.35, 0.2, 0.1, 0.05, 0.035, 0.0125, 0.0063, 0.0031, 0.00156 and one blank containing 10% ethanol with 90% water.

4.2.5 ROAT (I)

The skin areas were tested with a dose per application of 3.5 µg Ni/cm^2, 0.35 µg Ni/cm^2 or 0.035 µg Ni/cm^2. These doses per application correspond to an accumulated one-week dose of 50, 5 and 0.5 µg Ni/cm^2, respectively and an accumulated three-week dose of 150, 15 and 1.5 µg Ni/cm^2, respectively. The fourth area was exposed to 10% ethanol with 90% water. The control subjects were tested on two areas, one with 0.035 µg Ni/cm^2 and the other with 10% ethanol.

4.3 MDBGN dose-response study (II)

4.3.1 Test subjects (II)

18 patients from the Department of Dermato-allergology, Gentofte hospital, Denmark with a former positive standard patch test to MDBGN, tested within the last 6 years, were enrolled in the study after oral and written consent. Subjects were aged 24-63 years, mean age: 44 years. (Women: 15, men: 3).

4.3.2 Control subjects (II)

17 control persons with no MDBGN allergy were enrolled in the study after oral and written consent. Subjects were aged 19-69 years, mean age: 27 years. (Women: 11, men: 6).

4.3.3 Study design and materials (II)

The MDBGN was purchased from Bie&Berntsen, Rødovre, Denmark. The laboratory personnel at the laboratory at the Department of Dermato-allergology, Gentofte hospital, Denmark manufactured the test solutions (MDBGN in 20% ethanol with 80% water and the control: 20% ethanol with 80% water).

4.3.4 Patch test (II)

Dilution series of MDBGN in 20% ethanol with 80% water (µg MDBGN/cm^2): 100, 50, 25, 15, 10, 5, 3.57, 1.5, 1, 0.5, 0.357, 0.1786, 0.0893, 0.0357, 0.0179, 0.0089, 0.0045, 0.0022 and one blank containing 20% ethanol with 80% water. (Patients 1-5 were also tested with 150 µg MDBGN/cm^2).

4.3.5 ROAT (II)

The areas were tested with a dose per application of 3.57 μg MDBGN/cm^2, 0.357 μg MDBGN/cm^2 or 0.0357 μg MDBGN/cm^2. These doses per application correspond to an accumulated one-week dose of 50, 5 and 0.5 μg MDBGN/cm^2, an accumulated two-week dose of 100, 10 and 1 μg MDBGN/cm^2 and an accumulated three-week dose of 150, 15 and 1.5 μg MDBGN/cm^2, respectively. The fourth area was exposed to 20% ethanol with 80% water. The control subjects were tested on two areas, one with 0.0357 μg MDBGN/cm^2 and the other with 20% ethanol.

4.4 HICC dose-response study (III)

4.4.1 Test subjects (III)

17 patients from the Department of Dermato-allergology, Gentofte hospital, Denmark or from the skin clinic, Rødovre with a former positive standard patch test to HICC, tested within the last 6 years, were enrolled in the study after oral and written consent. Subjects were aged 22-64 years, mean age: 47.8 years. (women: 14, men: 3).

4.4.2 Control subjects (III)

17 control persons with no HICC allergy were enrolled in the study after oral and written consent; 2 withdrew before the start-up of the ROAT, leaving 15 who fulfilled the ROAT. Subjects were aged 23-66 years, mean age: 37.1 years. (Women: 8, men: 7).

4.4.3 Study design and materials (III)

The HICC was kindly donated by the Research Institute for Fragrance Materials (RIFM), New Jersey, USA and was produced by International Flavors and Fragrances Inc (IFF), Lot Number SM/8059062. The primary investigator (LAF) manufactured the test solutions (HICC in 70% ethanol with 30% water and the control: 70% ethanol with 30% water) at the laboratory at the Department of Dermato-allergology, Gentofte hospital, Denmark.

4.4.4 Patch test (III)

Dilution series of HICC in 70% ethanol with 30% water (μg HICC/cm^2): 1500, 300, 150, 100, 50, 25, 15, 10, 5, 3.57, 1.5, 1, 0.5, 0.357, 0.1786, 0.0893, 0.0357, 0.0179, 0.0022 and one blank containing 70% ethanol with 30% water.

4.4.5 ROAT (III)

The areas were tested with a dose per application of 35.7 µg HICC/cm^2, 3.57 µg HICC/cm^2, 0.357 µg HICC/cm^2 or 0.0357 µg HICC/cm^2. The highest dose (35.7 µg HICC/cm^2) was added to the study 3 weeks after the beginning of inclusion, because only a few test subjects reacted to 3.57 µg HICC/cm^2. For this reason those who already had a reaction to 3.57 µg HICC/cm^2 at the time that the highest dose was added to the study, (subjects: 501, 505, 506, 507, 510 and 511) were not tested with this dose. The ROAT doses per application corresponds to an accumulated one-week dose of 500, 50, 5 and 0.5 µg MDBGN/cm^2, an accumulated two-week dose of 1000, 100, 10 and 1 µg MDBGN/ cm^2 and an accumulated three-week dose of 1500, 150, 15 and 1.5 µg MDBGN/ cm^2, respectively. The fourth/ fifth area was exposed to 70% ethanol with 30% water. The control subjects were tested on two areas, one with 0.0357 µg HICC/ cm^2 and the other with 70% ethanol. The test subjects received 4-5 numbered containers every week containing numbered bottles with test solution. The containers were stored in refrigerators to minimize evaporation.

4.4.6 Statistics (III)

As 35.7 µg HICC/cm^2 was not present in the patch test, 50 µg HICC/ cm^2 was used for statistical comparison. Neither was 500 or 1000 µg HICC/cm^2 present in the patch test (corresponding to the accumulated 1- and 2-week doses for the ROAT); accordingly the nearest doses were used. The dose difference did not have any practical consequences because 100% of subjects had a positive patch test reaction to 150 µg HICC/cm^2 and would therefore also have had a positive reaction to 500 and 1000 µg HICC/cm^2. The subjects who already had a ROAT reaction, when the highest dose was included, and therefore did not test with this dose were counted as positive to the highest dose, because it is expected that they would have had reacted to this dose.

4.4.7 Evaporation (III)

In the ROAT the evaporation of HICC from un-occluded skin was estimated by the calculation-method developed by Kasting et al. [70]. Calculations were performed for the doses that were used in the ROAT and an indoor environment was assumed; furthermore no rub-off, wash-off or chemical degradation during the exposure time; and a skin temperature of 32°C was assumed. From the boiling point of 120-122 °C at 133 Pa (1 torr)[71] a vapour pressure of HICC of 5.045×10^{-3} mm Hg (torr) was estimated. The method used was Method 1, Eq. 14-25, from Handbook of Chemical Estimation Methods[72]. The CLOGP program Vers. 2.0 (BioByte, Inc., Claremont, CA, USA, 1999) was used to obtain a calculated log octanol/water partition coefficient (CLOGP) of 2.15. Water solubility was estimated to be 4.71 g/L [73]. A density of 0.994 g/cm^3 was obtained[71].

4.5 Relation between the patch test and the ROAT (IV)

4.5.1 Materials and methods (IV)

The equation was built on Study I and II. The investigation of the dose-response relation for the volatile fragrance constituents used the results from Study III and data from the testing of isoeugenol, which have been published previously [42].

4.5.2 Development of the model for non-volatile compounds (IV)

To develop a model to convert patch test data (response to x µg allergen/cm^2) in to ROAT data (response to x µg allergen/cm^2/application) the three following conditions (which had to be fulfilled in order to develop an equation) were investigated in the relation between the patch test and the ROAT: 1) the correlation between the two test methods by using Spearman's rank correlation, 2) the parallelism of the dose-response curves by performing a logistic dose-response analysis[74;75] and 3) the factor (F) describing the parallel displacement between the two dose-response curves for nickel and MDBGN on the log-dose axis. Then a common factor, F, for both allergens was determined.

Based on the following equation:

$$ED_{xx}(ROAT) = F \cdot ED_{xx}(patch\ test) \qquad (equation\ 1)$$

the model to convert the ED_{xx} for the patch test to the corresponding ED_{xx} for the ROAT was developed. The ED_{xx} describes the dose that will elicit a reaction in xx% of allergic individuals.

4.5.3 The patch test and ROAT relation for volatile compounds (IV)

Study III and the study with isoeugenol, identified in the literature were used to investigate the dose-response relation for volatile compounds. The study with isoeugenol was identified using Pubmed-medline and the following search terms: ROAT, use-test, repeated open application test, dose-response, and contact allergy. The following criteria were used to identify a study similar to Study I-III: 1) a series dilution patch test and a ROAT performed on the same allergic individuals, 2) for both test methods the concentration should be given in ug/cm^2, or it should be possible to calculate the ug/cm^2, for the ROAT it should be possible to calculate the ug/cm^2/application, 3) the duration of the ROAT should be at least three weeks, 4) at least five patch test concentrations and at least two ROAT concentrations had to be used, 5) the threshold concentration in the patch test should be defined as the weakest concentration giving a visible reaction in a continuous line of reactions starting from the highest concentration, and 6) the allergen tested had to be a volatile compound. For both studies logistic dose-response curves were determined; for both test methods,

and the patch test results were used to calculate a predicted ROAT dose-response curve, based on the developed equation for non-volatile compounds.

5. Results

5.1 Nickel dose-response study (I)

5.1.1 Dose-response findings

In the patch test 19 test subjects had at least one reaction to nickel, none had a reaction to the vehicle. One subject was all negative at patch testing, but reacted to the highest ROAT concentration. The lowest dose observed to give a reaction in the patch test according to the defined criteria was 0.5 μg Ni/cm^2. The dose which was calculated to elicit a patch reaction in 10%, the ED_{10}, was 0.78 μg Ni/cm^2. The calculated ED-values are given in Table 2.

Table 2
(Study I, nickel) Predicted doses (ED_{xx}), and the 95% confidence interval CI that will elicit a reaction in 50, 25, 10, 5 and 1% of allergic individuals respectively from the patch test (μg Ni/cm^2)

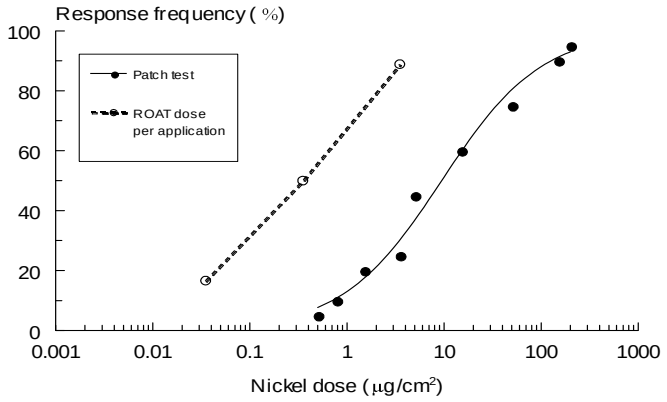
ED_{xx}	μg Ni/cm^2	95% CI
50	10.0	3.8-27
25	2.8	0.77-7
10	0.78	0.13-2.2
5	0.33	0.034-1.1
1	0.048	0.0018-0.24

In the ROAT, none had a reaction to the vehicle control site, and none of the control subjects had any reactions. When the dose per application in the ROAT was compared with the corresponding doses in the patch test, it was found that there was a significant higher response to the ROAT (after three weeks of exposure) at the two highest doses compared with the same dose in the patch test. The response rate to both test methods and the result of McNemars test are shown in Table 3 and the corresponding logistic dose-response curve for the patch test and the observed dose-response curve for the ROAT are shown in Figure 3.

Table 3
(Study I, nickel) The response frequency in the patch test compared with the response status in the ROAT (the dose per application) after 3 weeks.

Dose (μgNi/cm^2)	Patch test; response frequency in %	ROAT; response frequency in %	p-value (Mc Nemars test)
3.5	22.2	88.9	<0.001
0.35	0	50	0.004
0.035	0	16.7	0.250

Figure 3
(Study I, nickel) The fitted dose response curve for the patch test (N=18) (solid line) and the observed response after 3 weeks of exposure to the dose per application in the ROAT (broken line).

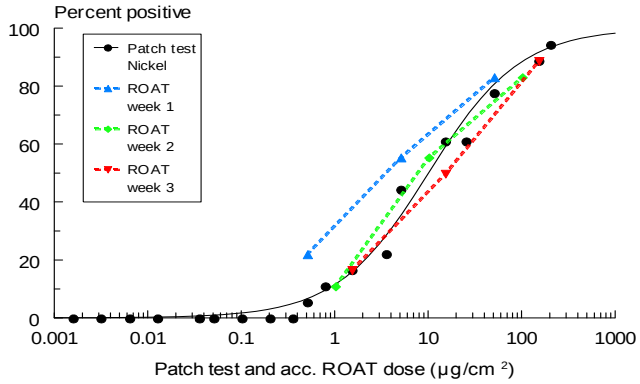


When the dose applied daily in the ROAT was totalled to the accumulated dose after 1 week and 3 weeks, and the response after 1 and 3 weeks was compared with the corresponding dose in the patch test, no statistically significant differences between the ROAT response and the patch test response were found. The responses and the result of McNemars test are shown in Table 4 and the resulting dose-response curves are shown in Figure 4. The dose-response curve for each ROAT week-dose is remarkable similar to the patch test dose-response curves.

Table 4
(Study I, nickel) The response frequency in the patch test compared with the response status in the ROAT (the accumulated dose after 1 and 3 weeks respectively).

Dose (µgNi/cm^2)	Patch test; response frequency in %	ROAT; response frequency in %	p-value (Mc Nemars test)
0.5	5.6	22.2	0.250
1.5	16.7	16.7	1
5	44.4	55.6	0.727
15	61.1	50	0.727
50	77.8	83.9	1
150	88.9	88.9	1

Figure 4
(Study I, nickel) The fitted dose response curve for the patch test (N=18) and the accumulated 1-week ROAT dose (blue), the accumulated 2-week ROAT dose (green) and the accumulated 3-week ROAT dose (red).



5.1.2 Drop outs and unexpected findings (II)

One test subject was excluded from the ROAT before the start-up because of eczema on the forearms, and another was excluded because two of the area-numbers was changed between two visits, and it was concluded that the subject was not capable of performing the ROAT correctly. In the ROAT 10/18=55.6% had a reaction within the first week of exposure to 0.35 µg Ni/cm^2, but in one subject the reaction (5 papules) disappeared after 3 weeks of exposure; accordingly, after three weeks 9/18=50% had a reaction to this concentration. 4/18=22.2% had a reaction to 0.035 µg Ni/cm^2 within the first week, but again the reaction (1 papule) disappeared in one subject the second week of exposure; accordingly, after the three weeks 3/18=16.7% had had a reaction. This observation was unexpected. It is possible that the two subjects developed tolerance; alternatively they may have had a positive ROAT if the exposure had been continued.

5.2 MDBGN dose-response study (II)

5.2.1 Dose-response study

All 18 test subjects had a reaction to at least one of the patch test concentrations. The two highest patch test concentrations gave the highest possible score in all tested subjects. The highest dose was therefore omitted from the patch test the second week of testing to minimize the discomfort in the patch testing. The lowest MDBGN concentration which gave a reaction according to the defined

criteria was 0.0357 μg MDBGN/cm^2. The ED_{10} was calculated to be 0.5 μg MDBGN/cm^2. All calculated ED-values are given in Table 5.

Table 5
(Study II, MDBGN) Predicted doses (ED_{xx}), and the 95% confidence interval CI that will elicit a reaction in 50, 25, 10, 5 and 1% of allergic individuals respectively from the patch test (μg MDBGN/cm^2).

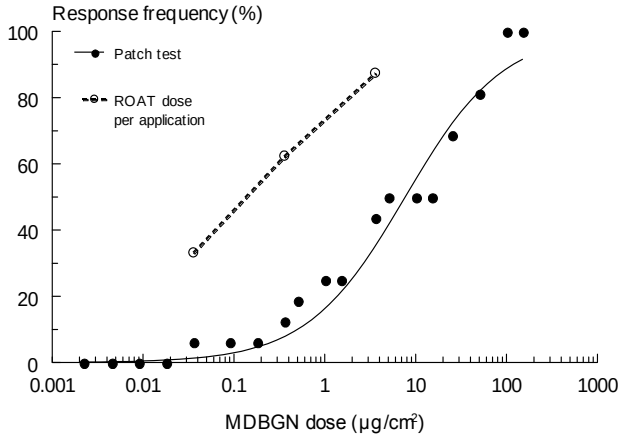
ED_{xx}	μg MDBGN/ cm^2	95% CI
50	7.68	2.44-22.1
25	1.96	5.52-0.4
10	0.5	1.69-0.052
5	0.2	0.8-0.0125
1	0.025	0.164-0.00049

When the response frequency to the dose per application in the ROAT was compared with the same dose in the patch test, there was a significantly higher response to the ROAT at the two highest ROAT doses compared with those of the patch test. The response frequencies and the result of McNemars test are shown in Table 6, and the dose-response curves are shown in Figure 5.

Table 6
(Study II, MDBGN) The response frequency in the patch test compared with the response status in the ROAT (the dose per application) after 3 weeks.

Dose (μgMDBGN/ cm^2)	Patch test; response frequency in %	ROAT; response frequency in %	p-value (Mc Nemars test)
3.57	43.8	87.5	0.016
0.357	12.5	62.5	0.0078
0.0357	6.7	33.3	0.13

Figure 5
(Study II, MDBGN) The fitted dose-response curve for the patch test (N=16) (solid line) and the observed response after 3 weeks of exposure to the dose per application in the ROAT (broken line).

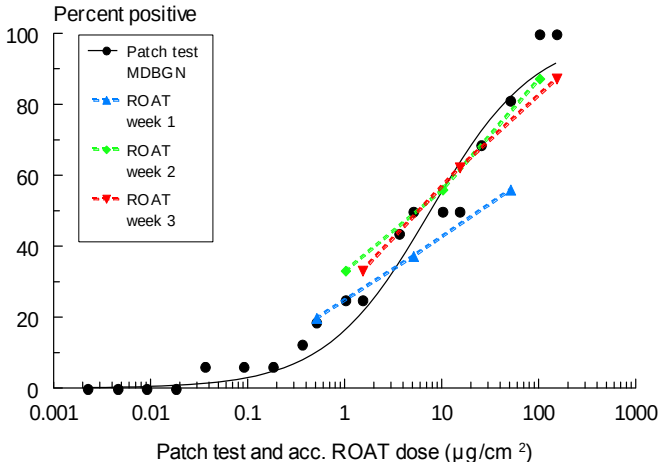


When the accumulated week doses in the ROAT were compared with the corresponding doses in the patch test there were no significant differences on the response frequencies (Table 7). In addition, the dose-response curves for the two test methods were indeed very similar (Figure 6).

Table 7
(Study II, MDBGN) The response frequency in the patch test compared with the response status in the ROAT (the accumulated dose after 1, 2 and 3 weeks respectively).

Dose (µgMDBGN/ cm^2)	Patch test; response frequency in %	ROAT; response frequency in %	p-value (Mc Nemars test)
0.5	20	20	0.5
1	26.6	33.3	0.5
1.5	26.6	33.3	0.5
5	50	37.5	0.5
10	50	50	0.5
15	50	62.5	0.5
50	81.3	56.3	0.13
100	100	87.5	0.5
150	100	87.5	0.5

Figure 6
(Study II, MDBGN) The fitted dose response curve for the patch test (N=16) and the accumulated 1-week ROAT dose (blue), the accumulated 2-week ROAT dose (green) and the accumulated 3-week ROAT dose (red).



5.2.2 Drop outs and unexpected findings (II)

One test subject had a weak reaction in the patch test to the vehicle site (score =1= few papules with no erythema, no infiltration). One test subject experienced intense itching on the back; therefore the patches were removed after 24 hours. The lowest concentration giving a patch test reaction in this person was 3.57 µg MDBGN/cm^2. This person had a severe allergic reaction, at the ROAT test site, to the highest concentration after 5 days (score=19) and chose to cease participation in the ROAT after 1 week. Two test subjects were excluded from the ROAT in the first week of exposure because of relapse of eczema. They did not develop any signs of reaction to the ROAT sites. One test subjects had a reaction in the ROAT to the vehicle area (score=2) with 4 papules on Day 14 and 2 papules on Day 21. All ROAT reactions to MDBGN had scores of 5 or above (which is proposed as a positive score by [49]). One control subject had erythema (score=3) at the ROAT site exposed to MDBGN on Day 16, and the exposure was terminated. This person had erythema on both arms and the chest; all erythema disappeared within 24 hours of exposure termination. The subject was not patch tested again with MDBGN, due to practical reasons. From the morphology of the reaction, it was concluded that it was not related to the exposure.

5.3 HICC dose-response study (III)

5.3.1 dose-response results

In the patch test all test subjects had a reaction to the highest HICC concentration. The lowest concentration giving a visible reaction, according to the defined criteria, was the lowest tested dose (score =1). The data from this person were not used for the comparison between the patch test and

the ROAT because the person was excluded from the ROAT, due to a developing flare-up. The calculated ED_{10} for the patch test was 0.662 μg HICC/cm^2. All ED values are shown in Table 8.

Table 8
(Study III, HICC) Predicted doses (ED_{xx}), and the 95% confidence interval CIthat will elicit a reaction in 50, 25, 10, 5 and 1% of allergic individuals respectively from the patch test (μg HICC/ cm^2).

ED_{xx}	μg HICC/ cm^2	95% CI
50	11.1	3.41-33.1
25	2.71	0.478-7.79
10	0.662	0.052-2.35
5	0.254	0.011-1.1
1	0.031	0.0003-0.225

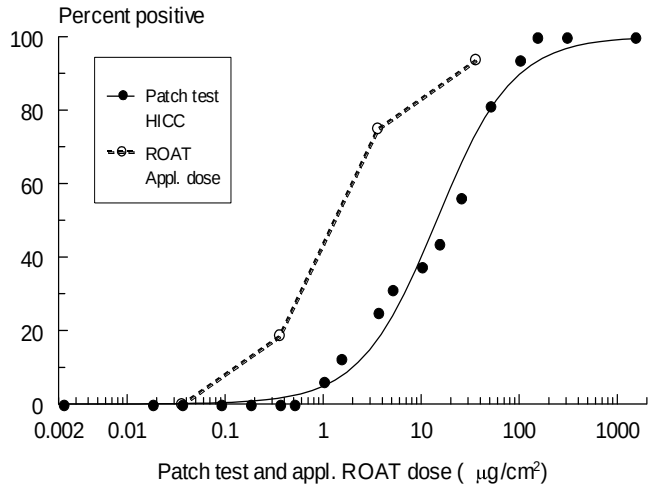
None of the control subjects had any reactions in the ROAT. When the response to the dose per application in the ROAT was compared with the response to the same dose in the patch test, there was a significantly higher response in the ROAT to 3.57 μg HICC/cm^2 than the response to this dose in the patch test. There was a higher response to all the doses in the ROAT (except for the lowest dose, which gave no response) compared to the patch test, but the difference was not significant (Table 9). When viewing the dose-response curves, it is seen that the dose-response to the ROAT (in dose per application) is displaced to the left compared with the patch test (Figure 7), as was also seen in Study I and II.

Table 9
(Study III, HICC) The response frequency in the patch test compared with the response status in the ROAT (the dose per application)after 3 weeks.

Dose (μg HICC/cm^2)	Patch test; response frequency in %	ROAT; response frequency in %	p-value (Mc Nemars test)
35.7*	81.3	93.8	0.32
3.57	25	75	0.0047
0.357	0	18.8	0.083
0.0357	0	0	n.d.

n.d. = not defined
* 50 μg HICC/cm^2 in the patch test

Figure 7
(Study III, HICC) The fitted dose response curve for the patch test (N=16) (solid line) and the observed response after 3 weeks of exposure to the dose per application in the ROAT (broken line).



When the response to the accumulated week-doses in the ROAT was compared with the corresponding doses in the patch test there was a significantly higher response in the patch test (at half of the doses), compared with the ROAT (Table 10). This is illustrated by Figure 8, where it is seen that the dose-response curves for the accumulated ROAT doses are displaced to the right compared with the patch test dose-response curve. The response to the accumulated ROAT dose is therefore not similar to the patch test response, as it was in Study I, and II (Figure 4 and Figure 6).

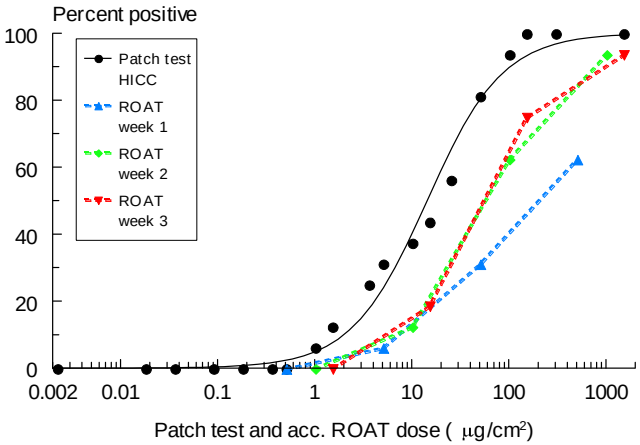
Table 10
(Study III, HICC) The response frequency in the patch test compared with the response status in the ROAT (the accumulated dose after 1, 2 and 3 weeks respectively).

Dose (µg HICC/cm^2)	Patch test; response frequency in %	ROAT; response frequency in %	p-value (Mc Nemars test)
0.5	0	0	n.d.*
1	6.3	0	0.32
1.5	12.5	0	0.16
5	31.3	6.3	0.046
10	37.5	12.5	0.046
15	43.8	18.8	0.046
50	81.3	31.3	0.0047
100	93.8	62.5	0.059
150	100	75	0.046
500	100**	62.5	0.014
1000	100**	93.8	0.32
1500	100	93.8	0.32

** n.d.=not defined

*: not tested, assumed 100%

Figure 8
(Study III, HICC) The fitted dose-response curve for the patch test (N=16) and the accumulated 1-week ROAT dose (blue), the accumulated 2-week ROAT dose (green) and the accumulated 3-week ROAT dose (red).



5.3.2 Evaporation

The calculations indicated that 72-75% of HICC applied to unoccluded skin every 12 hours would evaporate and 25-28% would be absorbed over the range of doses chosen in the ROAT.

5.3.3 Drop outs and unexpected findings (II)

One person developed a reaction to the lowest dose in the patch test. This person was excluded from the ROAT because of a developing flare-up (itching, no visible reaction) in the axillae, the original site of the HICC eczema. The concentration giving a reaction in this person corresponding to a 1+ reaction (according to the ICDRG criteria[34]) was 50 µg HICC/cm^2. This person had no reaction to the vehicle patch. Two test subjects had a reaction (score=1=few papules, no erythema, no infiltration) to the vehicle in the patch test.

5.4 Results in general (Study I-III)

It was a consistent finding for all three allergens that more patients reacted to the allergen, measured as dose per application, when applied repeatedly (in the ROAT) than with the single, occluded exposure in the patch test. This means that a person can be reactive at a repeated open application despite of a negative finding in the patch test to that dose. Furthermore, in Study I and II the response to the total applied dose in the ROAT was similar to the response in the patch test, which could indicate that the elicitation response is dependent on the total applied dose. In addition in Study III the response to the total applied dose in the ROAT was lower than the response in the

patch test, and this could be explained by evaporation of the allergen in the open test resulting in a lower total accumulated dose over time in the ROAT.

5.5 Relation between the patch test and the ROAT (IV)

Based on the analysis of the results from Study I, II and III it was concluded that there was a good correlation between the patch test and the ROAT (Table 11). There was also an acceptable parallelism between the two test methods (expressed by the value β, where no statistically difference was found between the patch test and the ROAT value and the visual impression of the dose-response curves), and the relation between the patch test and the ROAT was similar for nickel and MDBGN (factor F, Table 12).

Table 11
Spearman's rank correlation between the threshold concentrations in the patch test and ROAT

Allergen	Number of patients	Correlation coefficient	P-value
Nickel	18	0.45	0.033
MDBGN	15	0.76	0.0021
HICC	16	0.59	0.011

Table 12
Analysis of parallelism and calculation of the separate and combined factor F (bold), Study I and II

Allergen	Test	Separate analyses		Analyses of parallel dose-response curves				
		α	β	α	β	Δ	F=exp(-α/β)	**F combined**
Nickel	patch	-1.898	0.845	-1.852	0.824	2.811	0.0330	**0.0296**
	ROAT	0.925	0.793					
MDBGN	patch	-1.364	0.778	-1.183	0.684	2.483	0.0265	
	ROAT	1.063	0.542					

Based on the factor F for nickel and MDBGN, a combined factor F was calculated by taking the geometric mean value. (Table 12). The following equation was developed to use the ED values from the patch test to calculate the expected ED-values from the ROAT:

$$\mathbf{ED_{xx}(ROAT) = 0.0296 \cdot ED_{xx}(patch\ test)} \qquad \text{(equation 2)}$$

To control how well the combined factor predicts the ROAT outcome from Study I and II, ROAT dose-response curves were calculated using equation 2 (Figure 9 and 10). It was illustrated that for these two allergens the combined factor can be used with a reliable result.

Figure 9
Comparison of predicted and observed dose-response relations for ROAT of nickel. The predicted ROAT dose-response curve (red line) is obtained from the fitted patch test curve (solid black line) by multiplication of the patch test doses by a factor 0.0296, i.e. $ED_{XX}(ROAT) = 0.0296 \cdot ED_{XX}(patch\ test)$. The observed ROAT dose-response curve is shown as a broken black line.

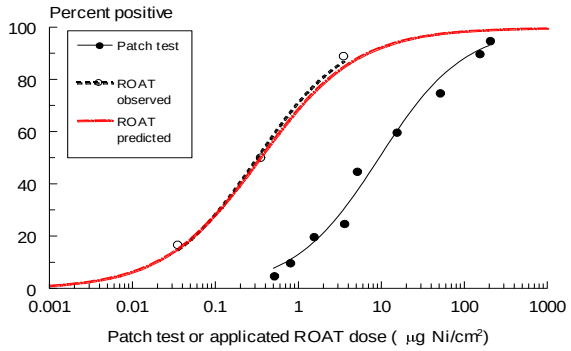
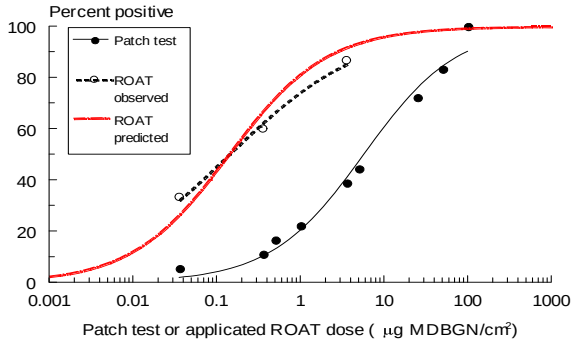


Figure 10
Comparison of predicted and observed dose-response relations for ROAT of MDBGN. The predicted ROAT dose-response curve (red line) is obtained from the fitted patch test curve (solid black line) by multiplication of the patch test doses by a factor 0.0296, i.e. $ED_{XX}(ROAT) = 0.0296 \cdot ED_{XX}(patch\ test)$. The observed ROAT dose-response curve is shown as a broken black line.



When the patch test and ROAT relation was analysed for the volatile fragrance constituents, it was found that the factor F describing the relation was very similar for HICC (F=0.0971) and isoeugenol (F=0.1157). As the study with isoeugenol was not performed with the scope of defining the relationship between the patch test and the ROAT, and only two test doses were applied in the ROAT, an equation for conversion between these two test methods is not suggested for volatile compounds. Nevertheless, based on the results presented here an equation for volatile compounds could be: $ED_{xx}(ROAT) = 0.1060 \cdot ED_{xx}(patch\ test)$, but further studies with volatile compounds are needed.

Using the patch test results from the volatile compounds the ROAT dose-response curve was estimated based on equation 2 (Figure 11 and 12). Equation 2 overestimates the response to the ROAT by a factor 3-4. This could be due to a lower total accumulated dose over time in the open test, when volatile compounds are used, because of evaporation, resulting in a lower response to the ROAT than expected, had the allergens not evaporated.

Figure 11
Comparison of predicted (red line) and observed (broken black line) dose-response relations for ROAT of HICC. The predicted dose-response curve is obtained from the fitted patch test curve(solid black line) by multiplication of the patch test doses by a factor 0.0296, i.e. $ED_{XX}(ROAT) = 0.0296 \cdot ED_{XX}(patch\ test)$.

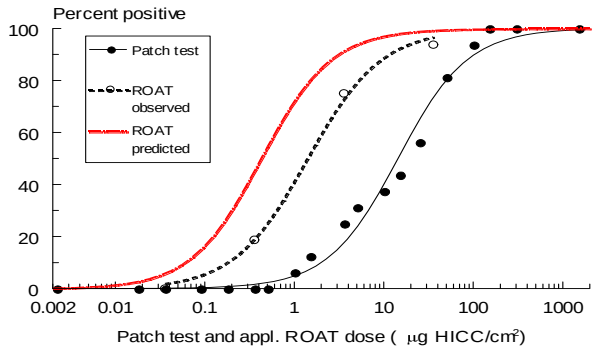
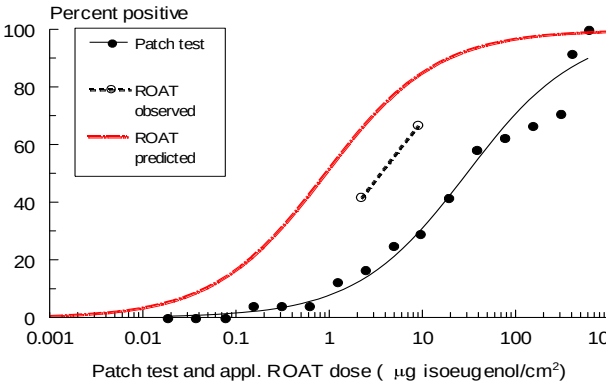


Figure 12
Comparison of predicted (red line) and observed (broken black line) dose-response relations for ROAT of isoeugenol. The predicted dose-response curve is obtained from the fitted patch test curve (solid black line) by multiplication of the patch test doses by a factor 0.0296, i.e. $ED_{XX}(ROAT) = 0.0296 \cdot ED_{XX}(patch\ test)$.



6. Discussion of methods and results

6.1 Discussion of results

In all three studies the dose applied twice daily in the ROAT (µg allergen/cm^2/application) resulted in a higher response than when the same dose was applied in the patch test (this was significant at two doses, Study I and II, and at one dose, Study III). Consequently, the elicitation doses derived by patch testing cannot be used directly to define the safe elicitation exposure level. The reason for this difference in response could either be accumulation of allergen in the skin upon repeated exposure or that the immune system reacts to the repeated stimulation. Indeed in Study I and II it was found that the response to the accumulated exposure in the ROAT was not significantly different from the response to the corresponding dose in the patch test. These results are consistent with findings from other dose-response studies. One study with MDBGN found approximately the same elicitation response to both 0.01% MDBGN applied 4 times a day and 0.04% applied once a day[76]. In the study with isoeugenol, 63% of persons reacting in a ROAT to 9 µg/cm^2 had a reaction to 2.2 µg/cm^2 on a later date[42], thus they reacted to the accumulated exposure of the low concentration. Other studies with PPD have also shown that the accumulated exposure over time influences the elicitation response[77;78], and PPD was shown to accumulate in the skin of rats upon repeated exposure[78]. A sensitization study with PPD also showed that more people became sensitized to a low concentration of PPD applied once a week for a short period than to a higher concentration applied for a longer period, but only once a month[79]. In Study III the response to the accumulated week doses in the ROAT was lower than the response to the same dose in the patch test. This finding was different than the findings in study I and II. A possible explanation for this phenomenon could be that HICC is a volatile compound, so, evaporation of HICC from the skin in the open test could result in a lower actual accumulated dose over time (compared to nickel and MDBGN), and therefore the response-frequency is lower in the ROAT than in the patch test. We estimated the evaporation of HICC from the skin, using an equation developed previously[70]. The estimation was based on the physico-chemical properties of HICC, and it was estimated that approximately 75% of the applied HICC would evaporate from the skin surface during 24 hours. In an in vitro study, the concentration of other fragrance ingredients was shown to decrease rapidly when stored in Finn Chambers at room temperature[80], and in another in vitro study several fragrance ingredients were shown to evaporate at different ratios[81]. However, yet another in vitro study with HICC showed that only 13.8% evaporated in an open chamber compared with 9.5 % in the occluded chamber[82]. Further studies with volatile compounds are needed to clarify the evaporation effect in open exposures. Applications in the axilla is likely to limit evaporation; this could be why patients with fragrance allergy report deodorant exposure as the most frequent site of first-time rash[83], and why fragrance allergy is associated with axillary dermatitis[4].

When the results from the three studies were used to define the relation between the patch test and the ROAT it was obvious that non-volatile and volatile chemicals should be described separately. It was found that the factor F, describing the relation between the patch test and the ROAT, was very similar for the non-volatile compounds (Study I and II); accordingly, an equation could be built based on these two studies. Furthermore the factor F describing the relation between the patch test and the ROAT in study III and in another study using the fragrance ingredient isoeugenol, was also very similar. It was chosen not to develop an equation for volatile compounds because volatile substances have different evaporation rates[81], only two ROAT doses were present in the isoeugenol study and the isoeugenol study was not performed with the scope of defining the relationship between the two test-methods. When using the equation derived from the non-volatile data to estimate the outcome of the ROAT based on volatile patch test data, it was found that the equation did predicted that the response to the dose per application in the ROAT would be higher than the response to the corresponding dose in the patch test, but the response was overestimated.

As the patch test is an easy test to perform and the ROAT is much more time consuming, the relation between these two test methods could be an important tool in the elicitation risk assessment. Additionally, it shows that elicitation patch test data should not be used directly to set safe elicitation exposure levels, since the patch test elicitation threshold is not a safe dose. Several factors other than the repeated exposure should also be considered when safe exposure levels are established. For example, the effect of combining several allergens in one product (e.g. fragrance mixtures), can give an additive or synergistic effect, so that lower concentrations of allergens are tolerated[67;84;85]. The vehicle in which the allergen is present also has an impact on the threshold[22;48;66].

A quantitative risk assessment has been suggested by industry and is aimed at protecting the healthy non-allergic population. However, as approximately 20% of the population is already sensitized to different allergens[1], it can be concluded that the risk assessment has not succeeded. The quantitative risk assessment is based on the LLNA, here the allergen (25 µl) is applied to the ear of CBA/Ca strain mice (on 1cm^2) for 3 consecutive days, ^{3}H-methyl thymidine is injected intravenously, the animals sacrificed and the lymphocyte proliferation in the local lymph node is measured. The sensitizing potential of a chemical is described by the concentration of chemical needed to produce a three-fold increase in the proliferation of the lymph node cells compared with vehicle-treated controls, described by the EC3 value[11;63]. Test chemicals can thus be classified according to the EC3-value[16;17]. By dividing the "no expected sensitization induction level" (the EC3 value or human sensitization data, if these are available and lower than the EC3 value) with different "sensitization assessment factors" (from 10-1000) that account for inter-individual variability in the user of the product, vehicle/product matrix and use considerations of the product the "acceptable

exposure level" can be determined[14;15]. The "acceptable exposure level" derived from the EC3-value by dividing the EC3-value with the maximal "sensitization assessment factors" (1000), is considerably higher than the dose per application that will elicit a reaction in 10% of a sensitized population in a ROAT (Table 13). It can be concluded from Table 13, that the "acceptable exposure level" is not acceptable concerning elicitation.

Table 13
All values are in µg allergen/cm^2
The numbers in bold show how many times higher the exposure level is in the acceptable-non-sensitizing area dose based on the EC3 value compared with the concentration that will elicit a reaction in 10% of allergic individuals in a ROAT (the ED_{10} ROAT). The sensitization assessment factors used are the highest possible (1000). MDBGN: the EC3 is given in percentage: 1.3% ref [86] , which equals a dose per area of: ((0.013x 25 µl)x 1000 µg/µl)/ 1 cm^2) ref [63].

	EC3	Acceptable exposure level (based on EC3)	ED_{10} patch test	ED_{10} ROAT	Relation between the Acceptable exposure level (based on LLNA) and the ED_{10} ROAT
Calculation	Nickel[63] MDBGN[86] HICC[87]	EC3/1000	Nickel MDBGN HICC	Ni &MDBGN: ED_{10} patch test x 0.0296 HICC: ED_{10} patch test x 0.0971	Acceptable exposure level/ ED_{10} ROAT
Nickel	140	0.14	0.78	0.023	**6**
MDBGN	325	0.325	0.5	0.0148	**22**
HICC	4275	4.275	0.662	0.064	**67**

It is seen that there is a substantial difference between the relationship of the sensitization dose and the elicitation dose between the different allergens (6-67). To a great extend this is caused by the difference in the EC3 values. Conversely, the doses that will elicit a reaction in 10% of allergic individuals in a ROAT resemble each other more than expected for each allergen when considering the difference in sensitization potency. An explanation of this could be that, even though allergens have different sensitization potencies, once an individual is sensitized, the dose needed to elicit eczema does not differ greatly, because the memory of the allergen has already been generated.

In retrospect a dose that elicits a reaction in only 10% of allergic individuals in a ROAT seems to be an effective dose for prevention. For example, according to the nickel directive the maximal permitted nickel-release from products intended to come in to direct and prolonged contact with the skin were 0.5 µg Ni/cm^2/week[88], and has recently been changed to 0.2 µg Ni/cm^2/week[89] in piercing post assemblies and the 1-week accumulated ED_{10}ROAT is 0.023 µg Ni/cm^2 x 2 daily exposures x 7 days = 0.322 µg Ni/cm^2/week. Seemingly, the maximum permitted level in the nickel directive is close to the ED_{10}ROAT, and this dose has proven effective in the prevention of nickel allergy[28-30]. Another approach could be prohibition of the allergen in question, this has been done for MDBGN[90], following an increase in allergy to MDBGN over a 10-year period[91], a limiting in the use of MDBGN to rinse-off products only[92] and subsequently a difficulty in establishing a safe non-eliciting dose[22;39;76]. Prohibition of all allergens causing allergic contact dermatitis is not a realistic approach, because it is not be possible to eliminate all allergens in the environment, as, for example,

preservatives in some types of cosmetics are needed to avoid the growth of microorganisms. Nonetheless, if 90% of the people with allergy could be protected from developing allergic contact dermatitis, it would have a major impact on the daily life of a large number of sensitized individuals, and a decrease in the incidence of contact allergy would be expected. Regulation of allergen exposure by legislation has for example proven effective in decreasing the prevalence of allergy to chromium [93], nickel[28-30] and methyldibromo glutaronitrile[94] , and is thereby an important, useful tool in the prevention of allergic contact dermatitis[95]. Based on the data presented in this thesis, it would be desirable if elicitation data from individuals already sensitized could be used to define the acceptable exposure level of allergens in the environment.

An independent post-marketing surveillance system should be developed based on case-reports of allergy towards new substances and on epidemiological data of the prevalence of allergy to allergens. These data should be based on international network observations, to promote prompt discovery of new allergens and potential epidemics. This system should evaluate the risk of elicitation from allergens causing allergic contact dermatitis, and the endpoint should be regulation of allergen exposure, preventing eczema among the sensitized population, and also preventing new sensitizations. The relationship between the patch test and the ROAT and the conversion of patch test ED-values to ROAT ED-values, as introduced in this thesis, could be used in this process of establishing a safe exposure level based on elicitation patch test data.

6.2 Methodological considerations

6.2.1 The test material

The choice of ethanol-water as the vehicle might have resulted in a lower threshold than if petrolatum had been chosen[22;66]. Furthermore, the ethanol solutions might have led to decreased skin hydration[96], thereby lowering the threshold; however, as the same vehicle was used for both the patch test and the ROAT, we do not expect the choice of vehicle to have influenced the relation between the two test methods.

6.2.2 The application procedure

All concentrations were randomised, which introduced a risk of accidentally applying the doses on a wrong area. In the ROAT, the test-subjects performed the applications at home. It would have been ideal if all exposures had taken place at the hospital, but this was not practical. The subjects were tested with several doses and with both test methods simultaneously. This could result in the excited skin syndrome[97], which could lead to reactions to concentrations that would have been negative if tested alone. Furthermore, in the nickel study, the patches were placed as close to one another as in an ordinary Finn chamber patch test (in the two other studies every other Finn

chamber removed), which could result in an enhanced reaction in a patch adjacent to a high patch test concentration. However, in a study using nickel sulphate, positive reactions to high doses did not enhance reactions to the adjacent patches[68] and yet another study with nickel sulphate showed similar results[98]. In the studies presented in this thesis two test-subjects had a developing flare-up of eczema in the MDBGN study and one subject had a developing flare-up in the HICC study. The subject in the HICC-study exhibited visible reactions to all tested HICC-patches, as well as itching in the axillae, even though; according to the labelling the deodorant being used did not contain HICC. This could be an indication of a developing systemic reaction to the HICC exposure. The itching disappeared shortly after the subject was withdrawn from the study.

6.2.3 The reading scales and readings

The chosen definition of thresholds included weaker reactions too. This posed a problem as it was not possible to distinguish between irritant reactions and allergic reactions. In the nickel study, a few test subjects had reactions in the ROAT to the lower concentrations. The reactions later disappeared and were below the cut-off point (5 points) proposed by Johansen et al[49]. In the MDBGN-study one person had a weak reaction to the vehicle site; this reaction was also below the defined cut-off point (5 points). We included weaker reactions in the ROAT because the patch test reactions included weaker reactions too, and as we wanted to compare the two test methods, concordance between the reading scales including weaker reactions, was desirable. For practical reasons all other ROAT-reactions, other than those mentioned here, were above 5 points; accordingly, for future studies it is recommended to count only reactions above 5 points in a ROAT. All readings, which were blinded (to the applied dose), were done by the principal investigator (LAF), assisted by the nurses who usually perform the patch test readings at the Department of Dermato-allergology at Gentofte hospital.

6.2.4 Selection bias

The test subjects were recruited among the patients from the hospital, and those who had earlier been referred to the hospital might have represented the most allergic part of the allergic population. However, when looking at the former patch test results, the reactions were equally distributed among 1+ and 2+ reactions, whereas only one 3+ reactions were recorded (according to the ICDRG-criteria[34]); this indicates that the most allergic patients did not participate.

6.2.5 Power

No statistically significant differences were found between the ROAT dose per application and patch test reaction for some of the doses, even though the observed response in the ROAT was

higher than the response in the patch test. The power of a test is the probability that a study of a given size will detect a statistically significant real difference[99]. To have achieved a power of 80%, with significant-level $P<0.05$, given the data obtained, the following number of patients should have participated:

Nickel; Table 3; dose: 0.035 µg Ni/cm^2; number of patients: 33

MDBGN; Table 6; dose: 0.0357 µg MDBGN/cm^2; number of patients: 39

HICC; Table 9; dose: 0.357 µg HICC/cm^2; number of patients: 39; dose: 35.7 µg HICC/cm^2; number of patients: 118.

It was, however, not possible to recruit more patients to the studies.

7. Conclusion

Allergic individuals react to lower doses (µg allergen/cm^2/application) in a repeated exposure than in the patch test. This applies for nickel, MDBGN and HICC. For non-volatile compounds (nickel and MDBGN), allergic individuals react to approximately the same dose in the ROAT and the patch test when the dose applied everyday in the ROAT is added up to the total accumulated dose per area over 1-3 weeks (accumulated µg allergen/cm^2). For HICC the response to the accumulated ROAT dose is lower than the response to the same dose in the patch test. This is probably due to evaporation. For non-volatile compounds the outcome of a ROAT can be expressed by: $ED_{xx}(ROAT) = 0.0296 \cdot ED_{xx}(patch\ test)$. The acceptable exposure level based on sensitization data (EC3) is not acceptable regarding elicitation and should therefore not be used as the endpoint in prevention of allergic contact dermatitis. The relation between the elicitation in the patch test and the ROAT could be a valuable tool used in the prevention of allergic contact dermatitis.

8. Future perspectives

The relationship between the allergen threshold after real life exposure to allergens in consumer products and patch test and ROAT thresholds, should be investigated in allergic patients with allergies to specific products. This would clarify the relevance of elicitation thresholds defined by experimental studies.

The effect of repeated exposures to allergens on the sensitization and elicitation thresholds should be clarified by the investigation of 1) the accumulation of allergen in the skin upon repeated exposure, 2) the immunological response to repeated exposure and 3) the evaporation of volatile compounds from the skin.

The accumulation of allergens in the skin in occupations with a high exposure to different allergens should be investigated on order to localize the sensitization and elicitation risk, and introduce prevention where necessary.

The effect of exposure to several allergens simultaneously on the elicitation thresholds should be investigated by studies carried out using allergic patients, exposed to a combination of allergens which is relevant to real-life exposure.

The effect of the total applied dose and the dose per area for elicitation should be investigated in studies using allergic patients, where the area of exposure is varied, and hence the total dose varied, but where the dose of allergen per skin area is kept constant.

Studies with the same design as those in this thesis, but using other non-volatile and volatile compounds could investigate whether the results presented here can be reproduced.

9. Reference List

1. Thyssen JP, Linneberg A, Menne T *et al*. The epidemiology of contact allergy in the general population--prevalence and main findings. *Contact Dermatitis* 2007; **57**: 287-99.

2. Rustemeyer T., van Hoogstraten I.M.W., von Blomberg B.M.E. *et al*. Mechanisms in Allergic Contact Dermatitis. In: *Contact Dermatitis* (Frosch,P, Menne,T, Lepoittevin,JP, eds), 4 edn. Springer, 2006: 11-43.

3. Veien N.K. General Aspects. In: *Contact Dermatitis* (Frosch,P, Menne,T, Lepoittevin,JP, eds), 4 edn. Springer, 2006: 201-54.

4. Nardelli A, Carbonez A, Ottoy W *et al*. Frequency of and trends in fragrance allergy over a 15-year period. *Contact Dermatitis* 2008; **58**: 134-41.

5. Cvetkovski RS, Rothman KJ, Olsen J *et al*. Relation between diagnoses on severity, sick leave and loss of job among patients with occupational hand eczema. *Br.J.Dermatol* 2005; **152**: 93-8.

6. Halkier-Sorensen L. Occupational skin diseases: reliability and utility of the data in the various registers; the course from notification to compensation and the costs. A case study from Denmark. *Contact Dermatitis* 1998; **39**: 71-8.

7. McCall BP, Horwitz IB, Feldman SR *et al*. Incidence rates, costs, severity, and work-related factors of occupational dermatitis: a workers' compensation analysis of Oregon, 1990-1997. *Arch.Dermatol* 2005; **141**: 713-8.

8. The European Surveillance System of Contact Allergies (ESSCA): results of patch testing the standard series, 2004. *J.Eur.Acad.Dermatol Venereol.* 2008; **22**: 174-81.

9. Skoet R, Olsen J, Mathiesen B *et al*. A survey of occupational hand eczema in Denmark. *Contact Dermatitis* 2004; **51**: 159-66.

10. Lachappelle JM, Wigger-Alberti W, Boman A *et al*. Prevention and Therapy. In: *Contact Dermatitis* (Frosch,P, Menne,T, Lepoittevin,JP, eds), 4 edn. Springer, 2006: 831-67.

11. Kimber I, Basketter DA. The murine local lymph node assay: a commentary on collaborative studies and new directions. *Food Chem.Toxicol.* 1992; **30**: 165-9.

12. Menne T, Wahlberg JE. Risk assessment failures of chemicals commonly used in consumer products. *Contact Dermatitis* 2002; **46**: 189-90.

13. McGarry HF. The murine local lymph node assay: regulatory and potency considerations under REACH. *Toxicology* 2007; **238**: 71-89.

14. Api AM, Basketter DA, Cadby PA *et al*. Dermal sensitization quantitative risk assessment (QRA) for fragrance ingredients. *Regul.Toxicol.Pharmacol.* 2008; **52**: 3-23.

15. Felter SP, Robinson MK, Basketter DA *et al*. A review of the scientific basis for uncertainty factors for use in quantitative risk assessment for the induction of allergic contact dermatitis. *Contact Dermatitis* 2002; **47**: 257-66.

16. Gerberick GF, Robinson MK, Ryan CA *et al*. Contact allergenic potency: correlation of human and local lymph node assay data. *Am.J.Contact Dermat.* 2001; **12**: 156-61.

17. Kimber I, Dearman RJ, Basketter DA *et al*. The local lymph node assay: past, present and future. *Contact Dermatitis* 2002; **47**: 315-28.

18. McNamee PM, Api AM, Basketter DA *et al*. A review of critical factors in the conduct and interpretation of the human repeat insult patch test. *Regul.Toxicol.Pharmacol.* 2008; **52**: 24-34.

19. Ford RA, Api AM, Letizia CS. Monographs on fragrance raw materials. *Food Chem.Toxicol.* 1992; **30 Suppl**: 1S-138S.

20. Liden C, Skare L, Nise G *et al*. Deposition of nickel, chromium, and cobalt on the skin in some occupations - assessment by acid wipe sampling. *Contact Dermatitis* 2008; **58**: 347-54.

21. Rasmussen K, Carstensen O, Ponten A *et al*. Risk of contact allergy and dermatitis at a wind turbine plant using epoxy resin-based plastics. *Int.Arch.Occup.Environ.Health* 2005; **78**: 211-7.

22. Jensen CD, Johansen JD, Menne T *et al*. Methyldibromoglutaronitrile in rinse-off products causes allergic contact dermatitis: an experimental study. *Br.J.Dermatol* 2004; **150**: 90-5.

23. Flyvholm MA, Mygind K, Sell L *et al*. A randomised controlled intervention study on prevention of work related skin problems among gut cleaners in swine slaughterhouses. *Occup.Environ.Med.* 2005; **62**: 642-9.

24. Held E, Mygind K, Wolff C *et al*. Prevention of work related skin problems: an intervention study in wet work employees. *Occup.Environ.Med.* 2002; **59**: 556-61.

25. Basketter DA, Andersen KE, Liden C *et al*. Evaluation of the skin sensitizing potency of chemicals by using the existing methods and considerations of relevance for elicitation. *Contact Dermatitis* 2005; **52**: 39-43.

26. Noiesen E, Larsen K, Agner T. Compliance in contact allergy with focus on cosmetic labelling: a qualitative research project. *Contact Dermatitis* 2004; **51**: 189-95.

27. Noiesen E, Munk MD, Larsen K *et al*. Difficulties in avoiding exposure to allergens in cosmetics. *Contact Dermatitis* 2007; **57**: 105-9.

28. Jensen CS, Lisby S, Baadsgaard O *et al*. Decrease in nickel sensitization in a Danish schoolgirl population with ears pierced after implementation of a nickel-exposure regulation. *Br.J.Dermatol.* 2002; **146**: 636-42.

29. Johansen J, Menne T, Christophersen J *et al*. Changes in the pattern of sensitization to common contact allergens in denmark between 1985-86 and 1997-98, with a special view to the effect of preventive strategies. *Br.J.Dermatol.* 2000; **142**: 490-5.

30. Schnuch A, Uter W. Decrease in nickel allergy in Germany and regulatory interventions. *Contact Dermatitis* 2003; **49**: 107-8.

31. Lachappelle JM. Historical Aspects. In: *Contact Dermatitis* (Frosch,P, Menne,T, Lepoittevin,JP, eds), 4 edn. 2006: 1-7.

32. http://www.videncenterforallergi.dk/Files/Filer/Patientinformation/StandardSerie2007.pdf . 13-3-0009.
Ref Type: Electronic Citation

33. Magnusson B, Hersle K. Patch test methods. II. Regional variations of patch test responses. *Acta Derm.Venereol.* 1965; **45**: 257-61.

34. Wahlberg JE, Lindberg M. Patch testing. In: *Contact Dermatitis* (Frosch,P, Menne,T, Lepoittevin,JP, eds), 4 edn. Springer, 2006: 365-90.

35. Hindsen M, Bruze M. The significance of previous contact dermatitis for elicitation of contact allergy to nickel. *Acta Derm.Venereol.* 1998; **78**: 367-70.

36. Hannuksela M, Salo H. The repeated open application test (ROAT). *Contact Dermatitis* 1986; **14**: 221-7.

37. Johansen J, Frosch P, Menne T. Allergic Contact Dermatitis in Humans - Experimental and Quantitative Aspects. In: *Contact Dermatitis* (Frosch,P, Menne,T, Lepoittevin,JP, eds), 4th edn. Springer, 2006: 189-98.

38. Johansen JD, Andersen KE, Menne T. Quantitative aspects of isoeugenol contact allergy assessed by use and patch tests. *Contact Dermatitis* 1996; **34**: 414-8.

39. Schnuch A, Kelterer D, Bauer A *et al*. Quantitative patch and repeated open application testing in methyldibromo glutaronitrile-sensitive patients. *Contact Dermatitis* 2005; **52**: 197-206.

40. Zachariae C, Hall B, Cottin M *et al*. Experimental elicitation of contact allergy from a diazolidinyl urea-preserved cream in relation to anatomical region, exposure time and concentration. *Contact Dermatitis* 2005; **53**: 268-77.

41. Zachariae C, Lerbaek A, McNamee PM *et al*. An evaluation of dose/unit area and time as key factors influencing the elicitation capacity of methylchloroisothiazolinone/methylisothiazolinone (MCI/MI) in MCI/MI-allergic patients. *Contact Dermatitis* 2006; **55**: 160-6.

42. Andersen KE, Johansen JD, Bruze M *et al*. The time-dose-response relationship for elicitation of contact dermatitis in isoeugenol allergic individuals. *Toxicol.Appl.Pharmacol.* 2001; **170**: 166-71.

43. Flyvholm MA, Hall BM, Agner T *et al*. Threshold for occluded formaldehyde patch test in formaldehyde-sensitive patients. Relationship to repeated open application test with a product containing formaldehyde releaser. *Contact Dermatitis* 1997; **36**: 26-33.

44. Johansen JD, Andersen KE, Rastogi SC *et al*. Threshold responses in cinnamic-aldehyde-sensitive subjects: results and methodological aspects. *Contact Dermatitis* 1996; **34**: 165-71.

45. Hannuksela M. Sensitivity of Various Skin Sites in the Repeated Open Application. *Am.J.Contact Dermat.* 1991; **2**: 102-4.

46. Johansen JD, Rastogi SC, Bruze M *et al*. Deodorants: a clinical provocation study in fragrance-sensitive individuals. *Contact Dermatitis* 1998; **39**: 161-5.

47. Fischer LA, Menne T, Johansen JD. Dose per unit area - a study of elicitation of nickel allergy. *Contact Dermatitis* 2007; **56**: 255-61.

48. Kynemund PL, Agner T, Held E *et al*. Methyldibromoglutaronitrile in leave-on products elicits contact allergy at low concentration. *Br.J.Dermatol* 2004; **151**: 817-22.

49. Johansen JD, Bruze M, Andersen KE *et al*. The repeated open application test: suggestions for a scale of evaluation. *Contact Dermatitis* 1998; **39**: 95-6.

50. Schnitzer A. Beitrag zur Frage das Mechanisms der Sensibiliserung. *Dermatologica* 1942; **85**: 339-47.

51. Magnusson B., Kligman AM. Induction of hypersensitivity. In: *Allergic Contact Dermatitis in the Guinea Pig, Identifications of Contact Allergens*. Charles C Thomas, 1970: 44-76.

52. Kligman AM. The identification of contact allergens by human assay. II. Factors influencing the induction and measurement of allergic contact dermatitis. *J.Invest Dermatol.* 1966; **47**: 375-92.

53. White SI, Friedmann PS, Moss C *et al*. The effect of altering area of application and dose per unit area on sensitization by DNCB. *Br.J.Dermatol.* 1986; **115**: 663-8.

54. Rees JL, Friedmann PS, Matthews JN. The influence of area of application on sensitization by dinitrochlorobenzene. *Br.J.Dermatol.* 1990; **122**: 29-31.

55. Friedmann PS. Contact hypersensitivity. *Curr.Opin.Immunol.* 1989; **1**: 690-3.

56. Friedmann PS. The immunology of allergic contact dermatitis: the DNCB story. *Adv.Dermatol.* 1990; **5**: 175-95.

57. Bruze M, Johansen JD, Andersen KE *et al*. Deodorants: an experimental provocation study with cinnamic aldehyde. *J.Am.Acad.Dermatol* 2003; **48**: 194-200.

58. Hannuksela M. Rapid increase in contact allergy to Kathon CG in Finland. *Contact Dermatitis* 1986; **15**: 211-4.

59. Johansen JD, Bernard G, Gimenez-Arnau E *et al*. Comparison of elicitation potential of chloroatranol and atranol--2 allergens in oak moss absolute. *Contact Dermatitis* 2006; **54**: 192-5.

60. Sosted H, Menne T, Johansen JD. Patch test dose-response study of p-phenylenediamine: thresholds and anatomical regional differences. *Contact Dermatitis* 2006; **54**: 145-9.

61. Friedmann PS, Moss C, Shuster S *et al*. Quantitative relationships between sensitizing dose of DNCB and reactivity in normal subjects. *Clin.Exp.Immunol.* 1983; **53**: 709-15.

62. Scott AE, Kashon ML, Yucesoy B *et al*. Insights into the quantitative relationship between sensitization and challenge for allergic contact dermatitis reactions. *Toxicol.Appl.Pharmacol.* 2002; **183**: 66-70.

63. Griem P, Goebel C, Scheffler H. Proposal for a risk assessment methodology for skin sensitization based on sensitization potency data. *Regul.Toxicol.Pharmacol.* 2003; **38**: 269-90.

64. Moss C, Friedmann PS, Shuster S *et al*. Susceptibility and amplification of sensitivity in contact dermatitis. *Clin.Exp.Immunol.* 1985; **61**: 232-41.

65. Carlsen BC, Fischer LA, Sosted H *et al*. Patch test dose-response study: polysensitized individuals do not express lower elicitation thresholds than single/double-sensitized individuals. *Br.J.Dermatol* 2009; **160**: 103-6.

66. Wahlberg JE, Skog E. Nickel allergy and atopy. Threshold of nickel sensitivity and immunoglobulin E determinations. *Br.J.Dermatol.* 1971; **85**: 97-104.

67. Johansen JD, Skov L, Volund A *et al*. Allergens in combination have a synergistic effect on the elicitation response: a study of fragrance-sensitized individuals. *Br.J.Dermatol* 1998; **139**: 264-70.

68. Andersen KE, Liden C, Hansen J *et al*. Dose-response testing with nickel sulphate using the TRUE test in nickel-sensitive individuals. Multiple nickel sulphate patch-test reactions do not cause an 'angry back'. *Br.J.Dermatol.* 1993; **129**: 50-6.

69. Frankild S, Volund A, Wahlberg JE *et al*. Comparison of the sensitivities of the Buehler test and the guinea pig maximization test for predictive testing of contact allergy. *Acta Derm.Venereol.* 2000; **80**: 256-62.

70. Kasting GB, Saiyasombati P. A physico-chemical properties based model for estimating evaporation and absorption rates of perfumes from skin. *Int.J.Cosmet.Sci.* 2001; **23**: 49-58.

71. http://www.chemblink.com/products/31906-04-4.htm, accessed on the 23rd of Feb.2009 . 2009.
Ref Type: Electronic Citation

72. Lyman, Rheel, and Rosenblatt. Handbook of Chemical Estimation Methods, American Chemical Society. 1990. Washington DC.
Ref Type: Serial (Book,Monograph)

73. Jain N, Yalkowsky SH. Estimation of the aqueous solubility I: application to organic nonelectrolytes. *J.Pharm.Sci.* 2001; **90**: 234-52.

74. Cox DR. *The analysis of Binary Data*. London: Menthuen, 1970: 100-1.

75. Finney DJ. *Statistical Method in Biological Assay*. London: Griffin C, 2009: 349-69.

76. Jensen CD, Johansen JD, Menne T *et al*. Methyldibromo glutaronitrile contact allergy: effect of single versus repeated daily exposure. *Contact Dermatitis* 2005; **52**: 88-92.

77. Hextall JM, Alagaratnam NJ, Glendinning AK *et al*. Dose-time relationships for elicitation of contact allergy to para-phenylenediamine. *Contact Dermatitis* 2002; **47**: 96-9.

78. White JM, Basketter DA, Pease CK *et al*. Intermittent exposure to low-concentration paraphenylenediamine can be equivalent to single, higher-dose exposure. *Contact Dermatitis* 2007; **56**: 262-5.

79. Basketter DA, Jefferies D, Safford BJ *et al*. The impact of exposure variables on the induction of skin sensitization. *Contact Dermatitis* 2006; **55**: 178-85.

80. Mowitz, M., Zimerson, E., Svedman, C., and Bruze, M. Stability of fragrance test preparations applied in test chambers. Contact Dermatitis (the 9th congress of the ESCD, Estoril, Portugal, 28-31 May), 34. 2008.
Ref Type: Magazine Article

81. Vuilleumier C, Flament I, Sauvegrain P. Headspace analysis study of evaporation rate of perfume ingredients applied onto skin. *Int.J.Cosmet.Sci.* 1995; **17**: 61-76.

82. Bhatia SP, Lalko J, Brain KR, Green DM, and Api AM. In-vitro Human skin penetration of the fragrance material HMPCC. Society of Toxicology 46th Annual Meeting and ToxExpo, Charlotte, North Carolina March 25-29, 2007 . 2007.
Ref Type: Magazine Article

83. Johansen JD, Andersen T.F., Kjøller M. *et al*. Identification of Risk Products for Fragrance Contact Allergy: A case-Referent Study Based on Patients´ Histories. *Am.J.Contact Dermat.* 1998; **9**: 80-7.

84. Krasteva M, Garrigue JL, Horrand F *et al*. Suboptimal non-inflammatory concentrations of haptens may elicit a contact sensitivity reaction when used as a mix. *Contact Dermatitis* 1996; **35**: 279-82.

85. McLelland J, Shuster S. Contact dermatitis with negative patch tests: the additive effect of allergens in combination. *Br.J.Dermatol* 1990; **122**: 623-30.

86. Basketter DA. Skin sensitization: strategies for the assessment and management of risk. *Br.J.Dermatol* 2008; **159**: 267-73.

87. GRA Expert Group, Api AM, Basketter, D., Cadby, P. A., Cano, M. F., Ellis, G., Gerberick, G. F., Griem, P., McNamee, P., Ryan, C. A., and Safford, B. J. Dermal Sensitization Quantitative Riak Assessmen (QRA) for Fragrance Ingredients, Technical Dossier, revised May 26, 2006, Revised June 22, 2006. 15-3-2006.
Ref Type: Hearing

88. European Parliament and Council Directive 94/27/EC of 30 June 1994 amending for the 12th time Directive 76/769/EEC on the approximation of the laws, regulations and administrative provisions of the Member States relating to restrictions on the marketing and use of certain dangerous substances and preparations. 1994.
Ref Type: Hearing

89. Commission Directive 2004/96/EC of 27 September 2004 amending Council Directive 76/769/EEC as regards restrictions on the marketing and use of nickel for piercing post assemblies for the purpose of adapting its Annex I to technical progress. 2004.
Ref Type: Hearing

90. Scientific Committee on Consumer Products European Commission. Opinion on Methyldibromo glutaronitrile. Adobted 15 March 2005/20 June 2006. Available in http://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_060.pdf. 2005.
Ref Type: Hearing

91. Wilkinson JD, Shaw S, Andersen KE *et al*. Monitoring levels of preservative sensitivity in Europe. A 10-year overview (1991-2000). *Contact Dermatitis* 2002; **46**: 207-10.

92. Opinion of the scientific commitee on cosmetic products and non-food products intended for consumers concerning Methyldibromo Glutaronitrile, Colipa no. P77 adopted by the SCCNFP during the 20th plenary meeting of 4 June 2002. 4-6-2002.
Ref Type: Hearing

93. Liden C, Bruze M, Menne T. Metals. In: *Contact Dermatitis* (Frosch,P, Menne,T, Lepoittevin,JP, eds), 4 edn. Springer Berlin Heidelberg New York, 2006: 537-68.

94. Johansen JD, Veien N, Laurberg G *et al*. Decreasing trends in methyldibromo glutaronitrile contact allergy - following regulatory intervention. *Contact Dermatitis* 2008; **59**: 48-51.

95. Liden C. Legislative and preventive measures related to contact dermatitis. *Contact Dermatitis* 2001; **44**: 65-9.

96. Loffler H, Kampf G, Schmermund D *et al*. How irritant is alcohol? *Br.J.Dermatol* 2007; **157**: 74-81.

97. Mitchell J, Maibach HI. Managing the excited skin syndrome: patch testing hyperirritable skin. *Contact Dermatitis* 1997; **37**: 193-9.

98. Memon AA, Friedmann PS. 'Angry back syndrome': a non-reproducible phenomenon. *Br.J.Dermatol* 1996; **135**: 924-30.

99. Altman DG. Clinical trials. In: *Practical Statistics for Medical Research*. London: Chapman & Hall, 1999: 440-76.

10. Summary in English

Allergic contact dermatitis is an inflammatory skin disease triggered by direct skin contact with skin sensitizers in the home environment and/or at the work place. It is a prevalent disease that may have consequences for both the individual and society by requiring treatment and sick-leave and in some cases affecting ability to work. Allergic contact dermatitis is a preventable disease, as reducing or avoiding exposures to the allergens in question will reduce the incidence of individuals becoming sensitized as well as morbidity among those who have already become sensitized.

Experimental data from already sensitized persons can be used to determine safe levels of allergen exposure. Two main methods exist by which data can be generated about thresholds for contact allergic reactions. One is the patch test, which is an easy test to perform. The other is the repeated open application test (ROAT), which is time consuming but mimics some real life exposure situations. A link between the two test methods could be a valuable tool for use in preventive strategies.

The aim of this thesis was to study the dose-response relationship for allergens with different physico-chemical properties in order to:

- examine if a relationship exists between the patch test and the ROAT results using both the dose per application of allergen and the total applied dose.
- examine if this relationship is independent of physico-chemical characteristics of the allergen.
- suggest a model for conversion of data from patch tests to ROATs.

Experimental studies were performed with three different allergens using identical methodology. A metal (nickel), a preservative (methyldibromo glutaronitrile, MDBGN) and a fragrance ingredient (hydroxyisohexyl 3-cyclohexene carboxaldehyde, HICC) was used. Test subjects were groups of eczema patients with contact allergy to one of the chosen allergens. A control group of individuals without allergies was included. A serial dilution patch test and ROAT was performed on the same allergic individuals simultaneously. Dose-response curves were drawn and the response to the different doses in the two test methods was compared statistically.

It was a consistent finding for all three allergens that more patients reacted to the allergen, measured as dose per application, when applied repeatedly (ROAT) as compared with the single, occluded exposure in the patch test. This means that a person can be reactive at a repeated open application in spite of a negative finding in the patch test to that dose. This is especially relevant in the context of testing with patients' own (cosmetic) products. When the doses applied everyday was added up in the ROAT to an accumulated one-week, two-week and three-week dose, it was found that in the nickel and MDBGN studies the dose-response for the patch test and the dose-response for the accumulated doses in the ROAT was approximately the same.

In the HICC study the response to the accumulated ROAT dose was lower than the response to the corresponding dose in the patch test. An explanation of this could be evaporation of HICC from the skin, resulting in a lower accumulated dose over time in the open test, compared with the occluded test.

Based on the two studies with the non-volatile compounds (nickel and MDBGN) it was possible to develop an equation that characterized the relationship between the patch test and the ROAT; the results for the volatile substance HICC were probably influenced by evaporation. The knowledge of this relationship can be used as a tool when risk assessment is based on human elicitation patch test studies and thus as basis for preventive strategies.

In conclusion

- Allergic individuals react to lower doses (µg allergen/cm2/application) in the ROAT than in the patch test.
- The relationship between the two test methods was probably dependent on the physico-chemical characteristics of the allergen.
- A model for conversion of patch test dose-response into ROAT dose-response was suggested for non-volatile compounds.

Investigation of the accumulation of allergen in the skin upon repeated exposure, the immunological response to repeated exposure and the evaporation of volatile compounds from the skin would be interesting as future research subjects.

11. Summary in Danish

Allergisk kontakteksem er en inflammatorisk hudsygdom, der opstår ved hudkontakt med allergifremkaldende stoffer i miljøet i hjemmet og/eller på arbejdspladsen.

Det er en hyppig sygdom, som kan have betydning ikke kun for den enkelte person, men også for samfundet pga. omkostninger til behandling, sygemeldinger og i nogle tilfælde tab af erhvervsevne. Allergisk kontakteksem kan forebygges, da man ved at nedsætte eller undgå eksponeringen for det pågældende allergen kan reducere incidensen af sensibiliserede personer samt morbiditeten blandt de, der allerede er sensibiliserede.

Eksperimentelle data fra allerede sensibiliserede personer kan anvendes til at fastlægge et sikkert allergeneksponeringsniveau. To primære metoder findes til at skabe data til fastsættelse af grænseværdier for kontaktallergiske reaktioner. Den ene er epikutantesten, som er en simpel test at udføre. Den anden test er den gentagne åbne applikationstest (ROAT), som er tidskrævende, men efterligner nogle eksponeringsformer i det virkelige liv. En forbindelse mellem disse to testmetoder kunne blive et værdifuldt redskab, der ville kunne anvendes til forebyggelse.

Formålet med denne afhandling er at undersøge sammenhængen mellem dosis og respons for allergener med forskellige fysisk-kemiske egenskaber ved at:

- undersøge, om der findes en sammenhæng mellem epikutantestresultater og ROAT-resultater med hensyn til dosis allergen per applikation og total mængde påført allergen.
- undersøge, om denne sammenhæng er uafhængig af de fysisk-kemiske egenskaber for allergenerne.
- foreslå en model, der kan oversætte epicutantestdata til ROAT-data.

Eksperimentelle studier med tre forskellige allergener blev udført med præcis samme metode. Der blev brugt et metal (nikkel), et konserveringsmiddel (methyldibromoglutaronitril, MDBGN) og et parfumestof (hydroxyisohexyl 3-cyclohexen carboxaldehyd, HICC). Forsøgspersonerne var alle eksempatienter med allergi over for et af de valgte allergener. En kontrolgruppe bestående af personer uden allergi blev inkluderet. Forsøgspersonerne blev testet simultant med epikutantest og en ROAT-fortyndingsrække. Dosis-responskurver blev beregnet, og responset på de forskellige doser i de to testmetoder blev sammenlignet statistisk.

Det var et gennemgående fund i alle tre studier, at flere patienter reagerede på den gentagne daglige applikation af allergenet (ROAT), målt som dosis per applikation, sammenlignet med den enkle okkluderede eksponering i epikutantesten. Dette betyder, at en person kan reagere på en gentagen åben eksponering på trods af et negativt fund ved samme dosis i epikutantesten. Dette er f.eks. relevant, når man tester med en patients egne (kosmetiske) produkter.

Når den dosis, der blev påført hver dag i ROAT´en, blev adderet til en akkumuleret en-, to- og treuges dosis, fandt man i nikkel- og MDBGN-studierne, at dosis-responskurven for epikutantesten og dosis-responskurven for den akkumulerede ROAT dosis var nogenlunde ens.
I HICC studiet var responset på den akkumulerede ROAT-dosis lavere end responset på den samme dosis i epikutantesten. En forklaring på dette kan være, at HICC er et parfumestof og fordamper fra hudoverfladen, hvilket vil resultere i en lavere akkumuleret dosis over tid i den åbne test sammenlignet med den okkluderede epikutantest.
På baggrund af de to studier med de ikke-flygtige stoffer (nikkel og MDBGN) var det muligt at udvikle en model, der forudsiger forholdet mellem dosis-responsresultater fra epikutantesten og ROAT´en. Viden om denne sammenhæng kan anvendes som et redskab til risikovurdering baseret på eliciteringsstudier med epikutantest foretaget på mennesker, og derved kan denne viden anvendes som basis for forebyggelse.
Det kan konkluderes, at

- allergiske personer reagerer på lavere doser (µg allergen/cm^2/applikation) ved gentagen åben applikation (ROAT) end ved en enkelt okkluderet applikation (epikutantest).
- forholdet mellem de to testmetoder formentlig er afhængig af fysisk-kemiske egenskaber for det pågældende allergen.
- en omregningsmodel der kan anvendes til at omsætte epikutantestdata til ROAT-data, kan udvikles for ikke-flygtige stoffer.

Det kunne være interessant i fremtidige forskningsprojekter at undersøge akkumulering af allergen i huden efter gentagne allergen eksponeringer, det immunologiske respons på gentagne allergen-eksponeringer samt undersøgelse af fordampning fra huden efter applikation af flygtige stoffer.

12. Manuscripts