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CONTACT ALLERGY

Occurrence and Susceptibility

Cynthia Catalina Aurora van Amerongen

2021

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Occurrence and Susceptibility

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LIST OF ABBREVIATIONS

ACD	Allergic Contact Dermatitis
AD	Atopic Dermatitis
AEL	Acceptable Exposure Level
AQ	Aqua
D	Day
DAPPD	diacetyl- <i>p</i> -phenylenediamine
DNCB	2,4-dinitrochlorobenzene
EBS	European Baseline Series
EC	Estimated Concentration
EDEN	European Dermato-Epidemiology Network
ESCD	European Society of Contact Dermatitis
ESSCA	European Surveillance System on Contact Allergies
EU	European Union
FLG	Filaggrin gene
FM	Fragrance Mix
GC	Gas Chromatography
GST	Glutathione S-Transferases
GWAS	Genome Wide Association Study
ICD	Irritant Contact Dermatitis
ICDRG	International Contact Dermatitis Research Group
IDEA	International Dialogue for the Evaluation of Allergens
IFRA	International Fragrance Association
IL	Interleukin
IR	Irritative Reaction
IPPD	<i>N</i> -isopropyl- <i>N'</i> -phenyl- <i>p</i> -phenylenediamine
LHC	Langerhans cells
LLNA	Local Lymph Node Assay
MAPPD	mono-acetyl- <i>p</i> -phenylenediamine
MI	Methylisothiazolinone
MS	Mass Spectrometry
NAT	<i>N</i> -acetyltransferase
NESIL	No Expected Sensitization Induction Level
PET	Petrolatum
PPD	<i>p</i> -phenylenediamine
RIFM	Research Institute for Fragrance Materials
ROAT	Repeated Open Application Test
SCCS	Scientific Committee on Consumer Safety
TDA	2,5-toluenediamine
TEWL	Transepidermal Water Loss
TLC	Thin-Layer Chromatography
TNF	Tumour Necrosis Factor
QRA	Quantitative Risk Assessment

Chapter 1

General introduction

INTRODUCTION

This thesis describes studies performed within the Expert Centre for Eczematous and Occupational Dermatoses at the Department of Dermatology, University Medical Centre Groningen, the Netherlands. The thesis focuses on important aspects of contact allergy. This general introduction section provides background information about the field of contact allergy and introduces specific concepts. Subsequently, an outline of the aims of the thesis will be provided.

PREFACE

Contact allergy or contact sensitization is defined as a delayed-type IV allergic reaction of the skin within the classification by Gell and Coombs.¹ Contact allergy is caused by exposure of the skin to certain substances in the environment. Most of these substances are harmless; however, repeated exposure to substances with sensitizing potential (or a single exposure to a potent allergen) can induce contact allergy. The clinical manifestation of contact allergy is a type of inflammation of the skin that causes eczema and is known as allergic contact dermatitis (ACD). Symptoms include erythema, papules, vesicles, oozing, excoriations, squamae, induration and lichenification. Contact dermatitis is not only caused by contact allergy. Another etiological subtype includes irritant contact dermatitis (ICD). ICD is caused by exposure to irritants, which may range from a single exposure to a toxic substance to more frequent exposure to non-toxic substances. In light of the current COVID-19 pandemic, frequent hand washing is recommended, which could lead to dry skin and subsequent eczema. The potential skin issues caused by frequent hand washing are an example of ICD that is caused by frequent exposure to water, soap and disinfectants without the presence of a contact allergy. The most important difference between ACD and ICD is that ACD involves a T cell-mediated immune response, whereas ICD involves a more general inflammation mechanism. Patients can have both ACD and ICD concomitantly. Therefore, dermatitis among patients with ACD may be partially explained by exposure to irritants as well.²

PATHOPHYSIOLOGY

The development of ACD occurs in two phases. First, an individual becomes sensitized, which is known as the sensitization phase. The sensitization phase is considered the induction phase of contact allergy and is characterized by the first recognition of an allergen by the immune system. Most contact allergens have a low molecular weight of fewer than 500 Daltons and are referred to as haptens, a term chosen to avoid confusion with aero-allergens.³ The small size of haptens enables them to penetrate the stratum corneum. More than 4000 chemicals can function as haptens, including those in cosmetics, fragrances, metals, rubbers and topical medications.⁴ Haptens themselves are too small to be recognized by the immune system; therefore, a hapten must form a complex with a carrier molecule (usually a protein) to become antigenic and able to activate the innate immune system. This process is known as the haptenization process and was first introduced by Landsteiner and Jacobs in 1936.⁵ In this process, hapten-protein complexes bind to Langerhans cells (LHC), which become activated and migrate while carrying the hapten-protein complex via afferent lymphatics to regional lymph nodes. The hapten-protein

complex is presented to naïve T cells within the lymph node. The proliferation of these T cells then differentiates into memory and effector T cells in the lymph node, after which the T cells are released into the blood circulation system, completing the sensitization phase.

Individuals who are not exposed again to the causative hapten may never manifest clinical symptoms their entire lives. Because of this, sensitization (without the clinical manifestation of dermatitis) is defined as an altered immune status and is not considered a disease. If repeated exposure to the causative contact allergen results in dermatitis, however, it is considered a disease. This represents the second phase of the development of contact allergy; the elicitation phase. This phase occurs after repeated exposure to the causative hapten, during which the hapten-protein complex is again presented by the LHC. This causes the memory T cells to secrete pro-inflammatory cytokines, which results in local dermatitis. The process of ACD development requires four days to several weeks for the induction phase and one to four days for the elicitation phase.⁶ Later in this thesis, the term “allergen” is used, the definition of this term includes haptens.

CROSS-REACTIONS

Cross-reactions in contact allergy can occur when an individual who is allergic to a specific allergen also reacts to another allergen to which the individual has not yet been exposed. This phenomenon may occur if a chemically similar hapten mimics the molecular structure of the causative hapten. If the immune system is not capable of distinguishing between the two haptens, activation of the causative hapten-specific T cells may result.⁷ A typical example is a contact allergy to *p*-phenylenediamine (PPD, CAS no. 106-50-3), which can cross-react with the hair dye allergen 2,5-toluenediamine (TDA, CAS no. 95-70-5), but also among others to the local anaesthetic benzocaine (CAS no. 94-09-7) and the rubber antioxidant *N*-isopropyl-*N'*-phenyl-*p*-phenylenediamine (IPPD, CAS no. 101-72-4).⁸ Additionally, it is possible for two non-chemically related haptens to cross-react. This phenomenon may occur if one of the haptens undergoes a transformation (e.g., is metabolized in the skin or oxidized outside of the skin) that produces a structure that is chemically similar to the other hapten.⁹ Cross-reactions should be considered in finding safe alternative products that do not contain potentially cross-reacting allergens. Cross-reactivity is difficult to distinguish from co-sensitization, which is defined as simultaneous sensitization to at least two haptens after exposure to both haptens has occurred in a specific product. For example, a contact allergy to nickel (CAS no. 7440-02-0) and cobalt (CAS no. 7440-48-4) may develop after exposure to metals.

DIAGNOSTICS OF PATCH TESTING

The diagnosis of contact allergy begins with a thorough anamnesis. Patients are generally referred to the dermatology department for evaluation of dermatitis. It is vital to assess the course of the symptoms to determine whether a relationship can be established between the development of symptoms and the individual's exposure to certain products. It is also important to assess possible irritant factors because, as previously mentioned, irritants can result in dermatitis without sensitization. The diagnostic gold standard of contact allergy detection is patch testing.¹⁰ This diagnostic procedure was introduced in 1895 by the dermatologist Josef Jadassohn, univer-

sally acknowledged as 'the father of patch testing'.¹¹ The aim of patch testing is to reproduce the elicitation phase of contact allergy.

During the patch test procedure, a small amount of a chemical is diluted in a vehicle consisting of petrolatum, aqua or ethanol. The choice of vehicle depends on the characteristics of the chemical. The different vehicles are attached in patch test chambers that vary in size and material. The physician applies a standardized amount of the chemical, using a micropipette or a syringe to minimize inter-individual variation in the amount of applied chemical.¹² In recent years, prefabricated, preloaded 'ready to apply' patch tests such as the T.R.U.E. TEST have been developed to standardize patch testing procedures.¹³ Virtually all chemicals can be patch tested. The history and localisation of dermatitis can indicate potential causative allergens; however, it is insufficient to test patients only for suspected allergens, because unsuspected allergens may turn out to be the cause of dermatitis.¹⁴

The European Baseline Series (EBS) of allergens is used throughout Europe to screen dermatitis patients for the most common contact allergens. Exposure to environmental allergens constantly changes; therefore, the EBS must be continuously adapted to ensure that new allergens are detected and allergens included in the EBS remains relevant.¹⁵ The inclusion of a new allergen in the EBS is warranted if results from consecutively patch tested patients indicate a positive patch test reaction prevalence of 0.5–1.0%. The EBS was most recently revised and updated in 2019 by Wilkinson *et al.*¹⁶ Patient history and course of dermatitis remain important considerations, because additional patch test series have been developed to address specific (occupational) exposure to certain contact allergens that are not present in the EBS. Some examples of occupations that involve specific allergen exposure are hairdressers, dentists and metal workers. In these specific cases, additional patch testing with the occupation-specific series may be considered. Other examples of additional patch test series include the cosmetics series and fragrance series. In addition to testing allergens at standardized concentrations, it is possible to patch test a patient's own product that is suspected to be responsible for the contact dermatitis. Notably, testing of a patient's personal products "as is" can be challenging. The concentration from the putative allergen in the patient's product may be insufficient to detect the contact allergy; conversely, such testing may lead to active sensitization or strong irritant reactions (i.e., false positive results). These challenges can be overcome by taking an extract from the patient's product and creating a dilution, as described in chapters 4 and 5 of this thesis. A dilution series of the extract can be tested in healthy controls to preclude false positive reactions and to distinguish an irritant reaction from a true allergic reaction.¹⁷

PATCH TESTING PROCEDURE

The European Society of Contact Dermatitis (ESCD) has developed guidelines for best practice in patch testing procedures.¹⁰ Patch tests are applied to the patient's back for practical reasons, including provision of a flat surface that is sufficiently large for good occlusion. If a patient requires additional patch test series and the back is not large enough to test all series, the outer surface of the upper arms or thighs may be used. These body surfaces can also be used if the patient's upper back is unsuitable for testing due to skin disease or a tattoo on the back. The occlusion time of a patch test is the duration for which the patch test is applied to the skin. The ESCD recommends an occlusion time of 48 hours. A longer occlusion time is not recommended because active sensitization may occur. After the patch test chambers are removed, visual

readings are conducted to determine the presence of positive patch test reactions. Patch test readings can be performed on the second day (D) after the patch test has been applied at least 15–60 minutes after the patch test chambers have been removed, allowing for the resolution of pressure effects. Readings can also be conducted on the third day (D3) or fourth day (D4) after patch test removal and around D7. At least two patch test readings are required, including one reading on D2, D3 or D4 and one reading on or around D7.¹⁰ A late patch test reading (i.e., a reading that occurs between D5 and D10) is advised to increase diagnostic sensitivity.¹⁸ Failure to perform a late patch test reading may cause clinicians to miss 7–30% of positive patch test reactions. Late patch test readings are particularly important to identify reactions to topicals, as discussed in chapter 2 of this thesis.^{19–21} The patch test reading is based on morphological features of the skin reaction, including erythema, infiltration, papules, vesicles and coalescing vesicles. The different morphology outcomes lead to a reaction outcome and score, which are based on globally acknowledged reading criteria established by the International Contact Dermatitis Research Group (ICDRG; see Table 1).^{22,23}

Table 1. International Contact Dermatitis Research Group (ICDRG) patch test reading criteria

Clinical features	Reaction outcome	Score
No reaction	Negative reaction	-
Faint erythema only	Doubtful reaction	?+
Erythema, infiltration, possibly papules	Weak positive reaction	+
Erythema, infiltration, papules, vesicles	Strong positive reaction	++
Intense erythema, infiltrate, coalescing vesicles	Extreme positive reaction	+++
Various morphologies, margins are sharply demarcated and the surface of the test area shows a silk paper structure or a shiny skin	Irritant reaction	IR

ADDITIONAL DIAGNOSTIC TESTS

In addition to patch testing, other diagnostic tests can be used to detect a contact allergy. If a patch test indicates a weak positive reaction to an allergen, it does not necessarily mean that this allergen is the cause of the dermatitis. The Repeated Open Application Test (ROAT) is a clinically relevant diagnostic test often used to complement patch testing. This test can also be useful if the patch test outcome does not show any positive reaction, despite a strong suspicion of a causal relationship between exposure to the product and the development of contact dermatitis.

In the ROAT, a substance is applied twice daily to a small surface area on the volar aspect of the forearm for at least seven days. The ROAT is performed without occlusion; therefore, the potential development of irritant and false-positive reactions is minimized.^{24,25} If dermatitis occurs at the ROAT test site, a contact allergy to the product is strongly suspected. Notably, a product may contain skin irritants that create an inflammatory environment, in which case the threshold for an elicitation reaction is lowered.

Many allergens are widely used in different products, and products can be insufficiently labelled; therefore, it is sometimes difficult to identify the exact ingredients in a product.²⁶ Demonstration of allergens in a product is an important step in identifying the causative allergen in a product and thus preventing ACD.²⁷ Chemical analysis can be performed to determine the exact composition of a product and its ingredients. Examples of such analyses include thin-layer chromatography (TLC), gas chromatography (GC) and mass spectrometry (MS). Chromatography is a separation technique that allows mixtures of different chemicals to be separated. This technique is based upon a moving phase (i.e., gas or liquid) and a stationary phase (i.e., liquid or solid).²⁷ GC is often conducted in combination with MS, which is used to determine the molecular structure and molecular weight of product components. The components produce signals and are recorded on a chromatogram as peaks.²⁷ The peaks correspond to mass spectra and are compared to known mass spectra from an existing library, providing valuable information about the product's chemical structure. Chemical analysis is not a common practise; only a few centres conduct chemical analyses in the field of dermatology to identify contact allergy. In TLC, the chromatogram can be patch tested to identify the culprit allergen, as demonstrated in chapter 4 of this thesis.

EPIDEMIOLOGY

Contact allergy is a common condition. Clinical surveillance of contact allergy has been established by multiple national and international contact dermatitis research groups. Surveillance in Europe is primarily based on patch testing results with the EBS. Relevant demographic and clinical data are also commonly collected. The goal of these surveillance strategies is to monitor the prevalence of contact allergies to identify emerging allergens, detect new allergens that exist beyond the local catchment area and evaluate trends over time.²⁸ An example of a large European surveillance meta-network is the European Surveillance System on Contact Allergies (ESSCA, <https://www.essca-dc.org>). Patch test results from the ESSCA network from 2015–2018 based on the EBS indicate a prevalence of 52.1% positive patch test reactions to at least one baseline series allergen in referred dermatological patients. Females were more frequently sensitized than males (67.1% and 32.9%, respectively).²⁹ Studies of the prevalence of contact allergy in the general population have also been conducted. For example, the European Dermato-Epidemiology Network (EDEN) Fragrance study consisted of a large cross-sectional epidemiological study of individuals from the European general population. The study findings indicate at least one positive patch test reaction in 27.0% of participants.³⁰ The patch tested population included in this cohort study is further investigated in chapter 3, specifically regarding their exposure to scented products and fragrance contact allergy. The prevalence of contact allergy in the general population has been studied in a recent systematic review and meta-analysis, which yielded a pooled prevalence of 20.1%, including a 16.5% prevalence in children and adolescents. As in other studies, the prevalence was significantly higher among females (27.9%) than among

males (13.2%) in the overall population.³¹ The high prevalence of contact allergy in the general population is primarily driven by contact allergy to nickel. In both the clinical population and the general population, nickel is the most common allergen (17.6% and 11.4%, respectively), followed by fragrance mix I (6.9% and 3.5%, respectively).^{29,31} About half of clinical dermatitis patients and 20% of the general population are sensitized to one or more contact allergens. The effects of ACD can be significant and may include occupational consequences and impaired quality of life.³² Contact allergy is considered a lifelong condition that cannot be cured. Therefore, the management of contact allergy focuses on identifying the culprit allergen and avoiding of the causative exposure.

EXPOSURE

The development of contact allergy cannot occur without exposure to a causative allergen. The estimation of skin exposure to substances in the environment is challenging. Studies have found that neither the total applied dose of a substance nor the size of the exposed skin area determines the sensitization rate. Rather, the measurement of 'dose per unit area' has been identified as the optimal quantitative approach to express skin exposure in relation to skin sensitization.^{33,34} Repeated and/or aggregated exposure to an allergen that may be present in multiple products used by the same individual should also be considered.³⁵ Exposure can be heightened for individuals working in specific occupations (e.g., hairdressers or health care workers). Therefore, occupation-specific patch test series have been developed to include the most frequent sensitizers from the corresponding industry, as mentioned previously. Heightened exposure to allergens is also observed among patients with specific skin conditions (e.g., high exposure to topical medication for leg dermatitis patients).³⁶ Among dermatitis patients, females are more often exposed to allergens than males, and females consequently have a higher prevalence of contact allergy.³⁷ The important topic of exposure will be further addressed in chapter 3 of this thesis.

INDIVIDUAL SUSCEPTIBILITY

Exposure is the most important determinant of whether an individual will develop a contact allergy; however, differences in individual susceptibility must also be considered. If multiple individuals are exposed to the same allergens to a similar degree, it remains unclear which individuals are more or less likely to develop a contact allergy. Several factors may contribute to differences in patch test outcomes between individuals, including differences in patch testing procedures and inter-individual differences. Sex, age and ethnicity have all been found to influence an individual's susceptibility to contact allergy, which will be further elaborated upon in the discussion section of this thesis.

Schnuch *et al.* reviewed the genetic factors involved in contact allergy and concluded that there are likely multiple genes that contribute to the development of contact allergy. Presumably, these genes act in combination and each has a relatively small effect.³⁸ Several genes have been investigated with regard to their role in contact allergy. Some areas in which polymorphisms may play a function-based role include the pathways that influence allergen availability to the organism, as well as inflammatory and immunological factors.³⁸ There is evidence that the genes that encode for tumour necrosis factor (TNF) and pro-inflammatory cytokines Interleukin (IL)-10

and IL-16 may be altered, and such alterations may be a risk factor in contact allergy development.^{39–41} Mutations in the filaggrin gene (FLG) have demonstrated inter-individual variation in transepidermal water loss and skin pH, and these variations may be associated with a decreased or increased susceptibility to contact allergy.^{42,43} Another study found that FLG mutations are associated with contact allergy among dermatitis patients.⁴⁴ The enzyme *N*-acetyltransferase (NAT) 1, encoded by the NAT1 gene, has been found to influence allergen metabolism. NAT1 is expressed among others in keratinocytes in human skin, and it is important in the biotransformation of aromatic amines, an important example being such as PPD, a hair dye molecule. As first described by Kawakubo *et al.*, unoxidized PPD that penetrates the skin is *N*-acetylated by NAT1 into mono-acetyl PPD (MAPPD) and diacetyl PPD (DAPPD).⁴⁵ In local lymph node assay these acetylated products have shown little to no sensitizing potential.⁴⁶ Based on the assumption that PPD acetylation by NAT1 can be considered a detoxification process with respect to sensitization, genotyping was performed in 147 PPD cases and compared to control. Fast-acetylation phenotypes were less prone to be sensitized to PPD, while slow-acetylator phenotypes were overrepresented in PPD sensitized individuals.⁴⁷ It is hypothesized that slow acetylation phenotypes are less able to *n*-acetylate PPD; therefore, a larger amount of PPD is available to induce sensitization. This topic is further discussed in chapters 6 and 7.

AIMS AND OUTLINE OF THIS THESIS

Several important aspects of contact allergy are reflected in the outline of this thesis.

Part I of the thesis explores the occurrence of contact allergy and examines various facets related to the occurrence of contact allergy, including exposure (specifically to fragrances) and diagnostic methods. **Chapter 2** presents a cross-sectional cohort study of clinical dermatitis patients. Patch test readings performed at D3 and D7 are analysed separately to investigate the additional value of the D7 patch test reading. **Chapter 3** presents a cross-sectional cohort study of individuals from the European general population. Exposure to multiple scented products used in daily life is estimated, and associations with fragrance contact allergy are explored. **Chapter 4** describes a chemical analysis of a hospital wristband used to identify the culprit allergen. **Chapter 5** reports two case studies of patients who used the same toothpaste brand and developed ACD to stannous (tin) fluoride.

Part II of this thesis comprises elements of individual susceptibility to contact allergy. **Chapter 6** investigates the expression pattern of NAT1 in human skin using immunofluorescence. Within the framework of a possible increased susceptibility based on a genetic trait, the *N*-acetylation status of two well-characterized highly PPD sensitive patients is investigated in **chapter 7**.

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PART I

Occurrence

Chapter 2

New positive patch test reactions on day 7-
The additional value of the day 7 patch test reading

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ABSTRACT

Background

Not performing a day (D) 7 patch test reading might result in positive patch test reactions being missed.

Objective

To investigate the added value of the D7 patch test reading for individual allergens, and to identify patient characteristics and allergen groups associated with new positive D7 reactions.

Methods

Data from patients patch tested between 2008 and 2018 with the extended European baseline series were analysed. Patch test readings were performed on D3 and D7. Positive reactions were categorized into positive on D3 or new positive on D7.

Results

A total of 3292 patients were consecutively patch tested with at least 43 allergens of the TRUE Test panels 1 and 2 supplemented with investigator-loaded allergens. In total, 447 (13.6%) patients showed new positive D7 reactions. In univariable regression analysis, age between 18 and 30 years showed a negative association with new positive D7 reactions. Significantly more D7 positive reactions were seen for topicals other than corticosteroids (odds ratio [OR] 2.60, 95% confidence interval [CI]: 1.92-3.51) and topical corticosteroids (OR 1.87, 95% CI: 1.09-3.21). No associations were found between sex, atopic dermatitis and occupational dermatitis and a new positive D7 reaction.

Conclusion

A D7 reading to identify new positive patch test reactions is of added value, especially for topicals other than corticosteroids and topical corticosteroids.

INTRODUCTION

According to the European Society of Contact Dermatitis (ESCD) guideline for diagnostic patch testing, it is recommended to perform at least two readings, on day (D) 2, D3, or D4, and around D7.¹ Most centres perform patch test readings on D2 to D4. A late patch test reading on D7 in addition to the D3 or D4 reading can identify new positive patch test reactions, ranging from 3% up to 34% in previous studies, which were found to be negative, doubtful or irritant on preceding readings.²⁻⁹ Multiple studies have reported the importance of an additional late patch test reading, especially for suspected contact allergies to metals, topicals other than corticosteroids, and topical corticosteroids. However, the allergens that have shown new positive reactions at an additional late patch test reading vary in the literature.^{7,10,11} It is unknown whether there are associated factors, for example, patient characteristics, that may enhance the tendency to develop new positive reactions at a D7 reading. Only one study evaluated sex and age as possible associated factors, and found a significantly higher rate of new positive D7 reactions in women than in men and in patients aged >40 years.⁷ The objectives of the current study were to evaluate the added value of the D7 patch test reading for individual allergens and allergen groups, and to identify factors associated with new positive D7 reactions.

METHODS

Study design and patch testing

We performed a retrospective data analysis on patch test data collected between January 2008 and July 2018 for consecutively patch tested patients who were routinely tested with our extended European baseline series (EBS). TRUE Test panels 1 and 2 (SmartPractice Europe, Reinbek, Germany) supplemented with additional investigator-loaded allergens (SmartPractice Europe, and Chemotechnique Diagnostics, Vellinge, Sweden) tested in Van der Bend square chambers (Van der Bend, Brielle, The Netherlands) were applied on the back for 48 hours under occlusion. Patch test readings were performed on D3 and D7. The readings were performed by experienced dermatologists according to the ESCD guideline.¹ Weak (+), strong (++) and extreme (+++) reactions were classified as positive reactions. Reactions reported as irritant, doubtful (?+) or follicular were counted as negative reactions in addition to the negative results. In addition, patch test results reported as irritant, doubtful or follicular at the D3 reading that became positive on D7 were evaluated separately. Reactions were evaluated as irritant if margins were sharply demarcated and the surface of the test area showed a silk paper structure or a shiny skin. Reactions were considered to be doubtful if erythema and/or infiltration did not cover the whole test area.¹²

Data analysis

Positive reactions were categorized into positive on D3 or new positive on D7 (labelled as negative on D3). For patients who had been tested more than once, only the most recent patch test result was evaluated. To evaluate patient characteristics, three groups of patients were defined: patients with only D3 positive reactions and no new positive D7 reactions; patients with only new D7 positive reactions corresponding to D3 negative reactions; and patients with both D3

positive reactions and new D7 positive reactions. The patient characteristics that were analysed were age, sex, atopic dermatitis in the patient's lifetime, and occupational dermatitis. Age was categorized into the following groups: <18, 18 to 30, 31 to 45, 46 to 60, and ≥ 61 years. To analyse the influence of type of allergen, allergens were grouped on the basis of chemical structure, cross-reactivity, and cosensitization, resulting in seven groups: metals, preservatives, fragrances, rubbers, dyes, topical medicaments ("topicals"), and topical corticosteroids (Table 1).^{13,14} A positive reaction to at least one of the allergens in the group was counted as an overall positive group reaction.

Table 1. Overview of the composition of each allergen group used for univariable regression analysis

Allergen group	Composition
Metals	Nickel sulfate, potassium dichromate, cobalt chloride
Preservatives	Paraben mix, MCI/MI, quaternium-15, formaldehyde, thiomersal, MI, MDBGN, 2-bromo-2-nitropropane-1,3-diol, diazolidinyl urea, imidiazolidinyl urea, 1,2-benzisothiazolin-3-one
Fragrances	FM I, Myroxylon pereirae resin (balsam of Peru), FM II, HICC
Rubber additives	Carba mix, black rubber mix, MBT, mercapto mix, thiuram mix
Dyes	p-Phenylenediamine, textile dye mix, Disperse Blue 106, Disperse Blue 124, toluene-2,5-diamine
Topicals	Neomycin sulfate, lanolin alcohols, caine mix, quinoline mix, sesquiterpene lactone mix, parthenolide, Amerchol L 101
Corticosteroids	Budesonide, tixocortol pivalate, hydrocortisone-17-butyrate

FM I, fragrance mix I; FM II, fragrance mix II; HICC, hydroxyisohexyl 3-cyclohexene carboxaldehyde (Lyrall); MBT, mercaptobenzo-thiazole; MCI, methylchloroisothiazolinone; MDBGN, methyldibromo glutaronitrile; MI, methylisothiazolinone

Statistics

Descriptive data are presented in tables as numbers with percentages and 95% confidence intervals (CIs). Prevalences are presented as both crude and age-standardized estimates with accompanying 95% CIs. The European standard population of 2013 was used as the reference for age and sex standardization.¹⁵ Univariable logistic regression was performed to analyse the association between patient characteristics and allergen groups on new positive D7 reactions, and these were expressed as odds ratio (ORs) with 95% CIs. Statistical analyses were performed with SPSS v.23 (IBM, Armonk, New York) and Excel 2013 (Microsoft, Redmond, Washington).

RESULTS

New D7 positive patch test reactions

A total of 3292 patients (67.0% female, 33.0% male, mean age 42.7 ± 16.9 years) were consecutively patch tested with at least 43 allergens of the TRUE Test panels 1 and 2 supplemented with investigator-loaded allergens. The sociodemographic characteristics of the total patch tested group and the subgroup of patients with at least one positive reaction are shown in Table 2.

Table 2. Sociodemographic characteristics of the patch tested population

	Total tested (n = 3292)			Reacted positively on D3 and/or D7 (n = 1653)		
	n	%	95% CI	n	%	95% CI
Sex						
Male	1087	33.0	31.4–34.6	453	27.4	25.3–29.6
Female	2205	67.0	65.4–68.6	1200	72.6	70.4–74.7
Age (y)						
<18	112	3.4	2.8–4.0	42	2.5	1.8–3.3
18–30	918	27.9	26.4–29.4	384	23.2	21.2–25.3
31–45	787	23.9	22.4–25.4	425	25.7	23.6–27.8
46–60	912	27.7	26.2–29.2	525	31.8	29.5–34.0
≥61	563	17.1	15.8–18.4	277	16.8	15.0–18.6
Atopic dermatitis (lifetime prevalence)						
Yes	1409	42.8	41.1–44.5	701	42.4	40.0–44.8
No	1883	57.2	55.5–58.9	952	57.6	55.2–60.0
Occupational dermatitis						
Yes	715	21.7	19.9–23.5	388	23.5	21.4–25.5
No	2577	78.3	76.9–79.7	1265	76.5	74.5–78.6

CI, confidence interval; y, year.

A total of 1653 patients (50.2%) had at least one positive reaction on D3 and/or D7. Of the total 3292 patch tested patients, 189 (5.7%) showed only new positive D7 reactions (i.e. read as negative, doubtful or irritant on D3) and another 258 (7.8%) patients showed positive D3 reactions with additional new positive D7 reactions. Thus, in total, 447 (13.6%) of the patch tested patients had new positive D7 patch test reactions. In Table 3 both crude and age-standardized and sex-standardized prevalences of the total number of positive reactions on D3 and D7 are

Table 3. Patch test results obtained with the baseline series with crude and age-standardized and sex-standardized prevalences of positive reactions on day (D) 3 and/or D7 accompanied by 95% confidence intervals (CIs)

New positive D7 reactions																			
Haptens	Concentration (µg/cm²)	Total patch tested patients (n = 3292)		Total crude positives on D3 and/or D7 (n = 1653)		Total age -standardized and sex- standardized positives on D3 and/ or D7				Total			Reaction type at D3 reading (n)			Reaction strength on D7 (n)			Proportion of total positive reactions
		n	%	n	%	n	%	95%CI	n	%	-	?	IR	+	++	+++	%		
Nickel sulfate	200	3172	607	19.1	424	14.5	12.2-14.6	104	3.3	64	22	18*	96	8	0			17.1	
Lanolin alcohols	1000	3218	44	1.4	41	1.4	0.9-1.7	10	0.3	4	6	0	9	1	0			22.7	
Neomycin sulfate	230	3209	27	0.8	25	0.9	0.5-1.1	22	0.7	21	1	0	20	2	0			81.5	
Potassium dichromate	23	3206	122	3.8	112	3.8	2.9-4.1	18	0.6	11	7	0	16	2	0			14.8	
Caine mix	630	3217	34	1.1	27	0.9	0.5-1.2	9	0.3	9	0	0	7	2	0			26.5	
Fragrance mix I	430	3216	138	4.3	115	3.9	2.9-4.2	21	0.7	16	4	1	21	0	0			15.2	
Colophonium	850	3204	110	3.4	95	3.2	2.4-3.6	17	0.5	15	2	0	15	2	0			15.5	
Epoxy resin	0.05% pet.	3216	91	2.8	92	3.1	2.3-3.4	28	0.9	20	7	1	24	3	1			30.8	
Quinoline mix	190	3218	7	0.2	9	0.3	0.1-0.5	2	0.1	2	0	0	1	1	0			28.6	

Myroxylon pereiirae (balsam of Peru)	800	3217	89	2.8	59	2.0	1.4-2.3	19	0.6	15	3	1	19	0	0	21.3
Ethylene diamine dihydrochloride	50	3218	29	0.9	34	1.2	0.7-1.4	3	0.1	1	1	1 ^a	3	0	0	10.3
Cobalt chloride	20	3211	175	5.5	160	5.4	4.2-5.7	25	0.8	17	6	2 ^a	23	2	0	14.3
PTBP-FR	40	3207	92	2.9	78	2.7	1.9-3.0	17	0.5	11	6	0	16	1	0	18.5
Parabens mix	1000	3218	9	0.3	9	0.3	0.1-0.5	2	0.1	1	1	0	2	0	0	22.2
Carbamix	250	3217	117	3.6	125	4.2	3.2-4.6	4	0.1	4	0	0	4	0	0	3.4
Black rubber mix	75	3218	34	1.1	28	0.9	0.5-1.2	7	0.2	6	1	0	7	0	0	20.6
MCI/MI	4	3219	331	10.3	312	10.6	8.7-10.7	23	0.7	18	5	0	20	3	0	6.9
Quaternium-15	100	3218	61	1.9	45	1.5	1.0-1.8	15	0.5	12	3	0	14	1	0	24.6
Mercapto benzothiazole	75	3217	34	1.1	34	1.2	0.7-1.4	1	0.0	1	0	0	1	0	0	2.9
p-Phenylenediamine	90	3210	105	3.3	83	2.8	2.0-3.1	13	0.4	7	6	0	11	2	0	12.4
Formaldehyde	180	3218	51	1.6	38	1.3	0.8-1.6	13	0.4	8	5	0	11	2	0	25.5
Mercapto mix	75	3217	43	1.3	46	1.6	1.0-1.8	2	0.1	1	1	0	2	0	0	4.7

Table continues on next page

Table 3 continued

New positive D7 reactions																
Haptens	Concentration (µg/cm ²)	Total patch tested patients (n = 3292)		Total crude positives on D3 and/or D7 (n = 1653)		Total age-standardized and sex-standardized positives on D3 and/ or D7		Reaction type at D3 reading (n)				Reaction strength on D7 (n)			Proportion of total positive reactions	
		n	%	n	%	n	%	Total	-	?	IR	+	++	+++		
Thiomersal	8	3209	40	1.2	30	1.0	0.6-1.3	9	0.3	7	2	0	9	0	0	22.5
Thiuram mix	25	3207	76	2.4	73	2.5	1.8-2.8	9	0.3	6	3	0	9	0	0	11.8
MI	0.2% aq.	466	32	6.9	31	7.2	4.4-8.9	3	0.6	2	1	0	2	1	0	9.4
Fragrance mix II	14% pet.	3275	246	7.5	181	6.0	4.7-6.3	33	1.0	14	18	1	33	0	0	13.4
HICC	5% pet.	3242	98	3.0	72	2.4	1.7-2.7	22	0.7	17	5	0	22	0	0	22.4
Sesquiterpene lactone mix	0.1% pet.	3277	43	1.3	40	1.3	0.8-1.6	13	0.4	10	3	0	11	2	0	30.2
Pathenolide	0.1% pet.	3233	42	1.3	37	1.2	0.8-1.5	14	0.4	12	2	0	9	5	0	33.3
MDBG	0.5% pet.	3247	127	3.9	134	4.5	3.4-4.8	17	0.5	7	10	0	17	0	0	13.4
Budesonide	0.1% pet.	3276	26	0.8	22	0.7	0.4-1.0	11	0.3	7	4	0	9	2	0	42.3

Tixocortol-21-pivalate	0.1% pet.	3277	27	0.8	29	1.0	0.6-1.2	4	0.1	2	2	0	4	0	0	14.8
Hydrocortisone-17-butyrate	1% eth.	3250	14	0.4	12	0.4	0.2-0.6	4	0.1	2	2	0	4	0	0	28.6
Textile dye mix	6.6% pet.	991	52	5.2	47	5.0	3.4-6.1	12	1.2	8	4	0	11	1	0	23.1
Disperse Blue 106	1% pet.	3250	31	1.0	27	0.9	0.5-1.1	9	0.3	8	1	0	9	0	0	29.0
Disperse Blue 124	1% pet.	3274	27	0.8	23	0.8	0.4-1.0	10	0.3	10	0	0	10	0	0	37.0
Toluene-2,5-diamine	1% pet.	2333	48	2.1	35	1.6	1.0-2.0	9	0.4	7	2	0	8	1	0	18.8
Sodium disulfite	1% pet.	395	35	8.9	35	9.6	6.1-11.7	6	1.5	2	4	0	6	0	0	17.1
2-Bromo-2-nitropropane-1,3-diol	0.5% pet.	3250	22	0.7	22	0.7	0.4-1.0	11	0.3	7	4	0	11	0	0	50.0
Diazolidinyl urea	2% pet.	3250	29	0.9	21	0.7	0.4-0.9	12	0.4	9	3	0	11	1	0	41.4
Imidiazolidinyl urea	2% pet.	3250	26	0.8	20	0.7	0.3-0.9	7	0.2	3	4	0	6	1	0	26.9
1,2-Benzisothiazolin-3-one	0.05% pet.	3230	21	0.7	25	0.8	0.5-1.1	2	0.1	0	2	0	2	0	0	9.5
Amerchol L 101	50% pet.	606	19	3.1	14	2.5	1.1-3.5	2	0.3	1	1	0	2	0	0	10.5

CI, confidence interval; D, day; H1CC, hydroxylisohexyl 3-cyclohexene carboxaldehyde (Lyrall); IR, irritant reaction; MCI, methylchloroisothiazolinone; MDBGN, methyl dibromo glutaronitrile (dibromodicyanobutane); MI, methylisothiazolinone; PTBP-FR, p-tert-butylphenol formaldehyde resin.

^a Follicular reactions: nickel sulfate, n = 18; ethylenediamine dihydrochloride, n = 1; cobalt chloride, n = 2.

shown together with 95% CIs. The allergens with the highest proportions of new positive D7 reactions (new positive D7 reactions/total positive reactions) in order of frequency were neomycin sulfate (81.5%, 22/27 patients), 2-bromo-2-nitropropane-1,3-diol (50%, 11/22 patients), budesonide (42.3%, 11/26 patients) and diazolidinyl urea (41.4%, 12/29 patients). For groups of allergens, the proportions of new positive D7 reactions were, in order of decreasing frequency, most frequent for topicals (33.3%, 72/216 patients), topical corticosteroids (28.4%, 19/67 patients), dyes (20.2%, 53/263 patients), fragrances (16.6%, 95/572), metals (16.3%, 147/904), preservatives (15.1%, 113/749), and rubber additives (7.6%, 23/304).

Regression analysis

A univariable logistic regression analysis was performed to investigate patient characteristics and allergen groups possibly associated with the occurrence of new positive D7 reactions. The results are shown in Table 4. For age, a significant negative association was found in the age group 18 to 30 years as compared with the age group ≥ 61 years (OR 0.58, 95% CI: 0.36–0.93). No significant associations were found for sex, atopic dermatitis in the patient's lifetime and occupational dermatitis and a new positive D7 reaction. Significant associations were found for the allergen groups of topicals (OR 2.60, 95% CI 1.92–3.51) and topical corticosteroids (OR 1.87, 95% CI 1.09–3.21) and a new positive D7 reaction. Rubbers showed the lowest prevalence (7.6%) of new positive D7 reactions, and were significantly negatively associated with new positive D7 reactions (OR = 0.37, 95% CI: 0.24–0.57). Table S1 shows the prevalence of new positive D7 reactions in each age group for each allergen group.

Reaction Strength

The reaction strength of the new positive D7 reactions and patch test results of the D3 reading are shown in Table 3. Of the 595 new positive D7 reactions in 189 patients, 548 (92.1%) were weak positive (+) and 46 (7.7%) were strong positive (++), especially to nickel sulfate, parthenolide, methylchloroisothiazolinone/methylisothiazolinone, colophonium, and epoxy resin. Only one extreme positive (+++) reaction was seen, namely, to epoxy resin. Of all new positive D7 reactions, 164 of 595 (27.6%) were regarded as doubtful and 4 of 595 (0.7%) were regarded as irritant at the D3 reading. Besides irritant and/or doubtful reactions, follicular reactions were seen to nickel sulfate (18 of 104 positive D7 reactions), cobalt chloride (2 of 25 positive D7 reactions), and ethylenediamine dihydrochloride (1 of 3 positive D7 reactions).

Table 4. Logistic regression analysis with new positive patch test reactions on day 7 as outcome

Factors ^a	Patients (n = 189)			
		n (%)	OR	95% CI
Sex	Male	56 (29.6)	1.00 (ref.)	
	Female	133 (70.4)	1.13	0.81-1.58
Age (y)	<18	6 (3.2)	0.96	0.38-2.42
	18-30*	35 (18.5)	0.58	0.36-0.93
	31-45	48 (25.4)	0.73	0.47-1.15
	46-60	59 (31.2)	0.73	0.48-1.12
	≥61	41 (21.7)	(ref.)	
Atopic dermatitis in lifetime	Yes	78 (41.3)	1.05	0.78-1.43
	No	111 (58.7)	1.00 (ref.)	
Occupational dermatitis	Yes	36 (19.0)	1.35	0.92-1.97
	No	153 (81.0)	1.0 (ref.)	
Allergen groups ^b	Allergens (n = 522)			
Metals (other = ref.)	Metals	147 (28.2)	0.94	0.76-1.16
Preservatives (other = ref.)	Preservatives	113 (21.6)	0.82	0.66-1.03
Fragrances (other = ref.)	Fragrances	95 (18.2)	0.99	0.78-1.26
Rubbers (other = ref.)	Rubbers**	23 (4.4)	0.37	0.24-0.57
Dyes (other = ref.)	Dyes	53 (10.2)	1.28	0.93-1.76
Topicals (other = ref.)	Topicals**	72 (13.8)	2.60	1.92-3.51
Corticosteroids (other = ref.)	Corticosteroids***	19 (3.6)	1.87	1.09-3.21

CI, confidence interval; OR, odds ratio; ref., reference; y, year.

^aUnivariable.^bUnivariable regression analysis controlled for age and sex.

*P = 0.025; **P < 0.001; ***P = 0.024.

DISCUSSION

The present analysis provided estimates of the prevalence of new positive D7 patch test reactions in patients consecutively patch tested with the extended European baseline series and evaluated possible associated factors. Contact allergies would have been missed in 13.6% of the patch tested patients without a D7 reading.

In the current study, the prevalence of new positive D7 reactions was significantly associated with the allergen groups topicals and topical corticosteroids. In multiple studies addressing the added value of a late patch test reading, neomycin sulfate has been the most frequently reported allergen associated with new positive reactions at a late reading.^{2,7,10,16} This is in agreement with our findings, in which neomycin sulfate was also the allergen with the highest proportion (81.5%) of new positive D7 reactions. A review by Macdonald *et al.* on the characteristics of neomycin sulfate reported slow local absorption through the skin and slow local immunological reactivity. Furthermore, the possibility of a reservoir of neomycin being retained in the stratum corneum for a long time could be a factor contributing to the high share of new positive reactions at a late patch test reading.¹⁷ Although neomycin sulfate showed the highest proportion (new positive D7 reactions/total positive reactions), the total prevalence of new positive D7 reactions in all patch tested patients was only 0.7% (22/3209).

There have been conflicting results of studies on topical corticosteroids and the association with new positive D7 reactions. In our patients, topical corticosteroids were significantly associated with new positive D7 reactions, and gave a proportion of 28.4% new positive D7 reactions for all positively tested patients. A recent study by Chaudry *et al.* reported that readings after D5 provided limited information, as no new reactions were seen to 0.01% budesonide, 1% clobetasol-17-propionate, 1% hydrocortisone-17-butyrate alcohol and 1% triamcinolone acetonide in 298 evaluated patients.¹⁰ It is interesting to note that budesonide was tested at a higher concentration of 0.1% in our baseline series. Davis *et al.* evaluated 135 patients, and only two patients showed positive patch test reactions to corticosteroids, on D7 and D9. In their experience, the extended reading was of limited value.¹⁸ A study by Higgins *et al.* presented 203 patients; no new positive reactions were observed to corticosteroids.⁶ Conversely, Madsen *et al.* found a second reading on D6/D7 to be important for hydrocortisone-17-butyrate and budesonide.⁷ Because of the anti-inflammatory effect, false-negative patch test reactions at early readings can be expected when corticosteroids are tested at high concentrations.^{19,20} Furthermore, topical corticosteroids induce local vasoconstriction with initial blanching and/or later vasodilatation. A possible positive patch test reaction could be confused with weak erythema resulting from vasodilatation. A further confounding issue is the "edge effect" phenomenon. In a classic positive patch test reaction, induration, erythema and infiltration cover the whole area. The edge effect is the manifestation of the skin reaction at the outer edge of the patch test site. It is believed that the anti-inflammatory effect of high concentrations predominates in the centre, and low concentrations prevail at the periphery. An earlier study investigating the edge effect in patch testing with budesonide found that these reactions become more clearly positive at later readings, further supporting the need for a late reading.²¹ The differences in patch test concentrations between our study and recent studies, in addition to the challenges of patch test reading resulting from the anti-inflammatory nature of corticosteroids, might explain the conflicting results in the literature.

In our cohort, a total of 607 patients reacted positively to nickel sulfate, and 104 (17.1%) of these reactions were new positive D7 reactions. It is interesting to note that 18 of these 104

new positive D7 reactions were labelled as follicular reactions at the corresponding D3 reading. Furthermore, nickel sulfate was the allergen with the highest number ($n = 22$) of doubtful or irritant reactions on D3 that became strong positive ($++$) by D7. A study by Thomas *et al.* showed nickel sulfate to be the allergen with the second highest number of new positive D6 reactions. Of the 32 positive reactions, 11 (34.4%) appeared on D6.¹¹ Ahlgren *et al.* found a significantly higher frequency of new positive D7 reactions to metals, especially to mercury, nickel, gold, and cobalt.⁵ Chaudry *et al.* reported late readings to be useful for metals, especially for gold sodium thiosulfate, which showed the highest prevalence of new positive reactions after D5.¹⁰ In our regression analysis, the group metals was not significantly associated with new positive D7 reactions as compared with other groups of allergens. However, our metal group consisted only of nickel sulfate, potassium dichromate and cobalt chloride from the European baseline series.

In the current analysis, the rubbers group was the only allergen group that was significantly less associated with new positive D7 reactions. Literature reports on the prevalence of rubbers showing new positive reactions at additional (late) patch test readings are scarce. A study by Madsen *et al.* evaluated a second patch test reading on D6/D7 in patients who were tested with at least TRUE Test panels 1 and 2. Mercaptobenzothiazole and carba mix (together with paraben mix) were the only allergens that did not show new positive reactions at an additional reading, which is in agreement with our findings.⁷

Associated factors

In general, contact allergies may be associated with several patient characteristics. These include sex, age, and occupational dermatitis.^{22–30} Women tend to have a higher prevalence of contact allergies than men.²⁶ Furthermore, several studies have evaluated the effect of age on the elicitation of contact allergy. Kwangsuksith *et al.* reported a decrease in reactivity upon primary exposure to new antigens in older individuals, owing to senescence of the immune system.²⁶ In our study, sex was not significantly associated with a higher prevalence of new positive reactions on D7. A study by Madsen *et al.* reported significantly more new positive reactions in women and in patients aged >40 years.⁷ In the current study, an age of 18 to 30 years were significantly less associated with new positive D7 reactions than an age of ≥ 61 years. However, allergens could be a confounding factor. The allergen with the highest proportion of new D7 reactions was neomycin sulfate. The use of neomycin sulfate in topicals in The Netherlands was reduced years ago. Consequently, it was mainly the elderly who were exposed, which could explain the lower proportion of new positive reactions in the age group 18 to 30 years. Logistic regression analysis was controlled for age and sex. Nevertheless, we found a significant association for age in all subgroups of allergens analysed. Thus, the effect of age can be only partly moderated by the exposure to allergens. For patients suffering from occupational dermatitis, it could be hypothesized that sensitization occurs earlier because of a high probability of impaired barrier function of the skin resulting from concomitant exposure to irritants, allowing for more rapid penetration of the contact allergen.^{23,30} In the current analysis, no differences were observed for the presence of occupational dermatitis between patients with new positive D7 reactions and patients with positive D3 reactions. Concerning the overall prevalence of contact allergy in atopic versus non-atopic dermatitis patients, Johansen *et al.* concluded that there were no significant differences.³¹ In the current analysis, the prevalence of new positive D7 reactions was also comparable between atopic and non-atopic dermatitis patients.

Strength and limitations

The added value of a D7 reading has been underscored by previous studies, but these investigations have mostly been performed on small populations, whereas the current study was performed on a large sample. A novel aspect was the investigation of patient characteristics in relation to late positive reactions. One limitation is the retrospective nature of the study. Furthermore, it is challenging to compare publications about new (delayed) positive patch test reactions at additional (late) patch test readings. Patch test materials and concentrations, vehicles, techniques and patch test interpretation differ between studies. Furthermore, variations in terminology and day of the additional patch test reading (D5-D9) do not always match in the compared studies.

CONCLUSION

The results of the present analysis support the importance of an additional late patch test reading on D7. Especially for topicals and corticosteroids, the share of new D7 reactions is high. Our results show that, in patients tested with True Test panel 1 and 2 and additional allergens, 13.6% of positive reactions are missed if no D7 reading is performed. Therefore, it is recommended to perform a D7 patch test reading to detect new, positive patch test reactions.

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Table S1. Relative number of new positive day (D) 7 reactions in each age group for each allergen group

		<div> <div>< 18 years</div> <div>18 – 30 years</div> <div>31 – 45 years</div> <div>46 – 60 years</div> <div>≥ 61 years</div> </div>				
		n	n (%)	n (%)	n (%)	n (%)
New positive D7 reactions to						
Metals	147	3 (2.0)	24 (16.3)	43 (29.3)	50 (34.0)	27 (18.4)
Preservatives	113	0 (0.0)	14 (12.4)	30 (26.5)	40 (35.4)	29 (25.7)
Fragrances	95	1 (1.1)	30 (31.6)	19 (20.0)	27 (28.4)	18 (18.9)
Rubbers	23	1 (4.3)	7 (30.4)	4 (17.4)	6 (26.1)	5 (21.7)
Dyes	53	2 (3.8)	12 (22.6)	15 (28.3)	13 (24.5)	11 (20.8)
Topicals	72	1 (1.4)	8 (11.1)	12 (16.7)	29 (40.3)	22 (30.6)
Corticosteroids	19	0 (0.0)	2 (10.5)	6 (31.6)	6 (31.6)	5 (26.3)

Chapter 3

Skin exposure to scented products used in daily life and fragrance
contact allergy in the European general population -
The EDEN Fragrance Study

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ABSTRACT

Background

Fragrances are widely used in scented products used in daily life with the potential to induce skin sensitization.

Objective

To evaluate exposure to scented products and to explore associations between exposure and fragrance contact allergy.

Methods

A cross-sectional study on individuals from 18 to 74 years of age, who were randomly selected from the general population in five European countries. A random sample (n= 3119) was patch tested and interviewed on exposure to scented products.

Results

Female participants were strongly associated with exposure to scented products relative to male participants. Participants age 40 years and older showed an inverse association with exposure to scented products. Compared to Sweden, The Netherlands followed by Germany showed the highest overall exposure to scented products. Sensitive skin was associated with exposure to scented products and with fragrance allergy. In univariable regression analysis, exposure to leave-on products and to specific scented product subgroups was significantly associated with fragrance allergy.

Conclusion

Exposure to scented products depends primarily on sex and age. Female sex and sensitive skin are relevant indicators for developing fragrance allergy. Because aggregate exposure, especially to scented leave-on products, may enhance the prevalence of contact allergy to fragrances, further investigations into exposure amounts and frequencies is warranted.

INTRODUCTION

Fragrances are used widely in scented products used in daily life. These products can be intended to be left on the skin such as creams and perfumes, intended to be rinsed off the skin such as shampoos or shaving products, or intended to be used as household items. Scented products contain low-molecular-weight chemicals that may have the potency to induce skin sensitization and subsequently, at sufficient exposure levels, cause allergic contact dermatitis.^{1,2} The most common allergen found at most centers after patch testing consecutive patients with the baseline series, after nickel, is Fragrance Mix I (FM I).³⁻⁶ In the European clinical population the prevalence of contact allergy to FM I was 7.8%.⁷ In the European general population, the prevalence of contact allergy to FM I was reported in a systematic review and meta-analysis yielding a prevalence of 3.5%.³ Another study from the general population showed that fragrance contact allergy was associated with self-reported cosmetic dermatitis.⁸ Multiple studies have evaluated the safety of chemicals in scented products and/or used quantitative risk assessment (QRA) to evaluate the induction of sensitization resulting from exposure to scented products.⁹⁻¹² These studies were carried out by calculating product use data using product information sheets or market information databases. Studies evaluating overall real life exposure to scented products are scarce.

The European Dermato-Epidemiology Network (EDEN) Fragrance Study is a large cross-sectional epidemiological study on a random sample of adults from the European general population.¹³⁻²⁰ The aim of this study is to investigate the overall real life exposure to scented products used in daily life and to explore a relationship between exposure to scented products and fragrance contact allergy.

METHODS

Study design and population

The EDEN fragrance study is a cross-sectional descriptive epidemiological study. Methods and study design have been published previously.^{13,14,19} In summary, a random sample of individuals from 18 to 74 years of age were selected from the general population and invited to a face-to-face interview in which a questionnaire was administered. The full phase of the study was conducted between August 2008 and October 2011. Additional patch testing was offered to a random sample of the included participants. If a participant was enrolled in the patch-test group, he or she was invited to patch testing after the interview had been completed.^{14,19} We performed a retrospective data analysis on the collected patch test data. Participants were included from Sweden, Germany (test sites: Heidelberg, Jena), The Netherlands, Italy, and Portugal. The study was approved by the ethics committee of each center.

Patch testing

A detailed description of the patch-testing procedures has been reported previously.^{16,17} Participants were patch tested with TRUE Test panels 1, 2, 3, hydroxyisohexyl 3-cyclohexene carboxaldehyde (HICC) 5% pet. and sesquiterpene lactone mix 0.1% pet.¹⁷ Two preparations of FM

I at 8% pet. were tested with the Finn chamber technique, one containing *Evernia prunastri* (oak moss) with a high content of atranol and chloratranol and the other containing *Evernia prunastri* (oak moss) with a low content of atranol and chloratranol. The corresponding test preparations with a low and high concentration of atranol and chloratranol were tested with the TRUE Test technique in panel 1 and as a Nixema test, respectively. In addition the eight separate ingredients of FM I were tested in pet. and FM II was tested in 14% pet., including its six separate ingredients. A detailed description hereof was published by Bruze *et al.*²⁰ Patch tests were applied on the back for 48 hours under occlusion. Patch-test readings were performed at day (D)3. A reaction was considered positive if a weak (+), strong (++), or extreme (+++) reaction was seen. Irritant (ir) and doubtful (? +) reactions were considered as negative reactions.

Data collection and analysis

Face-to-face interviews were conducted by trained interviewers and consisted of three parts.^{14,19} The sociodemographic characteristics included in the current analysis were sex, age (<40/≥40), physician-diagnosed contact dermatitis (including allergic- and irritant contact dermatitis), physician-diagnosed atopic dermatitis, self-reported dry or sensitive skin, and country. The question regarding presence of dry or sensitive skin was asked by interviewer in two separate questions: "Do you think that you have a dry, respectively, sensitive skin." A detailed history of exposure to different scented products in common use was reported. These products include the following: leave-on products (toiletry items that remained on the skin), rinse-off products (toiletry items that were rinsed off the skin), and household products (cleaning and freshener products).¹⁴ In the questionnaire, the estimated frequency of exposure during the last 12 months was scored after completion as follows: (0) never exposure, (1) exposure less than once a month, (2) monthly but not weekly exposure, (3) weekly but not daily exposure, and (4) daily exposure. For leave-on, rinse-off, and household products, respectively, 10, 5, and 5 different product subgroups were included in the questionnaire. Therefore, the overall exposure to scented products could be expressed as a continuous outcome. To study exposure to the different subgroups, the exposure outcome was categorized in which the response options (3) weekly but not daily exposure and (4) daily exposure were counted as exposure and the response options (0) never exposure was counted as no exposure. The response options (1) exposure less than once a month and (2) monthly but not weekly exposure were not included in the analysis. Therefore, exposure to the different subgroups could be expressed as a binary score.

To study a possible association between exposure and sensitization, all the 21 patch-test-fragrance allergens were included for analysis; FM I in pet. in high and low content of atranol and chloratranol, FM I with the TRUE Test and Nixema test, FM II, the eight and six separate components of FM I and FM II, respectively, Myroxylon pereirae, sesquiterpene lactone mix, and colophonium.

Statistics

Descriptive data are presented as numbers and percentages with 95% confidence intervals (95% CIs). For the overall exposure analysis, univariable linear regression analysis was performed and data are presented as standardized regression coefficients (beta [β]) with corresponding 95% CI.

To compare countries, Sweden was taken as reference. For the exposure analysis of the product subgroups, univariable binary logistic regression was performed. Effects of different variables on exposures are presented as odds ratio (ORs) with 95% CIs. A multivariable regression analysis including all variables that were significant in the univariable model was performed to control for potential confounders in both the linear and binary regression model. For the sub-analysis of associations between exposure and sensitization, univariable binary regression was performed and adjusted for age and sex. The Benjamini-Hochberg procedure was performed to control the False Discovery Rate (FDR) in multiple testing. The FDR was set to 0.10. Statistical analyses were performed with SPSS v.23 (IBM) and Excel 2013 (Microsoft).

RESULTS

Sociodemographic characteristics

Table 1 presents the sociodemographic characteristics of the 3119 patch-tested subjects included in the overall exposure analysis, together with the characteristics of the 224 subjects (7.2%) with at least one positive patch-test reaction to a fragrance allergen. Female subjects (54.9%) outnumbered male subjects (45.1%) and being ≥ 40 years of age (58.9%) outnumbered being < 40 years of age (41.2%). In the positive patch-test subsample, the female/male ratio shifted toward a predominantly female subpopulation (67.7% female compared to 32.3% male). For age, the percentage of subjects ≥ 40 years of age increased from 58.9% toward 69.6%. The proportion of subjects with contact dermatitis, dry, and sensitive skin was higher in the fragrance contact allergy subsample (16.1%, 59.6%, and 47.9%, respectively) compared to the total patch-tested population (8.1%, 52.1%, and 41.0%, respectively).

Overall exposure to scented products

Table 2 shows the results of the linear regression analysis with overall exposure to scented products as outcome. In a multivariable analysis, female subjects showed a significantly higher exposure to scented products (β 0.59, 95% CI 0.56–0.62) than male subjects. Subjects ≥ 40 years of age showed a significant inverse association with exposure to scented products (β -0.19, 95% CI -0.22 to -0.16). Small significant association effects were found between dry skin and overall exposure to scented products (β 0.04, 95% CI 0.01–0.07). Compared to Sweden, The Netherlands showed the highest overall exposure to scented products (β 0.14, 95% CI 0.11–0.17) followed by Germany (β 0.04, 95% CI 0.01–0.07).

Exposure to different scented products subgroups

In Figure 1, daily and/or weekly exposure to all scented product subgroups is presented. Mouthwashes or toothpastes, soaps, and shampoos are the most frequently used scented products, followed by deodorants and liquid detergents. Tables 3 and 4 show the results of the binary regression analysis of exposure to the different leave-on (Table 3) and the different rinse-off and household (Table 4) products subgroups. Only the significant results from the

Table 1. Sociodemographic characteristics of the patch-tested subjects and a subsample of subjects with a positive patch-test reaction to a fragrance allergen

	Total patch-tested subjects (n = 3119)			Positive patch-test reaction to a fragrance allergen (n = 224)		
	n	%	95% CI	n	%	95% CI
Sex						
Male	1405	45.1	43.3–46.8	72	32.3	26.2–38.4
Female	1712	54.9	53.2–56.7	151	67.7	61.6–73.8
Age (years)						
<40	1279	41.1	39.3–52.8	68	30.4	24.3–36.4
≥40	1836	58.9	57.2–60.7	156	69.6	63.6–75.7
Contact dermatitis	253	8.1	7.2–9.1	36	16.1	11.3–20.9
Atopic dermatitis	190	6.5	5.6–7.4	12	5.7	2.6–8.9
Dry skin	1626	52.1	51.8–55.3	130	59.6	53.1–66.1
Sensitive skin	1252	41.0	39.3–42.7	105	47.9	41.3–54.6

CI, confidence interval; N, number.

Included fragrance allergens: fragrance mix (FM) I 8% pet. containing *Evernia prunastri* (oak moss) with a high content of atranol and chloratranol, FM I 8% pet. containing *Evernia prunastri* (oak moss) with a low content of atranol and chloratranol, FM I True test and Nixema, FM I separate components; FM II 14% pet, FM II separate components, *Myroxylon pereirae*, sesquiterpene lactone mix, and colophonium.

multivariable analysis will be reported in this section. Female subjects showed a strong significant association in exposure to all scented product subgroups, except for exposure to men's cologne/aftershave and shaving products. As expected, male subjects were significantly associated with exposure to these two products subgroups. Age 40 years and older showed significant inverse associations for all leave-on products, except for exposure to men's cologne/aftershave and hygiene products. An additional stratified analysis by sex was performed and showed no statistical significant interaction. For rinse-off products, a significant inverse association for age 40 years of age and older was seen for exposure to bath oils, shaving products, and mouthwashes or toothpaste. Physician-diagnosed contact dermatitis was only associated with exposure to eye make-up (OR 1.43, 95% CI 1.06–1.92). Atopic dermatitis was only associated with exposure to lipsticks and lip balms (OR 1.88, 95% CI 1.13–3.12). Self-reported dry skin was associated with exposure to sunscreens (OR 1.30, 95% CI 0.96–1.75), skin creams (OR 2.69, 95% CI 2.11–3.44), eye make-up (OR 1.39, 95% CI 1.17–1.67), and lipstick and lip

Table 2. Linear regression analysis with exposure to scented products as outcome

Factors	Exposure to scented products			
	Univariable		Multivariable	
	β	95% CI	β	95% CI
Female	0.59	0.56–0.62	0.59	0.56–0.62
≥ 40 years	–0.18	–0.21 to –0.14	–0.19	–0.22 to –0.16
Contact dermatitis	0.06	0.02–0.10	0.00	–0.03–0.03
Atopic dermatitis	0.03	–0.01–0.07		
Dry skin	0.15	0.11–0.19	0.04	0.01–0.07
Sensitive skin	0.16	0.12–0.20	0.03	–0.01–0.06
Country (SE = ref)				
NL	0.13	0.08–0.18	0.14	0.11–0.17
DE	0.06	0.01–0.11	0.04	0.01–0.07
IT	0.02	–0.03–0.07		
PT	–0.01	–0.06–0.04		

Data are presented as standardized regression coefficients with corresponding standardized 95% CIs. Bold values indicate significant difference after Benjamini–Hochberg procedure for multiple testing with False Discovery Rate = 0.10.

β , standardized beta-coefficient; CI, confidence interval; DE, Germany; IT, Italy; NL, the Netherlands; PT, Portugal; SE, Sweden.

balms (OR 1.45, 95% CI 1.13–1.87). For exposure to household products, dry skin was associated with exposure to laundry detergents (OR 1.38, 95% CI 1.12–1.71), dishwashing detergents (OR 1.37, 95% CI 1.04–1.81), and scouring powders (OR 1.26, 95% CI 1.01–1.57). The presence of a sensitive skin was associated with exposure to different leave-on product subgroups: perfumes (OR 1.19, 95% CI 0.97–1.46), sunscreens (OR 1.62, 95% CI 1.20–2.18), skin creams (OR 1.31, 95% CI 1.01–1.70), and eye make-up (OR 1.83, 95% CI 1.53–2.18).

Table 3. Leave on products: Binary regression analysis with exposure to different subgroups as outcome for different subject characteristics

		Female, OR (95% CI)	≥ 40 years, OR (95% CI)
Perfumes	UVA	5.23 (4.34–6.29)	0.29 (0.24–0.35)
	MVA	5.83 (4.77–7.13)	0.23 (0.19–0.29)
Men's cologne/aftershaves	UVA	0.04 (0.03–0.05)	0.99 (0.84–1.16)
	MVA	0.04 (0.03–0.05)	
Sunscreens	UVA	4.26 (3.25–5.58)	0.32 (0.26–0.43)
	MVA	3.54 (2.64–4.73)	0.37 (0.27–0.50)
Skin creams, lotions, etc.	UVA	10.63 (8.29–13.64)	0.66 (0.54–0.81)
	MVA	9.74 (7.50–12.72)	0.59 (0.47–0.75)
Deodorants or antiperspirants	UVA	2.05 (1.61–2.61)	0.23 (0.17–0.32)
	MVA	2.16 (1.69–2.77)	0.22 (0.16–0.30)
Hair-care products	UVA	4.16 (3.53–4.90)	0.52 (0.44–0.61)
	MVA	4.50 (3.76–5.38)	0.45 (0.38–0.54)
Eye make-up	UVA	nt ^a	0.67 (0.58–0.78)
	MVA		0.69 (0.58–0.82)
Lipsticks and lip balms	UVA	27.76 (22.19–34.73)	0.34 (0.54–0.75)
	MVA	28.96 (22.49–37.27)	0.46 (0.35–0.59)
Other cosmetic products	UVA	82.06 (50.94–132.19)	0.73 (0.62–0.87)
	MVA	89.03 (54.21–146.21)	0.52 (0.42–0.65)
Hygiene products	UVA	84.21 (55.83–127.01)	0.85 (0.70–1.03)
	MVA	84.56 (55.16–129.65)	

Bold values indicate significant difference after Benjamini-Hochberg procedure for multiple testing with False Discovery Rate = 0.10.

CI, confidence interval; MVA, multivariable analysis; nt, not tested; OR, odds ratio; UVA, univariable analysis.

^aNot tested because <10 males were exposed and analysis could therefore not be performed.

Table 3 continued

Contact dermatitis, OR (95% CI)	Atopic dermatitis, OR (95% CI)	Dry skin, OR (95% CI)	Sensitive skin, OR (95% CI)
0.90 (0.66–1.21)	1.18 (0.83–1.70)	1.23 (1.04–1.46) 0.90 (0.74–1.11)	1.65 (1.38–1.97) 1.19 (0.97–1.46)
0.68 (0.49–0.93) 1.07 (0.69–1.65)	0.57 (0.39–0.83) 0.73 (0.45–1.19)	0.61 (0.51–0.71) 0.10 (0.88–1.39)	0.52 (0.44–0.62) 0.85 (0.67–1.07)
1.46 (0.93–2.27)	1.41 (0.83–2.38)	1.83 (1.41–2.37) 1.30 (0.96–1.75)	2.36 (1.82–3.06) 1.62 (1.20–2.18)
1.25 (0.86–1.80)	1.56 (0.99–2.58) 0.79 (0.46–1.37)	3.57 (2.88–4.43) 2.69 (2.11–3.44)	2.39 (1.92–2.98) 1.31 (1.01–1.70)
1.16 (0.74–1.83)	1.34 (0.78–2.30)	1.21 (0.95–1.53)	1.37 (1.07–1.76) 1.16 (0.89–1.50)
1.18 (0.88–1.56)	1.02 (0.74–1.41)	1.26 (1.07–1.47) 0.97 (0.81–1.18)	1.36 (1.16–1.59) 0.98 (0.81–1.18)
1.71 (1.30–2.25) 1.43 (1.06–1.92)	1.49 (1.09–2.05) 1.08 (0.77–1.51)	1.68 (1.43–1.96) 1.39 (1.17–1.67)	2.09 (1.78–2.46) 1.83 (1.53–2.18)
1.70 (1.24–2.31) 1.01 (0.65–1.54)	2.22 (1.53–3.23) 1.88 (1.13–3.12)	2.13 (1.80–2.52) 1.45 (1.13–1.87)	2.06 (1.74–2.45) 1.00 (0.78–1.30)
1.59 (1.19–2.15) 1.14 (0.80–1.62)	1.39 (0.99–1.94) 0.86 (0.56–1.30)	1.61 (1.35–1.92) 0.96 (0.76–1.21)	1.88 (1.58–2.23) 1.08 (0.86–1.36)
1.57 (1.12–2.20) 1.05 (0.93–1.63)	1.39 (0.93–2.08)	1.82 (1.49–2.21) 0.77 (0.57–1.03)	1.89 (1.56–2.30) 1.24 (0.94–1.63)

Table 4. Rinse-off and household products: Binary regression analysis with exposure to different subgroups as outcome for different subject characteristics

		Female, OR (95% CI)	≥ 40 years, OR (95% CI)
Bath oils, salts, beads, etc.	UVA	1.55 (1.30–1.83)	0.76 (0.64–0.90)
	MVA	1.56 (1.32–1.85)	0.74 (0.62–0.88)
Soaps, hand cleansers	UVA	1.80 (0.89–3.65)	0.33 (0.13–0.80)
	MVA	26.69 (8.33–85.53)	
Shampoos, conditioners	UVA	28.19 (8.85–89.87)	0.71 (0.43–1.17)
	MVA	26.69 (8.33–85.53)	
Shaving products	UVA	0.10 (0.09–0.12)	0.42 (0.36–0.50)
	MVA	0.09 (0.07–0.11)	0.30 (0.25–0.37)
Mouthwashes or toothpastes	UVA	2.66 (1.00–7.01)	0.27 (0.08–0.92)
	MVA	3.11 (1.17–8.32)	0.28 (0.80–0.95)
Laundry detergents	UVA	8.15 (6.70–9.93)	1.22 (1.02–1.46)
	MVA	7.75 (6.30–9.52)	1.14 (0.93–1.40)
Dishwashing detergents	UVA	4.95 (3.66–6.70)	0.88 (0.68–1.16)
	MVA	4.57 (3.36–6.22)	
Scouring powders	UVA	4.74 (3.86–5.83)	0.76 (0.62–0.93)
	MVA	4.51 (3.63–5.60)	0.66 (0.53–0.82)
Air fresheners	UVA	1.21 (1.02–1.43)	0.77 (0.65–0.91)
	MVA	1.20 (1.01–1.42)	0.77 (0.65–0.91)
Carpet shampoos, fresheners	UVA	3.10 (1.74–5.54)	1.19 (0.72–1.96)
	MVA		

Bold values indicate significant difference after Benjamini-Hochberg procedure for multiple testing with False Discovery Rate = 0.10.
CI, confidence interval; MVA, multivariable analysis; nt, not tested; OR, odds ratio; UVA, univariable analysis.

^aNot tested because expected count in one cell <5.

Table 4 continued

Contact dermatitis, OR (95% CI)	Atopic dermatitis, OR (95% CI)	Dry skin, OR (95% CI)	Sensitive skin, OR (95% CI)
1.09 (0.80–1.48)	1.18 (0.83–1.69)	1.20 (1.01–1.42) 1.10 (0.92–1.33)	1.19 (1.00–1.41) 1.08 (0.90–1.30)
0.47 (0.18–1.24)	nt ^a	1.23 (0.61–2.50)	0.85 (0.42–1.72)
1.15 (0.46–2.87)	0.51 (0.24–1.09)	1.44 (0.88–2.37)	2.00 (1.17–3.45) 1.24 (0.71–2.15)
0.69 (0.52–0.93) 1.05 (0.73–1.50)	0.99 (0.71–1.37)	0.73 (0.62–0.85) 1.11 (0.91–1.36)	0.75 (0.64–0.88) 1.13 (0.92–1.39)
0.25 (0.09–0.68) 0.22 (0.08–0.63)	1.26 (0.17–9.49)	2.32 (0.87–6.21)	1.51 (0.57–3.98)
1.76 (1.25–2.46) 1.39 (0.94–2.07)	1.24 (0.85–1.82)	1.72 (1.44–2.05) 1.38 (1.12–1.71)	1.44 (1.21–1.72) 0.86 (0.67–1.07)
1.63 (0.92–2.90)	0.87 (0.51–1.49)	1.77 (1.35–2.32) 1.37 (1.04–1.81)	1.19 (0.90–1.56)
1.51 (1.03–2.20) 1.30 (0.86–1.97)	1.08 (0.73–1.63)	1.60 (1.31–1.94) 1.26 (1.01–1.57)	1.41 (1.16–1.73) 1.98 (0.78–1.22)
0.79 (0.58–1.09)	0.80 (0.60–1.21)	1.00 (0.84–1.18)	1.01 (0.85–1.20)
0.54 (0.17–1.74)	1.18 (0.47–3.00)	1.27 (0.77–2.10)	1.25 (0.76–2.06)

Associations between exposure to scented products and fragrance contact allergy

Table 5 presents the results of the analysis of overall exposure to scented products and fragrance contact allergy stratified per scented product subgroup. In total, 224 subjects (7.2%) showed a positive patch-test reaction to at least 1 of the 21 allergens included in the association analysis. Univariable regression analysis showed a significant association between overall exposure to scented products and contact allergy to one of the included scented allergens (OR 1.70, 95% CI 1.14–2.52). Overall exposure to leave-on products showed a significant association with fragrance contact allergy (OR 1.79, 95% CI 1.21–2.64). Exposure to rinse-off products showed an inverse significant association with fragrance contact allergy (OR 0.61, 95% CI 0.44–0.81). Exposure to household products was not significantly associated with fragrance contact allergy. Stratified by product subgroups, significant associations between exposure and fragrance contact allergy were seen for exposure to skin creams (OR 1.80, 95% CI 1.15–2.82), eye make-up (OR 1.37, 95% CI 1.02–1.84), lipstick and lip balm (OR 1.41, 95% CI 1.03–1.93), other cosmetic products (OR 1.52, 95% CI 1.12–2.06), and hygiene products (OR 1.88, 95% CI 1.34–2.63). Significant inverse associations were seen between exposure and fragrance contact allergy for men's cologne/aftershave (OR 0.66, 95% CI 0.48–0.93) and shaving products (OR 0.60, 95% CI 0.44–0.81). The presence of a sensitive skin was associated with an increased risk of fragrance contact allergy in univariable regression analysis (OR 1.36, 95% CI 1.03–1.79). After adjusting for age and sex the outcomes were no longer significant.

Figure 1. Proportion of subjects who report daily or weekly exposure to different scented product subgroups among 3119 patch-tested subjects

Table 5. Binary regression analysis with fragrance contact allergy as outcome (n = 224)

Exposure to	Crude, OR (95% CI)	Adjusted ^a , OR (95% CI)
Scented overall	1.70 (1.14–2.52)	1.25 (0.69–2.29)
Leave on (including all subgroups)	1.79 (1.21–2.64)	2.43 (1.20–4.90)
Perfumes, liquid toiletries, or other highly perfumed products	0.96 (0.69–1.34)	0
Men's cologne/aftershaves	0.66 (0.48–0.93)	1.09 (0.70–1.69)
Sunscreens	1.45 (0.90–2.35)	1.55 (0.92–2.61)
Skin creams, lotions, etc.	1.80 (1.15–2.82)	1.47 (0.91–2.39)
Deodorants or antiperspirants	1.07 (0.67–1.70)	1.09 (0.68–1.76)
Hair-care products (sprays, tonics, gels, mousses—not shampoos, conditioners or other rinse-off products)	1.32 (0.98–1.79)	1.26 (0.91–1.75)
Eye make-up products	1.37 (1.02–1.84)	0.90 (0.61–1.34)
Lipsticks and lip balms	1.41 (1.03–1.93)	1.19 (0.78–1.82)
Other cosmetic products (foundation, blushes, powders, etc.)	1.52 (1.12–2.06)	1.20 (0.84–1.74)
Hygiene products (sanitary napkins, tampons, etc.)	1.88 (1.34–2.63)	1.64 (1.03–2.62)
Rinse-off (including all subgroups)	0.61 (0.43–0.89)	0.69 (0.46–1.04)
Bath oils, salts, beads, etc.	1.18 (0.85–1.62)	1.13 (0.82–1.57)
Bar soaps, liquid soaps, waterless hand cleansers, etc.	0.42 (0.16–1.09)	0.41 (0.16–1.09)
Shampoos, cream rinses, conditioners, etc.	0.58 (0.27–1.22)	0.41 (0.19–0.89)
Shaving products	0.60 (0.44–0.81)	0.86 (0.59–1.24)
Mouthwashes or toothpastes	1.39 (0.19–10.49)	1.36 (0.18–10.29)
Household (including all subgroups)	1.43 (0.94–2.16)	0.92 (0.56–1.52)
Laundry detergents, softeners, etc.	1.20 (0.85–1.69)	0.81 (0.55–1.22)
Liquid dishwashing detergents	1.31 (0.75–2.29)	1.06 (0.60–1.89)
Scouring powders, hard surface cleaners, etc.	1.33 (0.90–1.97)	1.09 (0.72–1.66)
Air fresheners, aerosol or liquid (including wicks)	1.05 (0.77–1.44)	1.03 (0.75–1.41)
Carpet shampoos, fresheners, etc.	0	0
Dry skin	1.30 (0.99–1.73)	1.16 (0.87–1.55)
Sensitive skin	1.36 (1.03–1.79)	1.23 (0.93–1.64)

Included fragrance allergens in sub-analysis: fragrance mix (FM) I 8% pet. containing Evernia prunastri (oak moss) with a high content of atranol and chloratranol, FM I 8% pet. containing Evernia prunastri (oak moss) with a low content of atranol and chloratranol, FM I True Test and Nixema, FM I separate components; FM II 14% pet, FM II separate components, Myroxylon pereirae, sesquiterpene lactone mix, and colophonium. Bold values indicate significant difference after Benjamini-Hochberg procedure for multiple testing with False Discovery Rate = 0.10. CI, confidence interval; OR, odds ratio.

^aAdjusted for age and sex.

DISCUSSION

Scented product exposure and fragrance contact allergy

This European study evaluated self-reported skin exposure to different scented products used in daily life. In addition, we explored associations between exposure to different leave-on, rinse-off, and household products and fragrance contact allergy using patch-test results in adults from the general population.

In this study, exposure to scented products varied with sex, age, the presence of a skin condition, and country of residence. We showed that females are significantly more exposed to scented products used in daily life than males. Several studies have been published on the effect of sex on the development of contact allergy.²¹⁻²⁴ It was assumed that increased female sensitivity to contact allergens is most likely due to a higher exposure to contact allergens than males.^{21,23} The current study shows that females are indeed more exposed to scented products and consequently may be more frequently sensitized to fragrance allergens than males. We also showed that subjects younger than the age of 40 years are more exposed to scented products than subjects 40 years of age and older, even though a positive patch-test reaction to one of the included fragrance allergens in this analysis was more frequently observed in subjects age 40 years of age and older. It is important to note that our questionnaire only addressed exposure in the 12 months prior to the interview. Repeated exposure over a period longer than 12 months was therefore not included in the analysis. This could explain the discrepancy in which younger subjects are more exposed to scented products during the last 12 months but show less positive patch-test reactions to fragrance allergens than older subjects, who may have a higher number of repeated exposures over a longer period. Diepgen *et al.* reported the clinical relevance of contact allergy to fragrance allergens from the current EDEN cohort.¹⁷ Clinical relevance was defined as a positive patch test in a subject who also reported a lifetime history of avoidance of any scented products because of any skin problem plus an itchy skin reaction lasting >3 days. The prevalence of clinically relevant fragrance allergy was 1.9% and increasing with age. Consequently, if older subjects have more clinically relevant reactions to fragrances, they might avoid contact with scented products and thus might be less exposed than subjects younger than 40 years of age.

In the current analysis, sensitive skin was associated with exposure to perfumes, sunscreens, skin creams and eye make-up. In addition, reporting a sensitive skin was associated with fragrance contact allergy in univariable regression analysis. Subjects with presumed sensitive skin probably use more skin care products. This could be a reason they are more frequently sensitized to fragrance allergens. Subjects with self-reported sensitive skin should therefore be advised to be cautious with exposure to scented products. Furthermore, a subdivision by country showed that The Netherlands followed by Germany were more exposed to scented products than Sweden. Diepgen *et al.*, however, reported no statistically significant difference for contact allergy to the different fragrance allergens between countries.¹⁷ Considering the study design, in which interviewers from the different countries were trained equally and different geographic areas from the different countries were taken into account (subjects participated from both rural and central areas), we could not find a clear explanation for the significant differences in exposure but not in the prevalence of fragrance contact allergy we found.

We found significant associations between overall exposure to scented products and fragrance contact allergy. For different leave-on product subgroups we found a positive association between exposure and fragrance contact allergy. A model for assessing the risk of

induction of skin sensitization to fragrances has been developed that resulted in Quantitative Risk Assessment (QRA) to determine safe levels of fragrance ingredients in consumer products.^{25,26} The QRA methodology follows four fundamental steps: hazard identification, dose–response assessment or hazard quantification, exposure assessment, and risk characterization.²⁵ Recently QRA1 has been refined, termed QRA2, which includes aggregate exposure to fragrances from multiple product use.¹² The design of the current study did not allow us to perform an aggregate exposure analysis for specific fragrance ingredients. However, we aimed to calculate exposure to scented products by calculating the total sum of the frequency of exposure to the included scented products, thereby creating a continuous outcome as described in the methods. Longitudinal clinical studies to evaluate the efficacy of QRA2 as a tool for prevention of fragrance contact allergy are required.¹²

Limitations

The measurement of exposure recommended by the Scientific Committee on Consumer Safety (SCCS) to use in skin sensitization risk assessment for fragrance ingredients is dose/area ($\mu\text{g}/\text{cm}^2$).²⁷ The measurement of exposure in the current study ranges between never exposure to daily exposure. The total dose applied to the skin including daily frequency, dose per unit, and application site was not reported and could therefore not be included in our analysis. In addition, another limitation is the cross-sectional design of the study: The questionnaire included only exposure during the last 12 months; therefore data about lifetime exposure is lacking. Subjects with high exposure to scented products prior to this last 12 months that resulted in contact dermatitis could have become current avoiders of the same scented products.

To decrease the false discovery rate and correct for multiple testing we applied the Benjamini-Hochberg procedure, which is less conservative than the family-wise error rate controlling procedures such as the Bonferroni correction. Because this was an exploratory study, we decided to use the Benjamini-Hochberg correction to identify possible associations to include for future research, which might otherwise not be found by applying more conservative correction procedures.

Conclusion

In the European general population, female subjects and subjects younger than 40 years of age are most exposed to scented products. High exposure to scented products may lead to fragrance contact allergy. Exposure to leave-on products can therefore be considered as a risk factor in developing fragrance contact allergy. Sensitive skin is associated with exposure to different leave-on product subgroups and with contact allergy to fragrances. Overall, exposure to scented products, especially to leave-on products, is associated with fragrance contact allergy. Because aggregate exposure, specifically to scented leave-on products, may enhance the prevalence of contact allergy to fragrances, further investigations into exposure amounts and frequencies are warranted.

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Chapter 4

Allergic contact dermatitis caused by
1,6-hexanediol diacrylate in a hospital wristband

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ABSTRACT

Background

1,6-Hexanediol diacrylate (1,6-HDDA) is a multifunctional acrylate and a potent sensitizer.

Objective

To report a case of allergic contact dermatitis caused by 1,6-HDDA in a hospital wristband.

Methods

A male patient presented with eczema on his wrist where he had worn a hospital wristband. Patch testing was performed with our extended European baseline series, additional series, and pieces of the hospital wristband. Thin-layer chromatography (TLC) was performed with extracts from the wristband and gas chromatography-mass spectrometry was used for chemical analysis.

Results

Positive reactions were found to pieces of the wristband, including adhesive rim (+++), inside (+++), and outside (++); to multiple allergens in the (meth)acrylates series; and to extracts of the wristband in acetone and ethanol. Chemical analysis of the ethanol extract showed presence of lauryl acrylate and 1,6-HDDA. Patch testing with TLC strips and subsequent chemical analysis showed that the substance causing the strongest reaction was 1,6-HDDA, to which the patient had a confirmed positive patch test reaction.

Conclusion

1,6-HDDA was identified as the culprit allergen responsible for allergic contact dermatitis caused by the hospital wristband.

INTRODUCTION

Patient identification (ID) wristbands are widely used in hospitals to provide patient information, thereby reducing the risk of patient misidentification. We report a patient with allergic contact dermatitis caused by 1,6-hexanediol diacrylate (1,6-HDDA) in a hospital patient ID wristband.

CASE REPORT

A 66-year-old non-atopic man was referred to the Department of Dermatology for evaluation of pruritic erythema and vesicles on the wrist at the site where he wore a hospital ID wristband (DuraSoft Laser Patient ID system; Precision Dynamics Corporation [PDC] Healthcare, Valencia, California) during hospitalization due to cardiac failure (Figure 1). The patient reported that the skin complaints on the wrist at the site of the wristband started a few hours after the wristband was put on. During a previous hospitalization, at which the same type of hospital wristband had been used, no skin complaints on the wrist were observed. He reported a history of similar skin complaints at the sites of application of electrocardiogram (ECG) electrodes. The patient was retired, and his previous job had been delivering parcels for 10 years. Before that he had been a butcher. He recalled possible relevant leisure exposure to two-component adhesive in the past, but it was unclear whether the adhesive contained acrylates.

Figure 1. (A), Pruritic erythema and vesicles on the wrist where a DuraSoft hospital wristband was worn; (B), Positive patch test results to extracts at day (D) 3; (C), Thin-layer Chromatography testing: Positive reactions on areas to spot 3 (+++), spot 2 (++), and spot 1 (++) at D7.
eth., ethanol; ac., acetone.

Patch testing

The patient was patch tested with our extended European baseline series (TRUE Test panels 1 and 2; supplemented with investigator-loaded allergens) as well as cosmetics, fragrances, (meth)acrylates, and a plastics and glues series (allergens from SmartPractice Europe, Barsbüttel, Germany, and Chemotechnique Diagnostics, Vellinge, Sweden). Furthermore, pieces of the DuraSoft hospital wristband were tested "as is". Because of the history of a skin reaction to ECG electrodes, various parts of these electrodes were also tested. Van der Bend Chambers were used (Van der Bend, Brielle, The Netherlands), which were fixed with Fixomull Stretch (BSN Medical, Hamburg, Germany). The patch tests were applied on the back for 48 hours under occlusion, and patch test readings were performed at day (D) 3 and D7, according to ESCD guidelines.¹

Strong positive patch test reactions were found to pieces of the wristband: adhesive rim (+++), inside (+++), and outside (++). Multiple strong positive reactions to the (meth)acrylates series were seen. Furthermore, the patient showed positive reactions to the gel and adhesive layer of several ECG electrodes. Results are shown in Table 1.

Thin-layer chromatography (TLC) and chemical analysis

Subsequently, extracts from the wristband were prepared in acetone and ethanol. Extraction was performed in an ultrasonic bath followed by concentration using a vacuum evaporator.² When the patient was patch tested, positive reactions to the extracts of the wristband were observed in acetone undiluted (+++) and diluted to 10% (+) as well as in ethanol undiluted (+++) and diluted to 10% (+) (Figure 1B and Table 1). The tested ethanol extract was analysed with gas chromatography-mass spectrometry (GC-MS) and showed presence of lauryl acrylate and 1,6-HDDA. The presence of these substances was confirmed by analysis of reference substances.

TLC was performed in order to separate the components of the extracts of the wristband material and to obtain TLC strips for patch testing.³ The spots, containing 65 µL of the undiluted ethanol extract, were applied to a sheet of thin-layer material with silica gel bound to a plastic carrier (TLC plastic roll, Silica Gel 60F 254; Merck, Darmstadt, Germany). A mobile phase consisting of heptane and ethyl acetate (50:50, vol/vol) was used for elution.³ After elution, spots visible in UV-radiation (254 and 366 nm) were marked with a pencil.

The patient was tested with the TLC strips (extract ethanol) and showed positive reactions below spot 3 (+++), spot 2 (++), and spot 1 (++) (Figure 1C and Table 1). Scrapings from reference TLC-plates did not demonstrate the presence of lauryl acrylate and 1,6-HDDA. Benzophenone could be identified in scrapings from spot 3. Eluting benzophenone together with higher concentrations of lauryl acrylate and 1,6-HDDA in the same type of TLC system as used before, followed by chemical analysis with GC-MS, revealed the presence of lauryl acrylate just above benzophenone, and 1,6-HDDA just below benzophenone. Hence, with an indirect identification method the substance causing the strongest reaction for the DuraSoft wristband TLC (below spot 3) was identified as 1,6-HDDA, for which the patient had a confirmed positive patch test reaction in earlier testing. The concentration of 1,6-HDDA in the patch test extract was approximately 0.003%, corresponding to 19 µg/g in the wristband.

Separate analysis of concentrated extracts of the adhesive rim and the other part of the band was performed. No acrylates in any detectable amount could be detected in the adhesive

rim. Analysis of the band showed that the present acrylates came from the glue used for fixation of the paper and plastic upper layer. The previously mentioned 1,6-HDDA and lauryl acrylate, as well as traces of isobornyl acrylate, were found in these paper/plastic samples. Additional patch testing was performed with isobornyl acrylate 0.1% and 0.01% pet. and with lauryl acrylate 0.1% pet. and 0.01% pet., which all were negative.

Table 1. Patch test results from patch test series, wristband product, extracts, substances, and thin-layer chromatography strips

Tested series, wristband product	Concentration, vehicle	D2	D3	D4	D7
Extended European baseline series	µg/cm ²				
p-tert-butylphenol formaldehyde resin (PTBP-FR)	40		++		+
Plastic/glue series	(%) pet.				
p-tert butylphenol	1		++		+
(Meth)acrylate series	(%) pet.				
Ethyl acrylate (EA)	0.1		+++		+++
Butyl acrylate (BA)	0.1		+++		++
2-Hydroxyethyl acrylate (2-HEA)	0.1		+++		+++
2-Hydroxypropyl acrylate (2-HPA)	0.1		+++		+++
2-Hydroxyethyl methacrylate (2-HEMA)	2		+		++
2-Hydroxypropyl methacrylate (2-HPMA)	2		+++		++
Triethylene glycol dimethacrylate (TEGDMA)	2		++		++
1,4-Butanediol dimethacrylate (BUDMA)	2		+++		++
1,4-Butanediol diacrylate (BUDA)	0.1		+++		+++
1,6-Hexanediol diacrylate (HDDA)	0.1		+++		+++
Diethylene glycol diacrylate (DEGDA)	0.1		+++		+++
Tripropylene glycol diacrylate (TPGDA)	0.1		+++		+++
Trimethylolpropane triacrylate (TMPTA)	0.1		+		++
Pentaerythritol triacrylate (PETA)	0.1		+		++
Oligotriacrylate (OTA)	0.1		+++		++
Epoxy acrylate	0.5		++		++
Triethylene glycol diacrylate (TREGDA)	0.1		+++		+++
Wristband product					
Wristband adhesive part	"as is"		+++		++
Wristband inside	"as is"		+++		++
Wristband outside	"as is"		+++		++

TLC spots 4, 5, and E (solvent front) negative.

ac., acetone; D, day; eth., ethanol; pet., petrolatum; TLC, thin-layer chromatography.

^aUsed for chemical analysis.

Table 1 continued

Tested series, wristband product		Concentration, vehicle	D2	D3	D4	D7
Wristband extracts						
Extract wristband ethanol		100% eth.	++	+++		++
Extract wristband ethanol		10% eth.	-	+		?+
Extract wristband acetone		100% ac.	++	+++		++
Extract wristband acetone		10% ac.	-	+		?+
TLC						
TLC below spot 3 ^a			+++	+++		+++
TLC spot 2			-	++		++
TLC spot 1			-	+		++
Substances identified on TLC scrapings		% pet.				
Isobornyl acrylate		0.1		?+	-	-
Isobornyl acrylate		0.01		?+	-	-
Lauryl acrylate		0.1		?+	?+	-
Lauryl acrylate		0.01		?+	-	-
ECG electrodes						
3 M Red Dot 2570	Gel	"as is"		++		+++
3 M Red Dot 2249-50	Adhesive layer	"as is"		++		++
3 M Red Dot 2249-50	Gel	"as is"		+++		++
3 M Red Dot 2255	Adhesive layer	"as is"		++		+++
3 M Red Dot 2255	Gel	"as is"		+++		+++

DISCUSSION

We present a patient with allergic contact dermatitis caused by 1,6-HDDA, which was probably a component of the glue used for fixation of the paper and plastic upper layer of his hospital wrist-band. 1,6-HDDA (CAS no. 13048-33-4) is a multifunctional acrylate. These acrylates, which are components of printing inks and coatings, are also used in dentistry. Contact allergy is mostly seen after occupational exposure.⁴

The multiple positive reactions to (meth)acrylates in our patient can be explained by cross-reactivity or co-sensitization. The patient reacted to the gel and adhesive layer of the ECG electrodes. According to the ingredient list, 3 M Red Dot 2570 contained an acrylate glue and polyethylene glycol dimethacrylate, and 3 M Red Dot 2249-50 and 3 M Red Dot 2255 contained an acrylate copolymer. Previous studies have shown that (meth)acrylates or acrylic acid were the culprit allergens in patients with allergic contact dermatitis caused by contact with ECG electrodes.⁵ Therefore co-sensitization could be another explanation for multiple reactivity to various (meth)acrylates in our patient. The positive reactions to spot 1 and 2 of the TLC probably corresponded to other (meth)acrylates that could not be identified. The positive reaction to p-tert-butylphenol formaldehyde resin (PTBP-FR) in our patient could also be explained by the ECG electrodes, which can contain this allergen in the adhesive layer and gel of the electrodes.⁶

In the current case, the acetone extract could not be tested with TLC because it was difficult to apply and did not produce good separations on the TLC; the wristband was made from vinyl, which contains a large number of plasticizers. Acetone dissolves many of these plasticizers, and their presence interferes with both application and separation of the sample. The limited presence of lauryl acrylate and 1,6-HDDA on the reference TLC-plates was probably because the concentration on the plates was too low.

A few cases of allergic contact dermatitis caused by wristbands have been reported in the literature.^{7,8} Tanahashi *et al.* reported three patients who wore the same type of amusement park wristband; positive photopatch test results to benzophenone 1% pet. were observed in the three cases and benzophenone was identified as the culprit allergen. Because 1,6-HDDA was also detected by GC-MS in the wristbands, patch testing was performed, but results were negative.⁷ Hills and Ive reported diisodecyl phthalate as the allergen responsible for allergic contact dermatitis in a patient who wore a polyvinyl chloride hospital ID wristband.⁸ It is interesting to note that the current case and the reported cases in the literature show that different contact allergens are responsible for allergic contact dermatitis caused by wristbands.

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Chapter 5

Cheilitis caused by contact allergy to toothpaste
containing stannous (tin) – two cases

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INTRODUCTION

Allergic contact cheilitis can be caused by contact allergy to different toothpaste ingredients. We report two patients with contact allergy to tin present as an ingredient in toothpaste.

CASE REPORT

Case 1

A 69-year-old atopic man (retired painter) was referred for evaluation of cheilitis. He reported recurrent swelling with small blisters and red spots intra-orally and on his tongue and, in addition, crusts on his lips for 6 months (Figure 1). He was using Sensodyne Rapid Relief toothpaste (GlaxoSmithKline, Brentford, UK).

Figure 1. Erythematous plaques with crusts located on the lips in patient 1

Table 1. Patch test results of two cases of allergic contact cheilitis

Tested series	Concentration, vehicle	Case 1		Case 2	
		Day 3	Day 7	Day 3	Day 7
Metal series 1st test: Ti^{IIIa} - CAS 7440-31-5 2nd test: Ti^{IIIab} - CAS 7440-31-5	50% pet.	+	+	+	++
	50% pet.	+	++	nt	nt
Fragrance series Hydroperoxides of limonene	0.3% pet.	nt	nt	+	-
Toothpaste product Sensodyne Rapid Relief toothpaste	"as is"	+++	+++	+++	+
	50% aqua	++	++	+	+
	30% aqua	++	++	nt	nt
	10% aqua	++	++	?+	+
	5% aqua	nt	nt	-	-
	3% aqua	+	+	-	-
	"as is"	nt	nt	+	+
Urtekram Aloe vera toothpaste	50% aqua	nt	nt	+	+
	10% aqua	nt	nt	+	+
	5% aqua	nt	nt	+	+
	3% aqua	nt	nt	?+	+

Additional substances

Tin^{bb} - CAS 7440-31-5

30% pet.

10% pet.

3% pet.

1% pet.

1% pet.

1% pet.

Stannous oxalate^{aa} - CAS 814-94-8Stannous chloride^{aa} – CAS 7772-99-8Stannous fluoride^{bb} – CAS 7783-47-3

0.5% pet. and aqua

0.15% pet. and aqua

0.05% pet. and aqua

0.015% pet. and aqua

Sodium fluoride^{cc} – CAS 7681-49-4 0.5/0.15/0.05/0.015% pet. and aqua

nt, not tested; pet., petiolatum.

^aChemotechnique Diagnostics.

^bRaw material from Sigma-Aldrich chemistry dilution series were prepared at the University Medical Centre Groningen (UMCG) pharmacy.

^cRaw material from Duchefa Farma, dilution series were prepared in the UMCG pharmacy.

-				-							
-				-							
-				-							
-				-							
++				++							
+++				++							

Pet.	Aqua	Pet.	Aqua	Pet.	Aqua	Pet.	Aqua
++	+	++	+	+++	?+	+++	+
+	-	+	+	++	+	++	+
+	-	+	-	++	-	++	-
-	-	-	-	?+	-	+	-
-	-	-	-	-	-	-	-

Case 2

A 62-year-old non-atopic housewife was referred for evaluation of erythematous gingiva. At consultation she recalled episodes of red and flaking skin changes on and below her lip. She switched from Sensodyne Rapid Relief toothpaste to Urtekram Aloe Vera toothpaste (Urtekram, Mariager, Denmark), after which improvement of her complaint occurred. Unfortunately, after a few months, this toothpaste also started to cause peri-oral and gingival symptoms.

Patch testing and results

Both patients were patch tested with our extended European baseline series (TRUE Test panels 1 and 2, supplemented with additional investigator-loaded allergens), as well as a dental and metal series. Patient 2 was also tested with a cosmetics and fragrance series (allergens from SmartPractice Europe, Barsbüttel, Germany and Chemotechnique Diagnostics, Vellinge, Sweden). Van der Bend Chambers (Van der Bend, Brielle, The Netherlands) were applied on the back for 48 hours under occlusion and were fixed with Fixomull Stretch (BSN Medical, Hamburg, Germany). Patch test readings were performed at day (D) 3 and D7.

The results of patch testing are shown in Table 1. Patient 1 showed a positive reaction (+) to tin 50% pet. at D3 and D7. Tin 50% pet. was retested and gave a positive reaction (+) at D3 reaction and a strong positive reaction (++) at D7. Patient 2 showed a positive reaction (+) at D3 and a strong positive reaction (++) at D7 to tin 50% pet. Also, a positive reaction (+) to hydroperoxides of limonene 0.3% pet. at D3 was found, which became negative at D7. Examination of the Sensodyne Rapid Relief toothpaste's ingredients revealed the presence of stannous (tin) fluoride.

Both patients were then patch tested with the Sensodyne Rapid Relief toothpaste "as is" and in a dilution series (50%, 30%, 10%, 5%, and 3% aq.), which yielded extreme positive reactions (+++) at D3 and D7 in patient 1 and at D3 in patient 2. Patient 1 showed positive reactions throughout the entire dilution series and patient 2 down to the 10% concentration. Patient 2 was also tested with Urtekram Aloe vera toothpaste and showed positive reactions (+) to pure toothpaste and all dilutions. Additional testing was also performed with a dilution series of tin (30%, 10%, 3%, and 1% pet.) and stannous fluoride (0.5%, 0.15%, 0.05%, and 0.015% in pet. and aq.) (raw material from Sigma-Aldrich Chemistry, Darmstadt, Germany; dilution prepared at the

University Medical Center, Groningen [UMCG]), stannous chloride 1.0% pet. and stannous oxalate 1% pet. (Chemotechnique Diagnostics); see Table 1. To exclude sodium fluoride as being the culprit allergen, sodium fluoride was tested (raw material from Duchefa Farma [Haarlem, The Netherlands], dilution 0.5%, 0.15%, 0.05%, and 0.015% in pet. and aq. prepared at the UMCG), which gave no positive reactions in either patient.

Three controls were tested with Sensodyne Rapid Relief toothpaste who all showed negative reactions. In 38 controls tested with stannous chloride 1.0% pet., two strong positive (++), five weak positive (+), three doubtful and seven irritant reactions were seen. Of 38 controls tested with stannous oxalate 1% pet., one positive reaction was seen; this subject also reacted positive (+) to stannous chloride. These reactions were clinically relevant in this subject, because she had periorbital eczema and tin was found in her eye shadow. After discontinuation of the use of the eye shadow her periorbital eczema resolved completely. Of these controls, 28 subjects were also tested with tin 50% pet. and showed no positive reactions.

DISCUSSION

We have described two patients with allergic contact cheilitis caused by tin present in Sensodyne Rapid Relief toothpaste (www.sensodyne.nl), which contains 0.454% w/w tin in the form of stannous (tin) fluoride. Stannous fluoride (CAS no. 7783-47-3) is a chemical compound that can be found as an ingredient in toothpaste.¹ Fluorides are considered to be the most active ingredient in toothpaste with beneficial effects on caries, dental plaque, gingivitis, and halitosis.^{1,2} It can also be beneficial for dentin hypersensitivity and is used for tubule occlusion causing nerve desensitization.^{2,3}

Patient 1 had used this toothpaste for over 20 years without complaints. After the diagnosis of contact allergy to tin was made, he switched to a toothpaste without tin and 2 weeks afterwards all skin complaints had resolved. Patient 2 had already switched from Sensodyne Rapid Relief toothpaste to Urtekram Aloe vera toothpaste with initial improvement of her gingival and skin complaints; however, these later recurred. This may well have been caused by her allergy to hydroperoxides of limonene; limonene was found to be an ingredient of the Urtekram toothpaste (www.urtekram.nl). After discontinuation of this toothpaste, the patient's peri-oral and gingival symptoms resolved completely.

Contact allergy to toothpaste and its ingredients has been critically reviewed by de Groot.² Symptoms usually manifest as cheilitis and dermatitis around the mouth can be present as well. Intra-oral symptoms are less common. Even though cheilitis is the main symptom of contact allergy to toothpaste ingredients, toothpastes may be under-recognized as a potential cause of cheilitis,⁴ possibly because contact allergy to toothpastes is infrequent.² In the literature, flavouring agents are the most frequently reported cause of toothpaste allergy.² Of the 34 critically reviewed reports by de Groot,² only one case report described contact allergy caused by stannous fluoride.⁵ However, the toothpaste itself was not tested and no controls were included.⁵ Recently, another case with allergic contact dermatitis caused by stannous fluoride in toothpaste has been reported.⁶

In the literature, little is known about testing with tin salts. Olivarius *et al.* reported 2206 patients patch tested with stannous chloride 1% pet. among whom 0.2%, 0.7%, and 0.6% patients showed positive, doubtful, and irritant reactions, respectively.⁷ We showed a high number of positive (7/38, 18%), doubtful (3/38, 8%), and irritant (7/38, 19%) reactions to stannous chloride 1%. In all but one control, there was no clinical relevance. It seems, therefore, that the 1% patch test concentration of stannous chloride is too high, given the frequently observed false-positive and irritant reactions. Nevertheless, we feel confident that in both our patients with cheilitis, contact allergy to tin is certain, based on all the other positive reactions to tin and tin salts. We suggest testing with the commercially available stannous oxalate 1% pet. when suspecting tin contact allergy. In both our patients strong (++) positive reactions were seen to stannous oxalate 1% pet. and in only one control a - relevant - positive reaction was found.

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Cheilitis caused by contact allergy to toothpaste containing stannous (tin)

PART II

Susceptibility

Chapter 6

The expression pattern of N-acetyltransferase 1
in healthy human skin

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ABSTRACT

Background

N-acetyltransferase 1 (NAT1) is an enzyme expressed among others in keratinocytes in human skin. NAT1 is important in the biotransformation of aromatic amines, an important example being *p*-phenylenediamine (PPD), a hair dye molecule. Unoxidized PPD penetrates the skin and is N-acetylated by NAT1.

Objective

To investigate in detail the expression pattern of NAT1 in human skin.

Methods

Cryosections obtained from healthy human skin were stained for NAT1 and expression patterns were observed. NAT1 double stainings were performed with antibodies against different cellular organelles to determine expression patterns.

Results

A speckled, granular expression of NAT1 was seen predominantly in the stratum basale. NAT1 was expressed in a cytoplasmic pattern, perinuclear, and in the nucleus. No colocalization was seen with the selected cellular organelles. Local differences in NAT1 expression patterns were observed between donors and between different biopsies obtained from the same donor.

Conclusion

NAT1 is expressed predominantly in the stratum basale and can be found in the cytoplasm, nucleus, and perinuclear in human skin. Further studies should be performed to investigate expression of NAT1 in a larger sample size.

INTRODUCTION

Arylamine N-acetyltransferases (NATs) are a group of enzymes involved in the biotransformation of aromatic amines. NAT is widely expressed among various species of animals and bacteria.¹ In humans, two isoforms of NATs are recognized, NAT1 and NAT2, encoded by the NAT1 and NAT2 genes. Studies have found that NAT1 is expressed in all investigated fetal² and adult tissues,³⁻⁸ among others, in keratinocytes in human skin.⁹ NAT2 is predominantly expressed in the liver and gastrointestinal tract.¹⁰ N-acetylation of aromatic amines in the skin is an important metabolic process that changes the chemical to immunologically inactive metabolites. A well-known example of an aromatic amine is the hair dye component *p*-phenylenediamine (PPD, CAS no. 106-50-3). Its low molecular weight and strong protein-binding potential make PPD a strong sensitizer, with the potency to cause severe contact allergic reactions.¹¹ In the hair dying process, most of the PPD oxidizes by air oxygen or via auto-oxidation into its oxidation products, including *p*-benzoquinonediimine, benzoquinone, or Bandrowski's base.¹² The majority of unoxidized PPD that penetrates the skin is N-acetylated by NAT1 into mono-acetyl PPD (MAPPD) and diacetyl PPD (DAPPD).^{9,13} There is one study in which two highly PPD sensitive cases were reported who showed weak positive reactions to MAPPD and DAPPD.¹⁴ This is rarely seen since these acetylated products are considered as nonsensitizers in local lymph node assay.¹² N-acetylation of PPD can, therefore, be considered as an important step for detoxification of this agent.

Until now, NAT1 has been mainly studied in the human reconstructed epidermis, human primary keratinocytes, HaCaT cells (immortalized human keratinocyte cell line), and neonatal human skin.^{9,15} The aim of this study is to investigate NAT1 expression in healthy human skin.

METHODS AND MATERIALS

Skin tissue

Eight skin biopsies of 4 mm were obtained from six healthy human volunteers (four females, two males, age 17–54 years), snap frozen in liquid nitrogen, and stored at –80°C until use. Skin biopsies were obtained from different body locations (Table 1). For immunofluorescence, microscopy cryosections of healthy skin biopsies of 4 µm thickness were mounted on poly-L-lisine coated glass slides and airdried for 30 minutes under a cold fan. All volunteers gave informed consent to the donation of their skin. The study was performed in accordance with the Declaration of Helsinki and received approval from the ethics committee (University Medical Centre Groningen).

Immunofluorescence analysis

After drying, cryosections were circled with a hydrophobic pen (Dako, Glostrup, Denmark), blocked with 1% ovalbumin (Serva, Germany) and incubated with a monoclonal rabbit antibody against human N-acetyltransferase 1 (1:100, EPR3221 [2]), Genetex; Irvine, CA, USA). This was followed by fluorescein isothiocyanate-labeled donkey anti-rabbit IgG (Invitrogen; Bleiswijk, the Netherlands). A control staining was added with a second NAT1 antibody; a polyclonal rabbit anti-human NAT1 (1:100, NAT1 antibody ES-195, a gift from Dr. Sim, Kings-

ton University, London).¹⁶ A negative control staining (Rabbit anti-Human collagen 14, COL14, MyBioSource, San Diego, CA, USA) and in addition omission of the first antibody was performed. For accessing organellar colocalization, double staining was performed with the EPR3221 (2) or ES-195 together with Mouse antibodies against early endosomes (Clone 14/EEA1, BD transduction laboratories, San Jose, CA, USA), lysosomes (H4A3, BioLegend, San Diego, CA, USA), the Golgi apparatus (Golgin97, Invitrogen, Carlsbad, CA, USA) and mitochondria (MTC02, Abcam, Cambridge, UK). In addition, double staining with keratinocyte membrane marker desmocollin 3 (U114, Progen, Heidelberg, Germany) or desmoglein 1 (P23, Progen, Heidelberg, Germany) was performed to investigate the cytosolic location of NAT1. Depending on the antibody employed, secondary antibodies used were chicken anti-Rabbit IgG conjugated with Alexa 488 and Alexa 568 labeled Goat-anti-Mouse IgG (Thermo Fischer, Waltham, MA, USA). Nuclei were made visible with Hoechst 33342. The mounting medium was SlowFade Gold (Thermo Fischer, Waltham, CA, USA) or for confocal imaging Prolong Diamond Antifade (ThermoFisher, Waltham, MA, USA).

Analysis of the sections were performed using a Leica DMRA fluorescence microscope and images were acquired by Leica DFC350 FX digital camera (Leica, Wetzlar Germany) and recorded using Leica Application Software (Leica). The double stainings were imaged with the Leica SP8X confocal microscope using a white light laser. Lifetime gating was used to eradicate the auto fluorescence background signal.

Table 1. Overview of the six donors, of which two donors (nos. 5 and 6) provided two biopsies

Donor no.	Sex (F/M)	Age (years)	Biopsy	Location biopsy
1	F	54	a	Wrist
2	M	42	b	Axilla
3	M	41	c	Glutes
4	F	17	d	Mamma
5 ^a	F	34	e	Axilla
		35	f	Axilla
6 ^b	F	54	g	Mamma
			h	Mamma

F, female; M, male.

^aBiopsies were obtained 1 year apart.

^bBiopsies were obtained on the same day.

RESULTS

NAT1 expression pattern

NAT1 expression in healthy human skin is shown in Figure 1. Stained cryosections with EPR3221 (2) showed a clear granular or speckled expression pattern, predominantly in the stratum basale and, to a lesser extent, in the upper epidermis. In all skin biopsies, expression was found in the nuclei. The levels of expression in the cytoplasm varied between donors. A control staining with a second NAT1 antibody, ES-195 (Figure 1H), showed a more granular expression pattern, predominantly in the stratum basale as well. The pattern was specific, as the negative control (omission of first antibody) did not show any specific staining pattern (Figure 1I).

Figure 2 shows the results from double staining for NAT1 with the keratinocyte membrane marker, desmoglein 1, to investigate the cytosolic location of NAT1. High resolution, confocal sequential Z-stack images clearly show that NAT1 is present free in the cytoplasm, and it does not colocalize with the membrane bound protein. In addition, NAT1 is clearly present perinuclear and in the nucleus as well. Another keratinocyte membrane marker, desmocollin 3, showed the same staining pattern (data not shown).

Double staining with selected organelles

On the primary single staining in Figure 1, a granular or speckled NAT1 expression pattern was seen, which could also be interpreted as vesicular. The observed expression pattern was suggestive of possible colocalization and, therefore, double staining for different cellular organelles were performed (Figure 3). The expression of the different organelles are seen both perinuclear and throughout the cytoplasm for mitochondria (Mito), in a cytoplasmic speckled pattern for early endosomes (EEA1) and lysosomes (Lys). For golgi apparatus (Golgi), expression is predominantly observed perinuclear. No obvious colocalization of NAT1 with any of these organelles was seen. The negative control (replacing the anti-NAT1 antibody with a non-epidermal Rabbit antibody) did not show any specific staining pattern (Figure 3E).

Figure 1. N-acetyltransferase 1 is expressed in different epidermal layers in healthy human skin. (A-E, G) Skin biopsies of six different donors were included (see Table 1). Two donors provided two biopsies each: donor 5 (E, F) and donor 6 (G, H). (A-G) Biopsies were incubated with monoclonal rabbit antibody EPR3221 (2) (green). (H) Biopsy included a control staining incubated with polyclonal rabbit antibody ES-195 (green). (I) Tissue section presents a negative control (omission of first antibody) from biopsy (G). In all skin tissues, a speckled/granular expression located primarily in the stratum basale and, to a lesser extent, in the upper epidermis were observed. In all skin biopsies expression was found in the nuclei (blue). The levels of expression in the cytoplasm varied between donors. White bar is 25 μm

Figure 2. N-acetyltransferase 1 (NAT1) is expressed in the cytoplasm. Double staining for NAT1 (red) and desmoglein 1 (green). Tissue sections were taken from biopsy E (see Table 1). Single focal plane scans at the height of z-stack (μm) from the glass slide shows NAT1 is present in the cytosol as it does not localize with membrane bound desmoglein 1. NAT1 is present perinuclear and nuclear (arrows) as well. White bar is 5 μm .

Figure 3. No colocalization of *N*-acetyltransferase (NAT1) and different organelle proteins. (A) mitochondria (Mito; MTCO2) are visible both perinuclear as well as free in the cytoplasm. (B) endosomes (EEA1; clone 14), (C) golgi apparatus (Golgin97) and (D) lysosomes (Lys; H4A3) have a cytoplasmic speckled pattern while (E) negative controls show no fluorescence in the presence of a nonsense 1st Ab (COL14). NAT1 (green, EPR3221 [2]) does not colocalize with either of these four organelle proteins (red, A-D). White bar = 0.5 μ m

DISCUSSION

In the current study we investigated the expression pattern of NAT1 in healthy human skin. We demonstrated that NAT1 was expressed in all six donors, predominantly in the stratum basale and, to a lesser extent, in the upper epidermis. N-acetylation is an important pathway in the metabolism of PPD. In the hair dying process, the majority of unoxidized PPD that penetrates the skin will be converted by NAT1 into its detoxification products MAPPD and DAPPD.^{17,18} Pot *et al.* investigated real-time detection of PPD penetration into human skin in two donors *in vivo* using raman spectroscopy.¹⁹ PPD penetration into the skin was fast and was detected in a high concentration in the stratum corneum and reached up to the viable epidermis in decreasing concentrations. The detection of PPD up to the viable epidermis supports our outcomes of the localization of NAT1 expression, which was predominantly in the stratum basale, where it is present to N-acetylate the PPD. Pot *et al.*, however, did not detect MAPPD and DAPPD in the viable epidermis. A possible explanation why these PPD derivatives could not be detected is that the formations did not occur in concentrations above the limit of detection of the experimental design. A study by Lichter *et al.* investigated the presence and localization of NAT1 in human reconstructed epidermis and in one tissue section from neonatal skin. NAT1 was expressed throughout the whole epidermis, without indicating a difference between layers and without a specific pattern.¹⁵ In addition, active PPD acetylation in normal human primary keratinocytes (NHEK) in four donors was seen in early, intermediate, and late states of keratinocyte differentiation. The same author reported a high NAT1 activity, particularly in proliferating cells, in HaCat cells and primary keratinocytes obtained from single donor cells.²⁰ Although the methods of detection of NAT1 expression and activity from the above mentioned studies differ from the current study, the reported results partly support our outcomes, as we found NAT1 expression predominantly in the stratum basale.

In all skin biopsies expression was found in the nuclei. The levels of expression in the cytoplasm varied between donors. The observed variation in expression pattern was also observed between different biopsies obtained from the same donor. We could not find a clear explanation for this, since the applied methods for staining was equal for all tissue sections. In donor no. 5, sampling of the skin biopsies occurred on separate days. It is known that NATs are unstable and are subject to a high turnover. This can be explained by their regulation with regard to their function – at least this applies to the circadian control of serotonin-*N*-acetyltransferase.²¹ The high turnover might explain the differences in expression pattern between the two skin biopsies from donor no. 5. In addition, although all skin biopsies were taken from healthy human skin and sampling procedures were equal for all biopsies, the biopsies were taken from different body locations. Future research is necessary to study whether this could affect the expression pattern. In order to confirm our results an additional control staining with another Rabbit anti-Human antibody (ES-195) was performed and showed NAT1 expression as well. NAT1 expression patterns were similar between staining with EPR3221 (2) and ES-195, allowing marginal differences in expression pattern. Since EPR3221 (2) is a monoclonal rabbit antibody and ES-195 a polyclonal rabbit antibody this could possibly contribute to the difference. In addition, ES-195 was originally made and used to visualize NAT2 expression during normal murine embryonic development.²² In a study validating NAT1 and NAT2 specific antibodies using the In-Cell Western method, ES-195 was two times more specific towards NAT1 than NAT2.²³

Several studies have shown that NAT1 activity is detectable in the cytosol in different tissues in humans and animals.^{9,16,24,25} In this study, we reconfirmed that NAT1 can indeed be considered as a cytosolic enzyme, as we showed NAT1 to be expressed in human skin cytosol.

Kawakubo *et al.* investigated the N-acetylation capacities of PPD for MAPPD and DAPPD in human cytosol and compared these findings in cultured human epidermal keratinocytes. PPD N-acetylation activity in human cytosol was slightly lower than N-acetylation activity in cultured keratinocytes. Their presented data suggests that interindividual variation can be observed in human skin and cultured keratinocytes and suggest that a larger study is necessary to confirm these findings.⁹ In the current study, NAT1 expression was not only seen in the cytosol, but also perinuclear and in the nucleus itself. In the context of the results from Kawakubo *et al.*, it could be hypothesized that N-acetylation capacity not only occurs in the cytosol, but can be found in other organelles as well. This is in line with earlier findings where NAT1 expression was found in muscular cytosol and nuclei,²⁶ and is in agreement with Lichter *et al.* who suggested that cellular changes may influence the course of substrate availability and conversion.¹⁵ In addition, Butcher *et al.* concluded that NAT1 is subject to substrate-dependent downregulation that is due to a loss of NAT1 protein. They suggest that further investigations are necessary to gain insight into the role of NAT1 in both xenobiotic activation and cellular metabolism.^{20,27} We found no previous studies in the literature investigating NAT1 colocalization with organelles in human skin. In the current study, we found a granular or speckled NAT1 expression pattern on the primary single staining, therefore, double stainings with different organelles were performed to study the possible presence of colocalization. No colocalization of NAT1 with early endosomes, lysosomes, mitochondria, and the trans-Golgi network was seen, indicating that NAT1 is not functionally present in any of these organelles. The absence of colocalization with early endosomes, lysosomes or, the trans-Golgi network could indicate that NAT1 is possibly present in other organelles, (e.g. intracellular storage vesicles, or shedding vesicles). The absence of colocalization with mitochondria was an interesting observation, considering acetyltransferases are assumed to have important functions in mitochondria, as NAT1 is involved in the folate catabolism and mitochondria contain as much as 40% of the cellular folate.²⁸

LIMITATIONS

The interindividual variations and variations in the same donor of NAT1 expression are not completely clear and could be explained by small-study effects. Larger studies including a higher number of biopsies obtained from the same donor, from the same donor site, and from multiple donors are needed.

CONCLUSION

NAT1 expression in healthy human skin is seen predominantly in the stratum basale and can be found in the cytoplasm, in the nucleus, and perinuclear. No colocalization with mitochondria, early endosomes, trans-Golgi network, or lysosomes was found. Further research should focus on the expression patterns of NAT1 in a larger sample size and should explore the possibility of a relation between differences in NAT1 expression patterns and NAT1 genotype, to gain a better understanding of NAT1 detoxification capacities in the development of contact allergy.

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Chapter 7

Evaluation of risk modification for *p*-phenylenediamine sensitization by *N*-acetyltransferase 1 and 2 for two highly sensitive cases

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INTRODUCTION

p-Phenylenediamine (PPD) (1,4-diaminobenzene; CAS no. 106-50-3) is well known as key allergen in hair dye-related allergic contact dermatitis. PPD can be *N*-acetylated to the non-sensitizing compounds mono-acetyl PDD (MAPPD) and diacetyl PPD (DAPPD) by *N*-acetyltransferase 1 (NAT1) in keratinocytes,¹ and outside the skin also by *N*-acetyltransferase 2 (NAT2).² When investigating the elicitation response modification by NAT1 and NAT2 genotypes, we showed that genotypes containing the rapid acetylator allele NAT1*10 and individuals homozygous for the rapid acetylator allele NAT2*4 were under-represented among PPD-sensitized cases.³ Here, we evaluated this finding by investigating two well-characterized highly sensitive cases with a long-standing history of allergic contact dermatitis caused by dyes.

CASE REPORTS

Case 1

Case 1 was a 41-year-old woman who had been dyeing her hair and eyelashes with permanent black hair dye 6 to 10 times a year for 5 years. She developed itching, erythema, vesicles, infiltration and oedema of her scalp, forehead, neck and ears after dyeing her eyelashes with permanent black hair dye.

Case 2

Case 2 was a 19-year-old female trainee hairdresser who had been dyeing her hair >10 times a year for approximately 4 to 6 years. She showed erythema and pruritus of her hands and scalp after working with hair dyes and dyeing her own hair and eyelashes with semi-permanent and permanent hair dyes, respectively. Furthermore, an eczematous skin reaction to a black henna tattoo in the past was recalled.

Both individuals had positive patch test reactions to PPD 90 µg/cm² (TRUE Test; SmartPractice Europe, Reinbek, Germany). Patch testing was performed with 20 mg of MAPPD 1% pet. and 20 mg of DAPPD 1% pet. (purity of >98.0% by high-performance liquid chromatography; supplied by Procter & Gamble, Mason, Ohio). Van der Bend Chambers were used (Van der Bend, Brielle, The Netherlands), fixed with Fixomull Stretch (BSN Medical, Hamburg, Germany). The patch tests were applied on the back for 48 hours under occlusion, and patch test readings were performed on day (D) 2, D3, and D7, according to ESCD guidelines. Case 1 showed a doubtful reaction to MAPPD 1% on D2, and a + positive patch test reaction to MAPPD 1% on D3. Case 2 showed doubtful reactions to MAPPD 1% on D2 and D3, and + positive reactions to DAPPD 1% on D2 and D3 (Figure 1).

Both patients were included in a previous study of 25 PPD-allergic subjects in whom cross-elicitation responses to 2-methoxymethyl-*p*-phenylenediamine were evaluated and compared with reactions to PPD in open use testing and diagnostic patch testing.⁴ Of all 25 patch tested patients, only the two cases that we describe in this report showed positive reactions to MAPPD and DAPPD. Table 1 shows the results of patch testing and open use testing.

Table 1. Positive patch test reactions in the routine diagnostic workup at first consultation and during study follow-up

Case 1				Case 2			
Positive test results May 2002: Routine diagnostic work-up first consultation				Positive test results March 2014: Routine diagnostic work-up first consultation			
	D2	D3	D7		D2	D3	D7
Nickel sulfate 200 µg/cm ²	+	+		PPD (free base) 90 µg/cm ²		++	+
PPD (free base) 90 µg/cm ²	+	+++		Toluene-2,5-diamine 1.0%		++	+
Disperse Orange 3 1.0% pet.	++	+++					
4,4-Diaminodiphenylmethane 0.5% pet.	-	+					
4-Aminoazobenzene 0.25% pet.	+	+++					
Toluene-2,5-diamine 1.0%	-	++					
Positive test results December 2015				Positive test results January 2016			
	D2	D3	D7		D2	D3	D7
Open use test PPD 2%	+++	+++	+++	Open use test PPD 2%	+++	+++	+++
Open use test ME-PPD 2%	++	++	++	Open use test ME-PPD 2%	+	+	+
ME-PPD 0.5%	+++	+++	+++	ME-PPD 0.5%	++	++	+
ME-PPD 1%	+++	+++	++	ME-PPD 1%	++	++	+
ME-PPD 2%	+++	+++	++	ME-PPD 2%	++	+++	+
MAPPD 1% pet.	?+	+	-	MAPPD 1% pet.	?+	?+	-
DAPPD 1% pet.	-	-	-	DAPPD 1% pet.	+	+	-

D, day; DAPPD, diacetyl-*p*-phenylenediamine; MAPPD, mono-*p*-phenylenediamine; ME-PPD, 2-methoxymethyl-*p*-phenylenediamine; PPD, *p*-phenylenediamine.

We studied the acetylation status of both cases, and analysed all known variant loci, including rare single-nucleotide polymorphisms, by using a DNA sequencing-based method and statistical haplotype reconstruction. For NAT1, we observed that both cases were homozygous for the NAT1 reference allele NAT1*4/*4 and for NAT2, we found NAT2*4/*5B for case 1 and NAT2*5B/*7A for case 2. These results give no indication for a fast acetylase phenotype. Thus, despite the limited capacity of N-acetylated PPD to reactivate T cells from allergic patients *in vitro*⁵ and *in vivo*,⁶ we confirmed our earlier results and found very sensitive individuals who also reacted to MAPPD (case 1) and MAPPD and DAPPD (case 2).

In line with our previous results, which showed rapid acetylators to be under-represented among PPD-allergic cases,³ we found only reference-type acetylators for NAT1 or slow and intermediate NAT2 acetylators among the cases.

Figure 1. (A), Case 1, positive patch test reaction (+) to mono-p-phenylenediamine 1% pet. on day 3. (B), Case 2, positive patch test reaction (+) to diacetyl-p-phenylenediamine 1% pet. on day 3.

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Chapter 8

General discussion and future perspectives

DISCUSSION

This discussion will focus on two important aspects of contact allergy, following the structure of this thesis, and consists of two parts. **Part I** addresses several aspects of the **occurrence** of contact allergy, including diagnosis of contact allergy and exposure to allergens (particularly scented products). **Part II** highlights aspects of individual **susceptibility** to contact allergy.

The occurrence of contact allergy is partially related to the way the diagnosis is established; the outcome of the patch test reading is essential. The European Society of Contact Dermatitis (ESCD) guideline for diagnostic patch testing recommends performing a patch test reading on the second, third or fourth day after patch test application (day (D) 2, 3 or 4), and around D7.¹ In **chapter 2**, we assessed patch test outcomes at the D3 and separately at the D7 reading. We found that in 13.6% of patients, a positive patch test reaction to at least one contact allergen would have been missed if no D7 reading was performed, especially for allergens found in topical medications other than topical corticosteroids, in topical corticosteroids, and in metals. Several studies have been published on the added value of a late patch test reading to increase diagnostic sensitivity.^{2–4} Interestingly, some patch test centres still prefer to perform only one patch test reading or perform two patch test readings before D5 due to logistical and practical considerations. A recent study by Dickel *et al.* provided additional recommendations for a late patch test reading after 7–10 days,⁵ again highlighting the value of performing at least two patch test readings at D3 and D7.

Contact allergy can only occur if an individual has become sensitized, which is caused by exposure to a hapten. Each subsequent exposure to the contact allergen can consequently lead to contact dermatitis. Several factors contribute to the occurrence of contact allergy. These can be categorized into three essential domains: (i) skin sensitization hazard; the intrinsic property of a hapten to cause harm; (ii) the extent of exposure, including dose per unit area and repeated/aggregated exposure; and (iii) individual susceptibility factors.⁶ Although it is beyond the scope of this thesis, the skin sensitization hazard will now be briefly addressed. Subsequently, assessment of the extent of exposure will be discussed. Finally, individual susceptibility factors will be addressed in Part II of this discussion.

The skin sensitization hazard of a chemical can be determined *in vivo* by using the local lymph node assay (LLNA).⁷ In the LLNA, repeated topical exposure to a chemical is measured by the proliferative response of lymphocytes in local lymph nodes.⁸ The amount required to induce the LLNA is called the estimated concentration (EC) required to result in a threshold positive response. Substances are classified as contact allergens if the EC value results in at least three-fold greater (stimulation index = 3) lymph node cell proliferation compared to controls, hence the EC3 value.⁹ The EC3 value is used to categorize the sensitization potential of the chemical into weak, moderate, strong, or extreme.¹⁰ The disadvantage of the LLNA is that it requires animal testing. As of 2013, it can no longer be applied for the assessment of the risk of induction of sensitization from fragrances, since animal testing for cosmetic ingredients has been banned.

Moving forward, the way in which the risk of induction of sensitization is assessed, specifically for fragrances, has developed based on general toxicological principles of quantitative risk assessment (QRA).¹¹ The induction of skin sensitization is a threshold-based phenomenon.¹² This implies that contact allergens show dose-response characteristics and can be incorporat-

ed into products at acceptable concentrations in which skin sensitization is not expected.¹² The Research Institute for Fragrance Materials (RIFM) and the International Fragrance Association (IFRA) adopted the QRA model to determine safe levels of fragrance concentrations in scented products.¹¹ This model follows the same fundamental steps as in toxicology risk assessment and integrates hazard identification, dose-response assessment or hazard quantification, exposure assessment, and risk characterization.¹¹ The aim is to calculate the acceptable exposure level (AEL). The first step is to determine a quantitative threshold exposure level that is considered unlikely to induce sensitization (the no expected sensitization induction level (NESIL). The NESIL can be derived from the LLNA and other information sources (for more details, see reference 13). As a consequence of the ban on animal testing for cosmetic ingredients, in-vitro methods are under development. The ultimate goal is that QRA forms a core strategy in the primary prevention of induction of skin sensitization caused by fragrances. Recently, the QRA model has been refined to include aggregate exposure to fragrances from the use of multiple products. This refined model is termed QRA2. This is supported by a study of 5588 cosmetic products, which showed that 85.5% and 73.9% products contained at least two or three fragrance ingredients, respectively.¹⁴ In addition, the QRA2 model includes new variables, such as product composition and skin site condition.^{13,15}

Chapter 3 aimed to assess one pillar of QRA, namely exposure to scented products. We analysed exposure to scented products in the European general population and explored associations with fragrance contact allergy. We found that exposure to scented products correlates strongly with female sex and age. Moreover, the prevalence of positive patch test reactions to fragrances in females was about twice as high compared to males, which was first reported by Diepgen *et al.*¹⁶ In multiple previous studies in patients with contact dermatitis, female sex was also significantly associated with fragrance contact allergy.^{17,18} We found the highest exposure to almost all included scented products in individuals younger than 40 years of age. This was an interesting observation, as we found that older subjects (40 years of age or older) were more frequently sensitized to fragrances. Cumulative lifetime exposure may be responsible for this outcome.

A set of 26 fragrances are mandatory to label within the European Union (EU) in cosmetic products if fragrance concentration exceeds 10 ppm (mg/L) in leave-on products and 100 ppm in rinse-off products.¹⁹ This results in a difficulty in assessing suspected fragrance-allergic patients, since many more existing fragrances are not necessarily labelled by regulation.²⁰ This also applies to substances other than fragrances. As an example, between 2008 and 2015, 172 new allergens have been identified, of which 119 were associated with contact allergy.²¹ Positive patch test reaction should be assessed on clinical relevance defined by the following criteria: certainty about the exposure to the contact allergen, whether the patient's dermatitis can be explained by localisation and course after exposure, and whether the patient's dermatitis resolves after exposure to the contact allergen has been discontinued.^{22,23} However, due to incomplete product labelling, the assessment of clinical relevance cannot always be performed. To identify the unrecognized potential culprit allergen in a product, chemical analysis can be performed. To illustrate this approach, in **chapter 4** we used chemical analysis methods by applying thin-layer chromatography (TLC) and gas chromatography-mass spectrometry on a hospital wristband. The aim was to identify the culprit allergen responsible for causing the patient's dermatitis. Chemical analysis is an effective method in the search for the culprit allergen in insufficiently

labelled products.²⁴ Notwithstanding the significant value of chemical analysis, some important limitations must be considered. It is necessary to have specific knowledge of chemistry as well as access to the appropriate equipment. Therefore, only a few centres worldwide perform chemical analysis in the field of contact allergy. In addition, false-negative results can arise, especially when simple methods are used. For example, if the TLC strips contain a limited extraction from the TLC silica plate, it may be below the detection limit to elicit an allergic skin reaction in patch testing.²⁵ For patient management, consumer safety and public health surveillance, product labelling should be enhanced.

As new substances and products are introduced to the consumer's market, exposure is regularly changing over time. Therefore, the spectrum of contact allergens is also changing. It is vital to remain vigilant to new contact allergens or well-known contact allergens found in new products. This is illustrated in **chapter 5**, where we reported two patients with contact cheilitis caused by the unexpected presence of stannous (tin) fluoride in toothpaste. Both patients were using the same brand of toothpaste containing tin. A review by de Groot concluded that previously, most cases of toothpaste allergy were caused by flavouring agents. However, in recent years, more diverse contact allergens have been identified as causative contact allergens in toothpaste.²⁶ We also assessed patch testing with tin and tin salts and recommended that testing with stannous (tin) oxalate is preferred over stannous (tin) chloride when suspecting contact allergy to tin. These cases demonstrate that in patients with cheilitis, contact allergy to toothpaste should be considered and that tin present in toothpaste could be the culprit allergen. The cases also contribute to patch test diagnostics for future research in testing with tin salts.

In the assessment of exposure leading up to contact allergy, an important element that must be considered is avoidance. Naldi *et al.* evaluated self-reported skin complaints and avoidance of common daily life consumer products in the European general population from the EDEN cohort study.²⁷ Of the 12337 included participants, 37.0% and 21.0% reported ever having avoided leave-on products and rinse-off products, respectively. The lifetime avoidance was higher in females than males (68.3% vs. 38.9%). A positive relationship was found between the location of an itchy rash and avoidance of specific products typically used in the areas of the itchy rash. Our analysis from **chapter 3** did not allow us to include avoidance as a parameter. It can be argued that individuals who showed positive patch test reactions to fragrances are currently avoiding a scented product, and exposure has occurred in the past. Hence, the association between exposure to scented products and fragrance contact allergy might have been stronger if lifetime exposure was assessed rather than exposure during the last 12 months.

To reduce the occurrence of contact allergy, restrictions in exposure have been implemented by national or European regulations. These restrictions have been shown to be effective. The Nordic European countries were the first to implement nickel regulation, starting in 1994.²⁸ The effects of these regulations can be seen in the results of the EDEN study, which evaluated patch test outcomes from five European countries.²⁹ Nickel contact allergy was the lowest in Sweden (8.3%) compared to the other countries in the study. Another example of effective regulation includes the EU's restriction of chromium (dichromate) in cement, after which a decrease in chromium contact allergy caused by cement exposure was observed.³⁰ Moreover, a substantial decrease by 50% in the prevalence of methylisothiazolinone (MI) contact allergy was seen after European regulatory action was taken in the unprecedented epidemic of contact allergy to MI.³¹ The

cosmetic industry (Cosmetics Europe) and the Scientific Committee on Consumer Safety recommended prohibiting the use of MI in leave-on cosmetics and restricted the maximum MI concentration to 15 ppm in rinse-off cosmetics.³² These outcomes show the importance of national and European regulations. An important way to reduce the risk of fragrance contact allergy is to reduce (aggregate) exposure by limiting fragrance levels in scented products. The prevalence of contact allergy to fragrance mix (FM) I is 7.8% in the European clinical population³³ and 3.5% in the European population.³⁴ An important finding from **chapter 3** is the prevalence of individuals with a positive patch test reaction to at least one fragrance, which was 7.2%. It appears that the QRA1 is not yet protective, and other measures are needed to reduce the incidence of sensitization to fragrances. However, as mentioned above, a proportion of subjects could have been sensitized prior to the implementation of the QRA1.

Part II of this thesis is devoted to the inter-individual variability in developing contact allergy, that is, the individual susceptibility. Contact allergy is considered an acquired immunological condition. The first part of this discussion showed that exposure is a major determinant in acquiring contact allergy. However, although exogenous factors (environmental and/or occupational exposure) may be equal in two individuals, some individuals will develop contact allergy, whereas others will not become sensitized. It is difficult to disentangle these topics. Some individuals may appear highly sensitive. The question of whether this is a reflection of individual susceptibility or repeated exposure that triggers the immune system remains unresolved. Susceptibility to induction of skin sensitization depends on multiple variables. These variables include sex, age, ethnic group, skin condition, and immunological and genetic predisposition. It is interesting to investigate the impact of these variables on the susceptibility to contact allergy, consequently making it possible to identify subgroups of individuals with an increased susceptibility to developing contact allergy.

Sex-related differences in the incidence of contact allergy are predominantly explained by differences in exposure. Limited evidence shows the influence of sex hormones on the elicitation phase. For nickel contact allergy, it has been reported that the ovular phase (high estrogen) shows an inhibitory effect and the progestagenic phase shows an increased immunological effect.³⁵ Sex hormones have also been shown to affect the skin barrier during the menstrual cycle or menopause.³⁶ Therefore, the latter can also contribute to a higher incidence of contact allergy with increasing age. Despite senescence of the immune system owing to a diminished capacity for (new) sensitization, an increase in the prevalence of contact allergy with age is seen, probably due to a high cumulative lifetime exposure.³⁷ The North American Contact Dermatitis Group conducted a large study on the influence of ethnicity on contact allergy that showed statistically significant differences in positive patch test reactions to specific allergens between black and white patients.³⁸ The etiology of these differences remains unclear, but may be caused by culturally determined differences in exposure rather than differences in susceptibility. It has also been suggested that a lighter skin type, rather than ethnicity, contributes to the difference in contact allergy incidence due to differences in epidermal barrier function.^{39,40}

Having considered the variables mentioned above, it should now be emphasized that the factors that most influence individual susceptibility are the skin barrier function and genetic polymorphisms in metabolic and immunogenic processes.^{11,15} A subgroup of patients that is considered to display increased susceptibility is polysensitized individuals. The term polysensitization is usu-

ally defined as at least three positive patch test reactions to contact allergens in the European Baseline Series (EBS)⁴¹ or to contact allergens in other tested series.⁴² Whether polysensitized patients acquire multiple allergies only because of a high degree of exposure to multiple allergens or whether this represents true increased susceptibility has not yet been fully elucidated. A landmark study by Moss and Friedmann indicated that polysensitized individuals were more easily sensitized to a lower concentration of 2,4-dinitrochlorobenzene, without concomitant exposure, than monosensitized individuals and healthy controls.⁴³ It cannot be excluded that a small fraction of polysensitization may be on account of cross-reactivity or false positives. Dittmar *et al.* formulated an additional requirement in the definition of polysensitization, counting concomitant sensitization caused by cross-reactions or co-sensitization as one positive reaction to account for this.⁴⁴ The authors' results showed a positive correlation between the number of positive reactions and reaction strength, indicating an increased reactivity and therefore increased susceptibility.⁴⁴ This is consistent with a previous review by Schnuch *et al.* who found that with increasing strength of a positive reaction to nickel or to fragrance mix (FM), the likelihood of additional positive reactions to unrelated contact allergens and the strength of these reactions increased significantly.⁴⁵ Therefore, polysensitized individuals are considered to be particularly suited to study the genetics of contact allergy.⁴⁶

The skin is a barrier preventing the entry of allergens and external irritants. Skin barrier dysfunction may therefore allow haptens to penetrate more easily into the epidermis and promote the development of contact allergy. Inter-individual differences in the skin barrier function can be found in loss-of-function mutations in the filaggrin gene. As a result, reduced hydration of the skin, an increased pH of the skin, and increased transepidermal water loss lead to activation of serine proteases. Consequently, intercellular connections are compromised and pro-inflammatory cytokines (interleukin [IL]-1 α and IL-1 β) are activated.⁴⁷ A reduced skin barrier function is inherent to atopic dermatitis (AD). Moreover, increased exposure (e.g., to topicals) in AD patients is observed. Therefore, enhanced susceptibility can be expected. However, in **chapter 3**, we showed that the prevalence of physician-diagnosed AD in the total patch tested sample was 6.5% and in the subsample of positive patch test reactions to at least one fragrance was 5.7%, indicating that the proportion of subjects with AD was slightly lower in the fragrance-allergic subsample. A systematic review including 436 manuscripts about positive patch test reactions in children with AD showed that in children with AD, contact allergy was a common problem. However, the prevalence of contact allergy in children without AD was significantly higher,⁴² illustrating that the discussion of whether AD patients are more susceptible to induction of sensitization remains inconclusive.

After a hapten has penetrated the skin, the induction of sensitization may depend on the skin's capacities to metabolize and detoxify the penetrating haptens. Two well-known examples in this line of defence are glutathione-S-transferases (GSTs) and N-acetyltransferases (NATs). In a case-control study involving cement workers, GSTT1 deficiency in chromate-sensitized individuals was observed (3.2% controls vs. 18.9% cases).⁴⁸ A strength of this study is that it controlled for allergen exposure. Moreover, in a case-control study in patients sensitized to mercury compounds, GSTT1 and GSTM1 deficiency was seen significantly more frequently in the sensitized patients.⁴⁹

NATs are enzymes involved in the biotransformation of aromatic amines. In the field of dermatology, the aromatic amine *p*-phenylenediamine (PPD) is the most studied contact aller-

gen concerning N-acetylation. In humans, two isoforms of NATs are recognized: NAT1 and NAT2, encoded by the NAT1 and NAT2 genes. It has been shown that after PPD penetrates the skin, it is enzymatically converted by NAT1 into its acetylation products, mono-acetyl PPD (MAPPD) and diacetyl PPD (DAPPD).⁵⁰ In local lymph node assay these acetylated products have shown little to no sensitizing potential.⁵¹ N-acetylation of PPD can therefore be considered an important step for the detoxification of this agent. Single-nucleotide polymorphisms in the NAT1 gene can result in the formation of slow or rapid N-acetylation alleles. In a study investigating the NAT1 genotype in PPD-sensitized individuals compared with controls, NAT1 genotypes containing the rapid acetylation allele were associated with reduced susceptibility to PPD sensitization.⁵² Hence, a slow acetylation allele might n-acetylate less of the penetrated PPD, consequently leading to less detoxification of this agent. A recent review by Hein *et al.* proposed a new phenotypic designation for NAT1 in which the use of “rapid” and “slow” acetylators should be discontinued and should be expressed as “increased activity” alleles, “decreased activity” alleles, and “no activity” alleles.⁵³ Although NAT2 was shown to be able to acetylate PPD in *in vitro* studies, NAT2 activity in the skin is limited.⁵⁰

NAT1 activity has been mainly studied in human primary keratinocytes and reconstructed epidermis, in neonatal human skin, and in HaCaT cells.^{50,51,56} In **chapter 6**, we have taken a first step by investigating the expression pattern of NAT1 in human skin. We showed that NAT1 is expressed predominantly in the stratum basale and to a lesser extent in the upper epidermis. In all skin biopsies, expression was found in the nuclei. The levels of expression in the cytoplasm varied between donors. We could not define a clear function for NAT1 in the nucleus. As we observed NAT1 expression mainly in the stratum basale, we assume that NAT1 synthesis occurs in the vital epidermis. It could be hypothesized that after synthesis, NAT1 is stored in the nuclei until required. Upon requirement, NAT1 might be transported to the upper epidermis, where it is available to detoxify the unoxidized PPD that penetrated the stratum corneum. This might explain why all subjects showed NAT1 expression in the nuclei and why expression in the cytoplasm varied between donors. It is known that NATs are unstable and are subjected to a high turnover.⁶ As we included biopsies taken from different body sites and taken on separate days in one donor, future research should focus on whether this could have affected the expression pattern. There is no literature on whether differences in NAT polymorphisms consequently lead to differences in NAT1 expression patterns in the skin. It could be hypothesized that individuals presenting different expression patterns, which were observed in our study, might correspond to different types of NAT1 activity alleles.

As mentioned above, the PPD acetylation products MAPPD and DAPPD possess little to no sensitizing potential. However, in a previous study from our research group investigating 25 highly sensitive PPD cases, two cases showed positive reactions to MAPPD and DAPPD.⁵⁴ We hypothesized that the MAPPD- and DAPPD-positive cases would have a slow/decreased or no activity acetylation allele, resulting in a higher susceptibility to develop MAPPD and DAPPD sensitization. Therefore, in **chapter 7**, we investigated the acetylation status of both cases for all known variant loci of the NAT1 and NAT2 genes, including single-nucleotide polymorphisms. We found no indications of either slow or fast acetylation alleles. In the literature, one study performed patch testing with the EBS in 455 dermatitis patients and found 16 subjects to be PPD sensitized. Only one polysensitized subject showed a positive reaction to DAPPD. No positive reactions were found to MAPPD.⁵⁵ Based on our two highly sensitive PPD cases, it might be tempting to question the non-sensitization potential of MAPPD and DAPPD. This should be investigated in future studies that include a larger sample size.

The question remains as to what degree differences in NAT1 acetylation status contribute to the susceptibility of induction of contact allergy. Although PPD contact allergy plays a less prominent role in allergic contact dermatitis (ACD), other aromatic amines are still widely used and exposure to the skin occurs frequently. Occupational exposure can occur in the production of pharmaceuticals,⁵⁸ pesticides,⁵⁹ and rubbers.⁶⁰ Therefore, studies investigating the function of NAT1 in the context of susceptibility to develop contact allergy remain of great importance.

Besides the above-mentioned genes encoding the metabolizing enzymes, polymorphisms in genes encoding for pro-inflammatory cytokines have been reported. Polymorphisms of IL-16 and IL-10 have been reported to be associated with an increased susceptibility to develop contact allergy. Another important pro-inflammatory cytokine is tumour necrosis factor (TNF). TNF plays an important role in both the sensitization phase by inducing Langerhans cells migration as well as the elicitation phase. A study in PPD-sensitized subjects found TNFA A allele carriers to be more prevalent, indicating an increased TNF production resulting in an increased immunological reaction.⁴⁶

FINAL REMARKS AND FUTURE PERSPECTIVES

Fragrance contact allergy is considered to be of high concern. Despite regulations by the European Commission and the introduction of QRA1, so far, no reduction in fragrance sensitization trends has been observed, as opposed to the effective regulations illustrated by reducing exposure to nickel in Nordic countries and the MI pandemic. Our findings from **chapter 3** address the question of whether the concentration in leave-on products may still be too high and highlight the need for further investigation. Future studies investigating exposure to leave-on products in particular and fragrance contact allergy are warranted. Reliable exposure data is essential for reliable safety evaluation. Since 2010, the Research Institute for Fragrance Materials has been co-operating with Crème Global (<http://www.cremeglobal.com>) and has developed a probabilistic model to estimate aggregate exposure in scented products.⁶¹ This model is now being used in QRA2. To evaluate the efficacy of QRA2 as a tool for the prevention of fragrance contact allergy, longitudinal clinical studies are required.¹³ An important challenge that must be overcome is distinguishing between fragrance sensitization prior to and after QRA2 implementation. Therefore, it would be of great interest to repeat our study from **chapter 3** in the same population to assess the frequency of newly fragrance-sensitized individuals.

A key method of monitoring the prevalence of contact allergy has relied on patch test outcomes on a set of patch test substances. Novel fragrance ingredients with possible sensitizing potential are introduced into the consumer market each year. However, the set of eight fragrances (FM I) and six fragrances (FM II) tested in the EBS have essentially remained unchanged over the last 35 and 15 years, respectively. The International Dialogue for the Evaluation of Allergens initiated a well-monitored project implemented by the Extended Fragrance Ingredients Surveillance Study. The aim is to evaluate the frequency of fragrance contact allergy to existing fragrance ingredients as well as seven new fragrances with high consumer exposure.⁶² Despite the ESCD guidelines for diagnostic patch testing, patch test outcomes may still differ between centres nationally and internationally due to differences in patch test-related factors. Harmonizing patch test procedures is of great importance, enhancing the possibility for comparing data and subse-

quently performing meta-analyses. For patch test readings, we advocate for the performance of two patch test readings at D3 and D7, respectively, as outlined in **chapter 2**.

Who will develop contact allergy and who will not remains a key question. We have taken a first step in investigating the expression patterns of NAT1 in healthy human skin. A further step towards NAT1 research is to study NAT1 expression pattern in a larger sample size and compare PPD sensitized with healthy subjects before and after exposure to PPD. In addition, associations between NAT1 expression patterns and NAT1 polymorphisms should be explored. Most genetic research in contact allergy is focussed on candidate genes, i.e., genes encoding specific potentially relevant to the pathophysiology of contact allergy. The next step forward is to perform a genome-wide association study (GWAS). This may result in obtaining an insight into sensitized individuals and their polymorphisms. Contact allergy has been considered to be a quantitative trait (e.g., not simply present or absent but expressed as a measured continuum).⁴⁶ Therefore, it has been suggested that a GWAS should be performed at the extreme tail of the distribution of sensitization, namely as polysensitization.⁴⁶ Another approach would be to control subjects for allergen exposure. The ultimate goal is to identify responsible genes contributing to this complex trait, leading to a roadmap for primary prevention.

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Chapter 9

Summary

SUMMARY

Contact allergy is a commonly acquired immunological condition. It is caused by exposure of the skin to certain substances in the environment. Besides, individual susceptibility also plays a role. The effects of contact allergy can be significant and may include occupational consequences and impaired quality of life. Contact allergy is considered a lifelong condition, and no cure currently exists. Therefore, the management of contact allergies focuses on identification of the culprit allergen and avoidance of causative exposure. The first section of this thesis focuses on the diagnosis of contact allergy and exposure to allergens (particularly scented products). The second section focuses on an element of individual susceptibility to contact allergy.

The European Society of Contact Dermatitis (ESCD) recommends performing two patch test readings for contact allergy diagnosis. The first reading on the second day (D), or third, or fourth day after patch application (D3 or D4), and the second reading occurs around D7. Failure to perform a late patch test reading (after D5) may result in missed positive patch test reactions. Regardless, some centres perform only one patch reading or perform two readings prior to D5. **Chapter 2** of this thesis presents an analysis of patch test outcomes of 3292 patients who were consecutively patch tested using the extended European baseline series (EBS). Patch test readings were performed on D3 and D7. Positive reactions were categorized as positive on D3 or new positive on D7. The aims of this analysis are to investigate the added value of the D7 patch test reading for individual allergens and to identify patient characteristics and allergen groups that are associated with new positive D7 reactions. The results indicate that for 13.6% of patients, a positive patch test reaction to at least one contact allergen would have been missed if no D7 reading was performed. New positive reactions on D7 were especially found to topical corticosteroids, other topical medications and metals. No associations were found between a new positive D7 reaction and patient sex, atopic dermatitis or occupational dermatitis. These findings indicate that a D7 patch test reading does contribute added value to contact allergy diagnosis.

Contact allergy can only occur if exposure to a hapten has resulted in skin sensitization by the causative hapten. A major source of exposure to substances in our environment are fragrances in scented products. Fragrances are widely used in scented products and have the potential to induce skin sensitization. The second most common allergen (after nickel) is fragrance mix I (FMI). **Chapter 3** evaluates exposure to scented products used in daily life and explores associations between exposure and a contact allergy to fragrances. A cross-sectional study was conducted among individuals aged 18–74 years of age who were randomly selected from the general population in five European countries. A random sample ($n = 3119$) was patch tested and interviewed about their exposure to scented products. Being female was a factor strongly associated with exposure to scented products. Interestingly, participants aged 40 years and older had an inverse association with exposure to scented products, despite the fact that this group was more frequently sensitized to fragrances. Cumulative lifetime exposure may explain this outcome. In addition, self-reported sensitive skin was associated with exposure to scented products and with fragrance contact allergy. Furthermore, exposure to leave-on products was also significantly associated with fragrance contact allergy.

Chapter 4 describes a patient who developed dermatitis on his wrist where he had worn a hospital wristband. Patch testing was performed using the extended EBS, additional series and pieces

of the hospital wristband. Positive reactions to pieces of the wristband and to multiple allergens in the acrylates series were identified. Due to incomplete product labelling of the wristband, an assessment of the clinical relevance of the positive patch test reactions could not be completed. Therefore, a chemical analysis was conducted on the hospital wristband using thin-layer chromatography (TLC) and gas chromatography-mass spectrometry. The aim of this analysis was to identify the culprit allergen that had caused the patient's dermatitis. Chemical analysis of an extract of the wristband indicated the presence of lauryl acrylate and 1,6-Hexanediol diacrylate (1,6-HDDA). Patch testing using TLC strips and subsequent chemical analysis confirmed that the substance causing the strongest reaction was 1,6-HDDA, to which the patient had a confirmed positive patch test reaction.

Exposure patterns change over time as new substances and new products are introduced to the consumer market. Therefore, the spectrum of contact allergens is also constantly changing. **Chapter 5** presents a case study of two patients who developed contact cheilitis, which was caused by the unexpected presence of stannous (tin) fluoride in their toothpaste. Both patients were using the same brand of toothpaste. Cheilitis is one of the main symptoms of contact allergy to toothpaste; however, toothpaste contact allergy is insufficiently recognized as a possible cause of cheilitis. Patch testing options with tin and tin salts are assessed, and the results indicate that testing with stannous (tin) oxalate is preferable to testing with stannous (tin) chloride in the case of a suspected contact allergy to tin.

After a hapten has penetrated the skin, the induction of sensitization depends on the skin's capacity to metabolize and detoxify the penetrating hapten. The enzyme *N*-acetyltransferases (NAT) is a well-known example in this line of defence. Two isoforms of NATs are recognized in humans: NAT1 and NAT2, which are encoded by the NAT1 and NAT2 genes, respectively. NAT1 is expressed among others in keratinocytes in the human skin. NATs are involved in the biotransformation of aromatic amines. The aromatic amine *p*-phenylenediamine (PPD), a potent hair dye molecule, is the most studied contact allergen concerning *N*-acetylation in the field of dermatology. **Chapter 6** examines the expression pattern of NAT1 in human skin. Cryosections obtained from healthy human skin were stained for NAT1, and expression patterns were observed. NAT1 double stainings were performed with antibodies against different cellular organelles to determine the expression patterns. A speckled, granular expression of NAT1 was predominantly observed in the stratum basale. NAT1 was expressed in a cytoplasmic pattern, perinuclear and in the nucleus. No colocalization was observed in the selected cellular organelles. Local differences in NAT1 expression patterns were observed between donors and between different biopsies obtained from the same donor. Further NAT1 research could study NAT1 expression patterns among a larger sample and compare PPD-sensitized subjects with healthy subjects before and after exposure to PPD.

It is known that PPD is enzymatically converted by NAT1 into its acetylation products, mono-acetyl PPD (MAPPD) and diacetyl PPD (DAPPD). In local lymph node assay these acetylated products have shown little to no sensitizing potential. However, a previous investigation of 25 highly sensitive PPD cases identified two cases with positive reactions to MAPPD and DAPPD. Therefore, we hypothesized that these cases may have a NAT-allele with decreased or no activity, which would result in heightened susceptibility to MAPPD and DAPPD sensitization. **Chapter 7** investigates the acetylation status of both cases for all known variant loci of the NAT1 and NAT2

genes, including single nucleotide polymorphisms. No indications were found for slow or fast acetylation alleles.

Appendices

Appendix I

Nederlandse samenvatting

SAMENVATTING

Contactallergie wordt beschouwd als een verworven immunologische aandoening. Het wordt veroorzaakt door blootstelling van de huid aan bepaalde stoffen in de omgeving. Daarnaast spelen individuele gevoeligheidsfactoren een rol. Het is een veelvoorkomende aandoening. De gevolgen van het hebben van een contactallergie kunnen aanzienlijk zijn en kunnen problemen geven in de werkomgeving. Daarnaast kan het leiden tot een verminderde kwaliteit van leven. Contactallergie wordt beschouwd als een levenslange aandoening die niet kan worden genezen. De behandeling ervan is daarom gericht op het identificeren van het verantwoordelijke allergeen en het vermijden van de oorzakelijke blootstelling. Dit proefschrift is gericht op de diagnostiek van contactallergie en de blootstelling aan allergenen, met name aan geparfumeerde producten, besproken in het eerste deel van dit proefschrift. Het tweede deel van dit proefschrift richt zich op een aspect van de individuele gevoeligheid.

De Europese richtlijn voor het uitvoeren van plakproeven raadt aan om ten minste twee plakproefaflezingen te verrichten, op de tweede dag na het aanbrengen van de plakproeven (dag (D) 2), D3 of D4 en rondom D7. Het niet uitvoeren van een tweede, late, plakproefaflezing kan resulteren in het missen van nieuwe positieve reacties. Echter, er zijn centra die slechts één plakproefaflezing verrichten of twee aflezingen verrichten voor D5. In **hoofdstuk 2** is daarom de toegevoegde waarde van de D7-aflezing onderzocht. We analyseerden plakproefuitslagen van 3292 patiënten die een plakproef ondergingen met de uitgebreide Europese standaardreeks. Plakproefaflezingen werden uitgevoerd op D3 en D7. Positieve reacties werden onderverdeeld in positief op D3 of nieuw positief op D7. Het doel was om de toegevoegde waarde van de D7-aflezing voor individuele allergenen te onderzoeken en om patiëntkenmerken en allergeengroepen te identificeren die geassocieerd zijn met nieuwe positieve D7-reacties. Het bleek dat bij 13.6% van de patiënten een positieve plakproefreactie op ten minste één contactallergeen zou zijn gemist als er geen D7-aflezing zou zijn uitgevoerd. Dit gold vooral voor allergenen die worden aangetroffen in topicale medicatie anders dan topicale corticosteroïden, in topicale corticosteroïden en in metalen. Er werden geen associaties gevonden met geslacht, constitutioneel eczeem, werkgerelateerd eczeem en een nieuwe positieve D7-reactie. Hieruit concluderen wij dat een D7-aflezing van toegevoegde waarde is.

Contactallergie kan alleen optreden nadat blootstelling aan een contactallergeen heeft geleid tot sensibilisatie van de huid. Een belangrijke bron van blootstelling aan stoffen in de omgeving zijn geparfumeerde producten. Geparfumeerde producten worden veelal in het dagelijks leven gebruikt en hebben de potentie om huidsensibilisatie te veroorzaken. Het meest voorkomende allergeen, na nikkel, is parfum mix I. In **hoofdstuk 3** is de blootstelling aan geparfumeerde producten geëvalueerd en wordt de associatie tussen blootstelling aan geparfumeerde producten en het voorkomen van parfumallergie onderzocht. In totaal zijn 3119 proefpersonen tussen 18 tot 74 jaar oud geïnccludeerd, die willekeurig werden geselecteerd uit de algemene bevolking in vijf Europese landen. Het vrouwelijke geslacht bleek sterk geassocieerd te zijn met blootstelling aan geparfumeerde producten. Deelnemers van 40 jaar en ouder vertoonden minder blootstelling aan geparfumeerde producten dan deelnemers onder de 40 jaar. Dit was een interessante uitkomst, omdat tevens zichtbaar was dat de deelnemers van 40 jaar en ouder vaker een contactallergie voor parfums hadden. Daarom werd de hypothese opgesteld dat cumulatieve levenslange blootstelling hier verantwoordelijk voor kan zijn. Daarnaast was een zelfgerapporteerde ge-

voelige huid geassocieerd met blootstelling aan geparfumeerde producten en parfumallergie. Blootstelling aan leave-on geparfumeerde producten was daarnaast significant geassocieerd met parfumcontactallergie.

In **hoofdstuk 4** is een patiënt onderzocht die zich presenteerde met eczeem op zijn pols op de plek waar hij een ziekenhuispolsband had gedragen. Er zijn plakproeven verricht met de uitgebreide Europese standaardreeks, aanvullende reeksen en onderdelen van de ziekenhuispolsband. Positieve reacties werden gevonden op onderdelen van de polsband en op meerdere allergenen in de acrylatenreeks. Vanwege onvolledige productietikettering van de polsband kon de beoordeling van de klinische relevantie van de positieve plakproefreacties niet worden uitgevoerd. Daarom zijn chemische analysemethoden toegepast, waaronder dunnelaagchromatografie (DLC) en gaschromatografie-massaspectrometrie. Het doel hiervan was om het allergeen te identificeren dat verantwoordelijk was voor het eczeem. Chemische analyse van een extract van het polsbandje toonde de aanwezigheid van laurylacrylaat en 1,6-hexaandioldiacrylaat (1,6-HDDA). Plakproeven met de DLC-strips en daaropvolgende chemische analyse toonden aan dat de stof die de sterkste reactie veroorzaakte 1,6-HDDA was, waarop de patiënt reeds een bevestigde positieve plakproefreactie had laten zien.

Blootstellingspatronen veranderen in de loop der tijd, omdat steeds nieuwe stoffen en nieuwe producten op de consumentenmarkt worden geïntroduceerd. Daarmee verandert ook het spectrum van contactallergenen. In **hoofdstuk 5** zijn twee patiënten gepresenteerd met klachten aan het mondslijmvlies en de lippen, geduid als contactcheilitis, veroorzaakt door de onverwachte aanwezigheid van tinfluoride in hetzelfde merk tandpasta dat zij gebruikten. Hoewel cheilitis een van de belangrijkste symptomen van contactallergie in de mond is, wordt een contactallergie voor tandpasta onvoldoende herkend als mogelijke oorzaak van cheilitis. Daarnaast wordt in dit hoofdstuk beargumenteerd dat tinoxalaat de voorkeur heeft boven tinchloride voor het testen van tinzouten bij het vermoeden van een contactallergie voor tin.

Nadat een allergeen de huid is binnengedrongen, is de inductie van sensibilisatie afhankelijk van het vermogen van de huid om dit penetrerende allergeen te metaboliseren. Een bekend voorbeeld van een enzym dat betrokken is bij dit proces, is N-acetyltransferase (NAT). Bij mensen worden twee isovormen van NAT's herkend, NAT1 en NAT2, gecodeerd door de NAT1- en NAT2-genen. NAT1 komt onder meer tot expressie in keratinocyten in de menselijke huid. NAT's zijn enzymen die betrokken zijn bij de biotransformatie van aromatische amines. Op het gebied van dermatologie is het aromatische amine p-fenyleendiamine (PPD), een kleurstof in haarverf, het meest bestudeerde contactallergeen met betrekking tot N-acetylering. In **hoofdstuk 6** is het expressiepatroon van NAT1 in de menselijke huid onderzocht. Immunofluorescentieonderzoek liet een gespikkelde, korrelige expressie van NAT1 zien, voornamelijk in het stratum basale. NAT1-expressie werd gevonden in het cytoplasma, in de kern en perinucleair. NAT1-dubbelkleuringen werden uitgevoerd met antilichamen tegen verschillende cellulaire organellen om expressiepatronen te bepalen. Hierbij was geen co-lokalisatie zichtbaar met de geselecteerde cellulaire organellen. Lokale verschillen in NAT1-expressiepatronen werden waargenomen tussen donoren en tussen verschillende bipten van dezelfde donor. Een volgende stap in NAT1-onderzoek is het bestuderen van het NAT1-expressiepatroon met een grotere steekproefomvang en het vergelijken van expressiepatronen tussen PPD-gesensibiliseerde en gezonde proefpersonen, zowel voor als na blootstelling aan PPD.

PPD ondergaat naast oxidatie ook acetylatisie door NAT1, waarbij het wordt omgezet in mono-acetyl-PPD (MAPPD) en diacetyl PPD (DAPPD). MAPPD en DAPPD worden als niet of nauwelijks sensibiliserend beschouwd. Echter, in een eerdere studie van de huidige onderzoeksgroep, die 25 zeer gevoelige PPD-patiënten onderzocht, bleek dat twee patiënten positieve reacties vertoonden op MAPPD en DAPPD. Met de wetenschap dat er verschillende polymorfismen van NAT1 voor kunnen komen, is de hypothese opgesteld dat deze patiënten mogelijk een verminderde of geen activiteit van het NAT-allel hebben, wat resulteert in een grotere kans om MAPPD- en DAPPD-sensibilisatie te ontwikkelen. In **hoofdstuk 7** is de acetylatisiestatus van beide patiënten onderzocht voor alle bekende variant-loci van de NAT1- en NAT2-genen, inclusief single-nucleotide polymorfismen. Er werden geen aanwijzingen gevonden voor langzame of snelle acetyleringsallelen.

Appendix II

Over de auteur

OVER DE AUTEUR

Cynthia Catalina Aurora van Amerongen werd op 25 december 1990 geboren te Amsterdam. Enkele maanden nadien verhuisde zij met haar zus en moeder naar Ibagué, Colombia, de stad waar haar moeder opgroeiende. Op haar derde keerden ze samen terug naar Nederland. In 2009 behaalde ze haar VWO diploma met het profiel Economie en Maatschappij aan de Rijks Scholengemeenschap Tromp Meesters te Steenwijk. Na haar eindexamen besloot Cynthia geneeskunde te willen studeren. Hiervoor heeft zij in 2010 de benodigde aanvullende vakken (profiel Natuur en Gezondheid) behaald aan het Deltion Sprint Lyceum te Zwolle.

In 2014 behaalde zij haar bachelor geneeskunde en sloot ze deze mooie periode af met een reis van vijf maanden door Zuid-Amerika. In het eerste jaar van haar coschappen kwam ze in aanraking met de afdeling dermatologie in het UMCG en was direct enthousiast over dit mooie vakgebied. Het tweede jaar coschappen vond plaats in Enschede. Ze liep aansluitend een tropenstage op de afdeling algemene chirurgie, plastische chirurgie en dermatologie in Medellín (Colombia). Het laatste jaar van haar studie bestond uit een wetenschappelijke stage op de afdeling Mond- Kaak- en Aangezichtschirurgie in het Amsterdam Universitair Medisch Centrum en het Instituto Maxilofacial te Barcelona. Haar semi-arts stage liep zij in het Centro Dermatologico Federico Lleras Acosta te Bogota (Colombia) en op de afdeling Dermatologie in het UMCG.

Na het behalen van haar artsenbul begon ze als arts-onderzoeker op de afdeling dermatologie in het UMCG. Dit heeft geresulteerd in een promotietraject en het tot stand komen van dit proefschrift. Vanaf mei 2021 is Cynthia gestart met de opleiding tot dermatoloog in het UMCG.

Appendix III

Dankwoord

DANKWOORD

Dit proefschrift zou niet tot stand zijn gekomen zonder de hulp van een groot aantal mensen. Ik sluit hiermee een intensieve en leerzame periode af waarbij ik naast mijn eigen onderzoeken ook voor een groot deel werkzaam was als trial-arts voor klinische studies. Graag wil ik, niet alleen voor deze periode maar ook daarbuiten, een aantal mensen bedanken.

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Appendix IV

Publicatielijst

PUBLICATIELIJST

van Amerongen CCA, Kramer D, Pas HH, Schuttelaar MLA. The expression pattern of N-acetyltransferase 1 in healthy human skin. *Contact Dermatitis*. 2021 Jul;85(1):1-6.

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Appendix V

Research Institute SHARE

RESEARCH INSTITUTE SHARE

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