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# Contact Allergy to Fragrance Substances –
## Epidemiological Aspects and Experimental Investigations

## PhD Thesis

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*“In God we trust; all others bring data”*

William Edwards Deming, American engineer and statistician

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**This PhD thesis is based on three original manuscripts. The manuscripts are referred to by the following Roman numerals throughout the thesis:**

I. Bennike NH, Zachariae C, Johansen JD. Trends in contact allergy to fragrance mix I in consecutive Danish patients with eczema from 1986 to 2015: a cross-sectional study. *British Journal of Dermatology* 2017: 176: 1035–41.

II. Bennike NH, Zachariae C, Johansen JD. Non-mix fragrances are top sensitizers in consecutive dermatitis patients - a cross-sectional study of the 26 EU-labelled fragrance allergens. *Contact Dermatitis* 2017: 77: 270–9.

III. Bennike NH, Palangi L, Bråred Christensson J, Nilsson U, Zachariae C, Johansen JD, Hagvall L. Allergic contact dermatitis to hydroperoxides of limonene and dose-response relationship – a repeated open application test (ROAT) study. Accepted for publication in *Contact Dermatitis* (October 28th 2018), DOI:10.1111/cod.13168.

## Preface

This thesis is based on scientific work carried out at the Danish National Allergy Research Centre, the Department of Dermatology and Allergy, Copenhagen University Hospital Herlev and Gentofte, Sahlgrenska University Hospital, Gothenburg, and Stockholm University between 2015 and 2018. The project was funded in its whole by the Danish Environmental Protection Agency. The project also received financial support from the Aage Bang's Foundation, the Liv Bryhn's Foundation, the Edward Welander Foundation, and the Finsen Foundation, all gratefully acknowledged.

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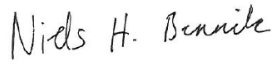

Niels Højsager Bennike

Copenhagen, October 2018

## Abbreviations

AEL: Acceptable Exposure Level

DNCB: Dinitrochlorobenzene

HICC: Hydroxyisohexyl 3-cyclohexene carboxaldehyde

IFRA: International Fragrance Association

EU: European Union

FMI: Fragrance mix I

FMII: Fragrance mix II

Lim-OOHs: Hydroperoxides of limonene

Lin-OOHs: Hydroperoxides of linalool

LLNA: Local Lymph Node Assay

MEC: Minimal Eliciting Concentration

MHC: Major histocompatibility complex

NESIL: No Expected Induction Sensitization Level

Pet.: Petrolatum

Ppm: Parts per million

QRA: Quantitative risk assessment

REACH: EU regulation concerning Registration, Evaluation, Authorisation and Restriction of Chemicals

ROAT: Repeated open application test

SAF: Sensitization Assessment Factors

SCCNFP: European Commission's Scientific Committee for Cosmetics and Non-Food Products

SCCP: European Commission's Scientific Committee on Consumer Products

SCCS: European Commission's Scientific Committee on Consumer Safety

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## Summary

### Background and aims

Fragrance substances are a frequent cause of contact allergy and more than 120 fragrance substances used in cosmetics have skin sensitizing properties. Among these, 26 fragrance contact allergens are mandatory to label in cosmetics within the EU. The use concentrations of most sensitizing fragrance substances in consumer products are based on the theoretical Quantitative Risk Assessment (QRA) model, introduced by the fragrance industry in 2008. In a clinical setting, screening for fragrance contact allergy is mainly done with fragrance mix I (FMI) and fragrance mix II (FMII), representing 14 of the 26 "EU-labelled" fragrances. Recently, oxidized limonene containing sensitizing hydroperoxides of limonene (Lim-OOHs) has emerged as a frequent sensitizer, with high rates of weak positive and doubtful patch test reactions.

The overall objectives of this thesis were:

- To investigate trends in contact allergy to FMI among consecutively patch tested dermatitis patients over a 30-year period, with special emphasis on the period 2006-2015.
- To estimate an overall prevalence of contact allergy to fragrance substances, as well as individual prevalence estimates and concomitant patch test reactions, in consecutive dermatitis patients patch tested with the 26 EU-labelled fragrance contact allergens as well as FMI and FMII.
- To investigate clinical relevance, elicitation threshold and dose-response relationship in patients with a positive patch test or a doubtful patch test to standard Lim-OOHs 0.3% in petrolatum (pet.).

### Methods

This thesis is based on three original manuscripts. Manuscripts I and II are cross-sectional studies based on patch test data from the Department of Dermatology and Allergy, Copenhagen University Hospital Herlev and Gentofte. Manuscript I investigates trends in contact allergy to FMI in 24,168 consecutive patients patch tested from 1986 to 2015. Manuscript II investigates contact allergy to FMI, FMII and the 26 EU-labelled fragrance contact allergens in 6,004 consecutive patients patch tested from 2010 to 2015. Manuscript III reports on an experimental repeated open application test (ROAT) study with Lim-OOHs carried out in 2017-18 at the Department of Dermatology and Allergy, Copenhagen University Hospital Herlev and Gentofte and the Department of Dermatology, Sahlgrenska University Hospital, Gothenburg. The study included subjects with a previous positive or doubtful patch test to Lim-OOHs 0.3% in pet. and healthy controls with no contact allergy to Lim-OOHs. In the study, participants were patch tested with standard Lim-OOHS 0.3% in pet., and a dilution series of Lim-OOHs in ethanol/water, before advancing to the ROAT. Here, participants were exposed to one (healthy controls) or three (allergic or doubtful allergic subjects) doses of Lim-OOHs in a simulated fine fragrance twice daily for up to 21 days.

**Results**

Manuscript I: Overall, contact allergy to FMI was diagnosed in 7.8% of consecutive dermatitis patients referred for patch testing between 1986 and 2015. A significant increase in FMI sensitization was observed among female dermatitis patients across the three decades. For the period 2011-2015, 10.4% of female dermatitis patients and 7.3% of male dermatitis patients were diagnosed with contact allergy to FMI, with both estimates being significantly higher than for the previous 5-year period. Contact allergy to FMI was of clinical relevance in 78% of patients, with no temporal changes between 2006-2010 and 2011-2015. Cosmetics constituted 95% of relevant exposures in patients with contact allergy to FMI.

Manuscript II: Contact allergy to FMI, FMII or one of the 26 EU-labelled fragrance contact allergens was found in 15.7% of consecutively patch tested patients between 2010-15. Among the 26 fragrance contact allergens, the highest rates of sensitization were seen for linalool hydroperoxides (Lin-OOHs) (3.9%), *Evernia furfuracea* (tree moss) (3.0%), and Lim-OOHs (2.5%). Only 30-50% of these had a concomitant positive patch test to FMI or FMII. Fewer patients sensitized to FMI were "mix positive and constituent positive" compared to FMII (32.7% vs 57.0%, $p<0.0001$). More patients were "mix negative but constituent positive" for FMII compared to FMI (12.4% vs. 3.2%, $p=0.0008$). The single constituents of FMI should be tested using increased concentrations (2%), except for cinnamal.

Manuscript III: In 11 subjects with a positive patch test to Lim-OOHs 0.3% pet., 11 (100%), 7 (64%) and 3 (27%) reacted in the ROAT to the applied doses of Lim-OOHs of 3.0 µg/cm$^2$ (1260 ppm), 0.99 µg/cm$^2$ (420 ppm), and 0.33 µg/cm$^2$ (140 ppm). No healthy controls reacted to the highest dose, and the difference in reactivity was statistically significant ($p < 0.0001$). In 13 subjects with a doubtful patch test to Lim-OOHs 0.3% pet., 2 (15%) reacted to the highest dose of Lim-OOHs in the ROAT ($p=0.36$ compared to the healthy controls). Of these, one also reacted to both the middle and lowest ROAT dose of Lim-OOHs.

**Conclusions**

Self-regulated risk management by the fragrance industry has failed in terms of establishing safe use concentrations of established fragrance contact allergens in scented consumer products. Effective primary prevention of contact allergy to individual sensitizing fragrance substances, preferably by a ban, requires substantial scientific data documenting continuous high rates of sensitization, quantitative exposure assessment, and clinical investigations of elicitation threshold and dose-response relationship.
Screening with the 26 EU-labelled fragrance contact allergens substantially improves the diagnosis of fragrance contact allergy. Full ingredient labelling of all sensitizing fragrance substances used in consumer products would improve the diagnosis even further as well as be of vital importance in terms of secondary prevention in those many individuals already sensitized. The results of this thesis substantiate the clinical relevance of contact allergy to Lim-OOHs. Further exposure quantification for the individual limonene hydroperoxides is important in the continued risk assessment for this common sensitizer.

# Dansk resumé


**Baggrund og formål**

Parfumestoffer er en hyppig årsag til kontaktallergi og mere end 120 parfumestoffer brugt i kosmetik har et allergifremkaldende potentiale. Blandt disse skal 26 parfumestoffer inden for EU deklareres med navn hvis de tilsættes til et kosmetisk produkt. Siden 2008 har de individuelle brugskoncentrationer af de fleste allergifremkaldende parfumestoffer været bestemt udfra risikovurderings-modellen "Quantitative Risk Assessment" (QRA), fremsat og udviklet af parfumeindustrien. Den primære screening for parfumeallergi i klinikken udgøres af lappetest med parfumeblandingerne "fragrance mix I" (FMI) og "fragrance mix II" (FMII). Disse indeholder 14 allergifremkaldende parfume-komponenter, der alle er blandt de 26 deklarationspligtige parfumestoffer. De seneste år er der rapporteret hyppige tilfælde af kontaktallergi over for parfumestoffet oxideret limonene, der indeholder sensibiliserende limonene hydroperoxider (Lim-OOHs). Ofte ses kun svagt positive og tvivlsomt positive lappetestreaktioner over for dette parfumestof.

De overordnede formål med denne afhandling var:

- At undersøge udviklingen over tid i kontaktallergi over for FMI blandt fortløbende eksempatienter lappetestet over en 30-årig periode, med særligt fokus på perioden 2006-2015.
- At estimere en samlet prævalens af kontaktallergi over for parfumestoffer, samt individuelle prævalensestimater og samtidige lappetestreaktioner, blandt eksempatienter lappetestet fortløbende med de 26 deklarationspligtige parfumestoffer, samt FMI og FMII.
- At undersøge klinisk relevans, tærskelværdier for provokation af allergisk kontakteksem samt dosis-respons sammenhæng hos patienter med enten en positiv eller tvivlsom positiv lappetestreaktion over for standard Lim-OOHs 0,3% i petrolatum (pet.).

**Metode**

Denne afhandling er baseret på 3 originale manuskripter. Manuskript I og II er tværsnits-registerstudier baseret på lappetestdata fra Hud- og Allergiafdelingen, Københavns Universitetshospital Herlev og Gentofte. Manuskript I undersøger udviklingen i kontaktallergi over for FMI over tid blandt 24.168 fortløbende eksempatienter for perioden 1986-2015. Manuskript II undersøger kontaktallergi over for FMI, FMII og de 26 deklarationspligtige parfumestoffer hos 6.004 fortløbende patienter for perioden 2010-15. Manuskript III omhandler et eksperimentelt "repeated open application test" (ROAT) studie med Lim-OOHs udført på Hud- og Allergiafdelingen, Herlev og Gentofte Hospital samt på Hudafdelingen på Sahlgrenska Universitetssygehus, Göteborg. Studiet inkluderede deltagere med enten en tidligere positiv eller tvivlsomt positiv lappetest over for Lim-OOHs 0,3% i pet, samt raske kontroller. I studiet blev deltagerne lappetestet igen med Lim-OOHs 0,3% i pet. samt en fortyndingsrække med Lim-OOHs i alkohol/vand, før avancement til ROAT-delen. Her blev deltagere eksponeret for én (raske kontroller) eller tre (allergiske og muligt allergiske individer) doser af Lim-OOHs i en simuleret parfume (alkohol/vand) to gange dagligt i op til 21 dage.

**Resultater**

Manuskript I: 7,8% af alle patienter i perioden 1986-2015 havde kontaktallergi over for FMI. En stigning i hyppigheden af FMI kontaktallergi blev observeret blandt kvindelige eksempatienter over de 3 årtier. I perioden 2011-15 fik 10,4% af kvindelige og 7,3% af mandlige eksempatienter konstateret kontaktallergi over for FMI, begge estimater var signifikant højere end for den forudgående 5-årige periode. En positiv lappetest over for FMI var af klinisk relevans hos 78% af patienterne uden nogen observeret ændring i denne andel mellem 2006-10 og 2011-15. Kosmetik udgjorde 95% af relevante eksponeringer hos patienter med aktuel relevans af deres kontaktallergi over for FMI.

Manuskript II: Kontaktallergi over for FMI, FMII eller et af de 26 deklarationspligtige parfumestoffer blev diagnosticeret hos 15,7% af de undersøgte eksempatienter fra 2010-15. Blandt de 26 parfumestoffer var der flest positive reaktioner over for linalool hydroperoxider (Lin-OOHs) (3,9%), *E. furfuracea* (eng. tree moss) (3,0%), og Lim-OOHs (2,5%). Kun 30-50% af disse patienter havde en samtidig positiv lappetest over for FMI eller FMII. Færre FMI-positive patienter var "mix positive og komponent positive" sammenlignet med FMII (32,7% mod 57,0%, $p<0.0001$). Flere patienter var "mix negative men komponent positive" for FMII sammenlignet med FMI (12,4% mod 3.2%, $p=0.0008$). Komponenterne af FMI bør testes i højere koncentrationer (2%), undtagen cinnamal.

Manuskript III: Blandt 11 deltagere med en positiv lappetest for Lim-OOHs 0,3% i pet. reagerede 11 (100%), 7 (64%) og 3 (27%) i ROAT studiet på de applicerede doser af Lim-OOHs på henholdsvis 3,0 $\mu g/cm^2$ (1260 ppm), 0,99 $\mu g/cm^2$ (420 ppm) og 0,33 $\mu g/cm^2$ (140 ppm). Ingen raske kontroller reagerede på den højeste dosis, og denne forskel i reaktion var signifikant ($p<0.0001$). Blandt 13 deltagere med en tvivlsomt positiv lappetest over for Lim-OOHs 0,3% i pet. reagerede 2 (15%) i ROAT-delen på den højeste dosis ($p=0.36$ versus raske kontroller). En af disse deltagere reagerede også på både den mellemste og laveste ROAT dosis af Lim-OOHs.

**Konklusion**

Parfumeindustriens selvregulering har svigtet i forhold til at etablere sikre brugskoncentrationer af allergifremkaldende parfumestoffer i forbrugerprodukter. Effektiv primær forebyggelse af kontaktallergi over for parfumestoffer, helst i form af forbud, kræver uafhængig videnskabelig dokumentation for vedvarende høj hyppighed af sensibilisering, kvantitativ eksponeringsvurdering og kliniske studier af tærskelværdier for provokation af allergisk kontakteksem samt dosis-respons forhold. Screening med de 26 deklarationspligtige parfumestoffer forbedrer markant diagnostikken af parfumeallergi. Fuld deklaration af alle allergifremkaldende parfumestoffer i kosmetik er kritisk for yderligere forbedring af diagnostikken samt sekundær forebyggelse. Resultaterne af denne afhandling underbygger den kliniske relevans af kontaktallergi over for Lim-OOH. Videre eksponeringskvantificering for de enkelte hydroperoxider i oxideret limonene er vigtig i den videre risikovurdering af denne hyppige årsag til kontaktallergi.

# 1. Introduction

Contact allergy is an acquired immunological disorder characterized by delayed type hypersensitivity to environmental, low molecular weight, organic chemicals or metal ions[1]. To date, more than 4,900 chemical compounds have been classified as potential contact allergens[2], and a recent study found that 27% of the adult general population in five European countries suffer from contact allergy[3]. The clinical manifestation of contact allergy is allergic contact dermatitis, an inflammatory skin disease characterized in the acute phase by erythema, itching, swelling, and possible presence of vesicles. If exposure to the culprit contact allergen is not ended, allergic contact dermatitis may become chronic, with scaling and development of painful fissures commonly seen[4]. Allergic contact dermatitis significantly impairs quality of life in those affected[5] and is a disease with high costs both for the individual and for society[6].

Fragrance substances are a heterogeneous group of low molecular weight organic compounds with the common property of being volatile, and hence able to be perceived as a scent. The International Fragrance Association (IFRA) defines fragrance ingredients or substances as "*any basic substance used for odour or malodour coverage"*[7]. In addition, some fragrance substances contain functional groups and can be used e.g. for their antibacterial effect[8]. A fragrance substance can be either a chemically defined substance of natural or synthetic origin, or a natural extract containing several, often less well-defined, ingredients[9]. More than 2,700 chemicals and natural extracts are registered for use as fragrance substances in cosmetic products within the EU[10]. In 2012, a comprehensive review on the topic of fragrance contact allergens in cosmetic products was published as an opinion by the Scientific Committee on Consumer Safety (SCCS) of the European Commission[11]. The SCCS opinion identified more than 120 fragrance substances with skin sensitizing properties, of which 82 fragrance substances were categorized as established contact allergens in humans. Several of these contact allergens are among the most used fragrance ingredients in consumer products such as personal care products and household detergents[12,13].

In the aforementioned study on contact allergy in the general population, 4.1% of adult Europeans were diagnosed with contact allergy to one of the investigated fragrance substances[14]. Among eczema patients suspected of contact dermatitis, as many as one in six are diagnosed with contact allergy to a fragrance substance[15]. However, due to the large number of known fragrance contact allergens, as well as continuous introduction of new potentially sensitizing fragrance substances to the market by industry, the true prevalence of contact allergy to fragrance substances is unknown.

This thesis, entitled "Contact Allergy to Fragrance Substances – Epidemiological Aspects and Experimental Investigations", explores contact allergy to fragrance substances with regards to prevalence of disease, diagnostic considerations, and experimental assessment of exposure required for the development of allergic contact dermatitis. The background for the three manuscripts (I-III) comprising the thesis is presented below.

# 2. Background

## 2.1 Immunology of contact allergy to fragrance substances

The development of allergic contact dermatitis, the clinical manifestation of contact allergy, is a two-step process involving a clinically unapparent sensitization phase and a symptomatic elicitation phase. For an individual to become sensitized to a reactive chemical following skin exposure, Landsteiner and Jacobs discovered more than 80 years ago that the chemical must react with protein structures in the skin, in order to stimulate a sufficiently strong immunological reaction[16]. As fragrance substances are volatile by definition, the molecular mass of most fragrance chemicals is small, usually around 200-300 Daltons. Combined with lipophilic properties possessed by many fragrance substances, the skin barrier is easily penetrated following cutaneous application such as by the use of a cosmetic product[17,18]. Most sensitizing fragrance chemicals are haptens, meaning that following penetration of the epidermal barrier, the fragrance chemical can interact directly with skin proteins owing to inherent chemical properties[19]. In the epidermis and superficial dermis, the immunogenic hapten-protein complex can be recognized and taken up by Langerhans cells, the professional antigen presenting cells of the skin. Activation, mediated by the hapten-protein complex, causes the Langerhans cells to express costimulatory molecules, which facilitates migration to the regional draining lymph nodes. Here, the processed hapten-protein antigen is presented to naïve T cells. The interactions between Langerhans cells and naïve T cells, in the presence of major histocompatibility complex (MHC) molecules, stimulate proliferation and formation of both antigen-specific effector T cells and antigen-specific memory T cells, resulting in sensitization of the individual. Elicitation of allergic contact dermatitis can occur upon subsequent sufficient exposure to the same, or a structurally similar, fragrance contact allergen. The innate immune system reacts to re-exposure by causing the release of T cell-attracting chemokines. This results in inflammation and infiltration of the exposed skin area with contact allergen-specific T cells. As a result of this inflammatory activation of the adaptive immune response, the clinical picture of acute allergic contact dermatitis emerges usually within 24 to 48 hours following re-exposure to the culprit contact allergen[20,21].

## 2.2 Exposure to fragrance substances

It is generally accepted that the use of cosmetics, defined broadly to include all personal care products used for hygiene and beautification purposes, represent the main source of dermal exposure to fragrance substances[22]. It has been reported that 75-80% of the worldwide production of fragrance substances is used in cosmetic products, with the remaining 20-25% being used in household products and less commonly other consumer products such as air fresheners and toys[23,24]. Aromatherapy and the use of herbal products and fragranced topical medicaments are less common among individuals from the general population[25]. Although the concentration of a single fragrance ingredient can be much higher in a product such as an air freshener, dermal exposure from such sources is low compared to exposure from cosmetics, such as body lotions and deodorants, which are applied directly to the skin[26].

A fragrance blend can consist of a mixture of a few up to several hundred different fragrance substances, and this blend can then be incorporated into the end user product. The fraction that the fragrance blend constitutes of a scented product differs. Cosmetic products such as fine fragrances or perfumes can contain 15-30% fragrance ingredients, colognes about 3-5%, and deodorants and creams/lotions around 0.5-1%[27]. In addition to being exposed to multiple fragrance substances in each cosmetic product, the daily use of more than one cosmetic product containing the same fragrance substance results in what is referred to as aggregate exposure. In a market survey conducted by the fragrance industry among 36,000 consumers from the US and Europe, 19% used a combination of deodorant, toothpaste/mouthwash and shower gel/shampoo on a daily basis. Further, 12% of consumers additionally used fine fragrances on a regular daily basis, while further 8% additionally used cosmetic styling products or moisturisers, respectively, on a daily basis[28].

Since 2005, 26 of the 82 well-established fragrance contact allergens in humans have been mandatory to label in cosmetic products within the EU, if present at 10 parts per million (ppm) or above in leave-on and 100 ppm or above in wash-off cosmetics or household detergents, respectively[29]. Hence exposure to these 26 "EU-labelled" fragrance contact allergens can be assessed by examining the ingredient labelling of cosmetic products. Among these, the fragrance terpene limonene has repeatedly been identified as one of the fragrance contact allergens most often labelled across various cosmetic product categories on the market in the UK[30], Germany[31], and Denmark[13]. Based on the German data, a high correlation has been established between actual usage volumes of the 26 fragrance substances in cosmetic products produced in Europe, and exposure estimates for these based on frequency of ingredient labelling[32].

## 2.3 Risk factors for sensitization to fragrance substances

The risk of developing contact allergy to a sensitizing fragrance substance is determined by 1) individual susceptibility, 2) the sensitizing potency of the fragrance substance, and 3) conditions of exposure to the fragrance substance.

It is believed that individual genetic susceptibility does play a role in developing sensitization to a contact allergen in general, albeit specific genetic risk factors have yet to be identified[33]. It has for long been speculated whether individuals with atopic dermatitis, and hence a compromised skin barrier, are at increased risk of developing contact allergy. However a recent meta-analysis found no significant association between atopic dermatitis and contact sensitization[34]. Among eczema patients suspected of contact dermatitis, increasing age and female sex are associated with contact allergy to fragrance substances. These observations are most likely due to accumulated exposure over time, and increased exposure in women through the use of more cosmetic products compared to men[35,36]. Although sensitization to fragrance contact allergens mainly occurs outside the workplace, allergic contact dermatitis caused by fragrance substances is associated with certain occupations, including those working as

healthcare workers, cleaning personnel, cosmeticians/beauticians, and hairdressers[36–38]. As opposed to the overall incidence of occupational allergic contact dermatitis, which has decreased significantly over the last 20 years, no significant decrease has been observed for the incidence of fragrance contact allergy contributing to occupational contact dermatitis[39].

### *2.3.1 Sensitizing potency of fragrance substances*

It is believed that the more protein-reactive a chemical is, the more potent it is at causing sensitization through recognition of the hapten-protein complex by the T-cell receptor[40]. Currently, there is no validated and generally accepted *in vitro* test available for assessing the skin sensitizing potential of chemicals[41]. Over the last decades, the preferred method for assessing the skin sensitizing potential of fragrance substances in consumer products, both by industry and regulatory bodies such as REACH under the European Commission, has been the local lymph node assay (LLNA), which is performed in mice[42–44]. For a chemical to be considered as a potential contact sensitizer in the LLNA, the chemical must induce at least a 3-fold increase, compared to a vehicle control, in the proliferation of lymphocytes in the regional lymph node draining the area of application of the chemical. The estimated concentration (in percentage) of the chemical needed to produce this 3-fold stimulation of lymphocyte proliferation is referred to as the EC3[44]. The European Commission's expert group on skin sensitization has proposed a further categorization of potential skin sensitizing chemicals, based on the LLNA, into extreme sensitizers (EC3-value $\leq$ 0.2%), strong sensitizers (EC3-value > 0.2% but $\leq$ 2%), and moderate sensitizers (EC3-value > 2%)[45].

The majority of sensitizing fragrance substances are haptens, and hence able to interact directly with skin proteins following penetration of the epidermal barrier. However, some fragrance substances require activation before having the potential to cause sensitization. Prehaptens are chemicals with negligent or very low apparent sensitizing potency which are transformed outside the body, without the requirement of specific enzymatic systems, to more potent sensitizing allergen(s). Among others, limonene has been established as a prehapten, with air exposure of pure limonene (a process referred to as autoxidation) causing the formation of specific oxidation products with a high sensitizing potential. This is evident in experimental studies, where the EC3-value for oxidized limonene is an order of magnitude lower than the EC3-value for pure limonene, corresponding to a significantly higher sensitizing potency for the oxidized fragrance substance[19]. Autoxidation of limonene causes the creation of several oxidation products, of which allergen-specific limonene hydroperoxides (Lim-OOHs) have been shown to be the most potent chemical structures with regards to risk of sensitization[46].

### *2.3.2 Exposure related risk factors*

Several exposure related factors can influence the risk of both sensitization and the elicitation threshold upon subsequent exposure to a contact allergen[47,48]. Animal studies and ethically obsolete human studies have established that the dose of a contact allergen per skin surface area, and not the total applied dose, is critical for induction of sensitization[49,50]. Using the experimental potent contact allergen dinitrochlorobenzene (DNCB), it has been shown that the risk of sensitization does not change when the area of application is halved or doubled respectively, if the dose per unit area is kept constant[51]. Below a critically small area of exposure, the area of application does however become an important factor with regards to risk of sensitization[52]. From an immunological point of view, the relationship between increased dose of exposure per unit skin area and increased risk of sensitization could be explained by more allergen being available per Langerhans cell, resulting in an increased stimulation of the immune system. On the other hand, below a critically small area of exposure, not enough hapten-protein complex bearing Langerhans cells are activated to sufficiently stimulate an immune response[53]. The exact relationship between the dose needed for sensitization and the dose needed for elicitation is unknown. However, experimental studies with DNCB have shown that the dose required for sensitization is higher than the dose required for elicitation of allergic contact dermatitis[54].

Several other exposure related factors are deemed important for both the induction and elicitation of contact allergy to fragrance substances. These include volatility of the fragrance substance[55], vehicle effects[25], concomitant exposure to irritants[56], concomitant exposure to multiple sensitizing fragrance substances[57,58], duration of skin exposure and frequency of application[59], anatomical skin region[60], and occurrence of occlusion[61] such as by the use of a cosmetic product in flexures or under clothing.

## 2.4 Diagnosing contact allergy to fragrance substances

### *2.4.1 The patch test procedure*

Contact allergy is diagnosed by patch testing, an *in vivo* test which aims to reproduce the elicitation phase of allergic contact dermatitis following exposure to a specific contact allergen. Patch testing is done by placing specified doses of a contact allergen under occlusion, usually in petrolatum (pet.) or water, on the skin under standardized conditions. An occlusion time of two days is recommended, followed by patch test readings of any possible reactions, which is optimally done at three examinations on day 2, day 3 or 4, and day 5 to 7 following application[62]. Reading of patch test reactions is done by inspection and palpation of any apparent reaction. According to globally acknowledged criteria, a weak positive patch test reaction (+) is characterized by the presence of erythema, infiltration, and possibly papules. The additional presence of vesicles defines a strong positive patch test reaction (++), and presence of coalescing vesicles (bullae) defines an extreme positive reaction (+++)[63,64]. All chemicals can induce skin irritation, if exposing an individual to sufficiently high doses. Irritant patch test reactions can assume various different

morphologies; however a general notion of sharp-edged margins and fine wrinkling of the exposed skin area are indicative of an irritant patch test reaction. Furthermore, extension of the observed reaction beyond the area exposed to the contact allergen can be used to clinically discriminate an allergic reaction from an irritant reaction[62,65]. Doubtful patch test reactions are characterized by weak erythema with no homogeneous infiltration. A doubtful patch test reaction is generally the most difficult to score clinically. The occurrence of (slight) erythema with no infiltration can represent either a weak allergic response or an irritant response[66]. In order to determine if a doubtful patch test reaction is indeed an allergic response, the patch test can be repeated with several concentrations / serial dilutions of the contact allergen, or a use test such as the repeated open application test (ROAT), which is described below, can be performed[62].

### *2.4.2 Patch testing with fragrance contact allergens*

Diagnosing contact allergy to fragrance substances is challenging due to the large number of known fragrance contact allergens. In the 1970s, Walter Larsen attempted to solve this matter by composing a mixture of fragrance substances that represented the fragrance contact allergens most often giving positive reactions among patch tested eczema patients suspected of fragrance dermatitis[67,68]. Initially named fragrance mix, now fragrance mix I (FMI), the mixture is composed of seven fragrance chemicals (amyl cinnamal, cinnamyl alcohol, cinnamal, eugenol, geraniol, hydroxycitronellal, and isoeugenol) as well as the natural extract *Evernia prunastri* (oak moss absolute). The composition of FMI has remained unchanged since 1984, where the concentrations of the individual fragrance ingredients in the mix were lowered from 2% to 1%, and the emulsifier sorbitan sesquioleate (SSO) was added at a 5% concentration[69]. To diagnostically support FMI 8% in pet. in detecting contact allergy to fragrance substances, a multicentre trial was set up in the beginning of the new millennium to investigate contact allergy to 14 frequently used fragrance contact allergens, among consecutively patch tested dermatitis patients[70]. Based on these investigations, fragrance mix II (FMII) 14% in pet. was introduced in 2005, consisting of six fragrance chemicals: Citronellol 0.5%, citral 1.0%, coumarin 2.5%, hydroxyisohexyl 3-cyclohexene carboxaldehyde (HICC, tradename Lyral®) 2.5%, farnesol 2.5%, and hexyl cinnamal 5.0%[71,72].

FMI and FMII are both part of the European baseline series of contact allergens and are hence patch tested in the majority of referred dermatitis patients. In addition, the European baseline series also contains HICC 5% pet., which is tested separately in addition to its presence in FMII. The European baseline series also contains the natural extracts balsam of Peru (*Myroxylon pereirae)* and colophonium as potential markers of fragrance contact allergy[62]. However, the importance of these natural extracts as screening markers of fragrance contact allergy varies to some extent. Crude balsam of Peru has been banned from use as a fragrance compound since 1982, however extracts and distillates of this natural extract are still used, and patients with contact allergy to balsam of Peru may react to these[73]. Patients sensitized to colophonium may react concomitantly to other natural extracts, possibly in relation to a fragrance contact allergy[73,74].

In patients with a positive patch test to either FMI or FMII, a more specific diagnosis of fragrance contact allergy can be established by performing breakdown patch testing, that is patch testing with the individual constituents, of the respective mix. For the individual constituents of FMI, the concentrations used in breakdown testing vary between manufacturers, and hence vary between patch test clinics, from 1% to 2%. On the contrary, breakdown testing with the constituents of FMII is standardized to being performed with double the concentration for each constituent compared to those used in the mix[75]. Often, breakdown testing is only performed in patients with either a positive patch test to the respective mix, or in patients suspected of contact allergy to fragrance substances[69,76–80]. However, if patch testing consecutive patients with the 26 EU-labelled fragrance contact allergens, breakdown testing of both FMI and FMII is performed consecutively, as all fragrance substances represented in the two mixtures are among these 26 fragrance contact allergens (see Table 1).

Among the 26 EU-labelled fragrance contact allergens not present in FMI or FMII, Lim-OOHs has emerged as an important allergen within recent years. Internationally, high rates of contact allergy have been reported among consecutive dermatitis patients patch tested with standard Lim-OOHs 0.3% in pet.[15,81–84]. However, patch testing with Lim-OOHs is complicated for two major reasons: Firstly, high rates of only weak positive patch test reactions, as well as high rates of doubtful and/or irritant patch test reactions to Lim-OOHs have been reported, which has led to some concern on the interpretation and clinical relevance of positive reactions[85,86]. Secondly, the actual content of the sensitizing Lim-OOHs in a consumer product containing limonene is not possible to quantify without the assistance of advanced chemical analyses[87].

| **Ingredients of Fragrance mix I (FMI) 8% pet.** | **Ingredients of Fragrance mix II (FMII) 14% pet.** | **Patch test concentrations (pet.) of the 26 EU-labelled fragrance allergens not present in FMI or FMII** |
|---|---|---|
| Amyl cinnamal 1.0% | Citronellol 0.5% | Alpha-isomethylionone 1.0% |
| Cinnamyl alcohol 1.0% | Citral 1.0% | Amyl cinnamyl alcohol 1.0% |
| Cinnamal 1.0% | Coumarin 2.5% | Anise alcohol 1.0% |
| Eugenol 1.0% | HICC/Lyral® 2.5% | Benzyl alcohol 1.0% |
| Geraniol 1.0% | Farnesol 2.5% | Benzyl cinnamate 5.0% |
| Hydroxycitronellal 1.0% | Hexyl cinnamal 5.0% | Benzyl salicylate 1.0% |
| Isoeugenol 1.0% | | Butylphenyl methylpropional (Lilial®) 10% |
| *E. prunastri* (oak moss absolute) 1.0% | | *E. furfuracea* (tree moss absolute) 1.0% |
| Sorbitan sesquioleate 5.0% (emulsifier) | | Hydroperoxides of Limonene (Lim-OOHs) 0.3% |
| | | Hydroperoxides of Linalool (Lin-OOHs) 1.0% |

*Table 1 Ingredients of FMI and FMII, and patch test concentrations of the 26 EU-labelled fragrance contact allergens not present in either mixes. Patch testing with the single constituents of FMI is done at either 1% or 2% (except cinnamal), while patch testing with the single constituents of FMII is always done with double the concentrations of those found in the mix.*

### *2.4.3 Repeated open application test*

The repeated open application test (ROAT) was developed by Hannuksela and Salo in 1986 to aid the diagnosis of allergic contact dermatitis in patients with either verified or suspected sensitization to a certain contact allergen or user product[88]. The ROAT is a standardized exposure test mimicking daily use of a product containing a (suspected) contact allergen of interest. Hence the ROAT can be used to investigate whether a positive patch test to e.g. a fragrance substance is of clinical relevance, that is, whether the patient develops allergic contact dermatitis when exposed to the allergen under simulated real-life conditions. Experimentally, the ROAT provides a method for eliciting allergic contact dermatitis, under standardized conditions, to known doses of a contact allergen, in order to determine the elicitation threshold and dose-response relationship in allergic individuals[89]. Originally, exposure in the ROAT consisted of twice daily application of a product for seven days. However a large body of evidence has shown that a longer period of allergen exposure is needed in order to detect more cases of relevant allergic contact dermatitis, especially to lower doses of exposure[90–97]. Accordingly, an exposure period of two to four weeks is recommended for experimental ROAT studies[62]. In addition to the potency and applied dose of the investigated contact allergen, as discussed above, the outcome of the ROAT is also influenced by anatomical localisation[60,98], size of the exposed area[99], and vehicle of the applied product[100]. A standardized reading scale has been developed for scoring of positive ROAT reactions, involving assessment of the proportion of involved skin area, presence and strength of erythema, and clinical signs of infiltration[101].

## 2.5 Legislation and prevention of contact allergy to fragrance substances

Within the EU, the European Parliament and Council's Regulation No. 1223/2009 is the main legislative framework for cosmetic products on the EU market[102]. Annex II of the regulation lists substances that are prohibited to use, while annex III lists substances with restricted use in cosmetic products, respectively. Building on an opinion by the European Commission's Scientific Committee for Cosmetics and Non-Food Products (SCCNFP) from 1999, the 26 EU-labelled fragrance contact allergens have been listed in annex III since 2005, as they are to be labelled on a cosmetic product if present at 10 ppm or above in leave-on cosmetics and 100 ppm or above in wash-off cosmetics or household detergents. This labelling was intended to "*improve the diagnosis of contact allergies among consumers and should enable them to avoid the use of cosmetic products which they do not tolerate"*[102]. For all other fragrance substances not listed in annex II (banned substances), their presence in cosmetic products are only to be referred to under the common International Nomenclature of Cosmetic Ingredients (INCI) labelling of "perfum", "aroma", or "fragrance". This includes the remaining of the more than 120 fragrance substances with skin sensitizing potential according to the 2012 SCCS opinion[25].

### *2.5.1 Quantitative Risk Assessment (QRA)*

In 2008, the Quantitative Risk Assessment (QRA) was introduced by IFRA and the fragrance industry as a predictive model for establishing safe use concentrations of sensitizing fragrance substances in consumer products, in order to prevent sensitization from these[103]. The basic principle of the original QRA is to derive an acceptable exposure level (AEL), expressed in $\mu g/cm^2$, to a given fragrance substance in a given fragranced consumer product that will not cause sensitization. The AEL is determined from a "no expected sensitization induction level" (NESIL) dose derived from dose-response studies in the LLNA, and confirmatory human repeat insult patch test (HRIPT) studies in healthy volunteers. In order to extract the AEL from the NESIL, the dose per unit area is divided by a set of safety factors, termed Sensitization Assessment Factors (SAF), in order to account for inter-individual variability in sensitization susceptibility, matrix effects of different products, and exposure related factors such as body area where the product is applied, as well as frequency and duration of use[103,104]. Since its introduction in 2008, IFRA and its associated members have based their standards for use concentrations of individual fragrance ingredients in different cosmetic products on the QRA. Currently, IFRA associated members supply 90% of the global market for fragrance substances, corresponding to an 8 billion US dollars industry[105].

Following its introduction, the QRA was heavily criticized in a 2008 opinion by the European Commission's Scientific Committee on Consumer Products (SCCP)[106]. The SCCP concluded that the QRA was a theoretical un-validated model with lack of in-depth method description. Furthermore, it was of concern to the SCCP that the QRA neither considered aggregate exposure to fragrance substances, nor considered occupational exposure. The SCCP also pointed out that estimation of an AEL through the use of the QRA would allow for exposure to certain fragrance substances, in higher concentrations than already known to cause allergic contact dermatitis in consumers[106]. Recently, IFRA and the fragrance industry have introduced QRA2, which however still, according to the SCCS, has scientific shortcomings with regard to being able to establish safe use concentrations of known fragrance contact allergens in consumer products[107].

# 3. Thesis objectives

## 3.1 Study part 1a (manuscript I)

- To investigate temporal trends in contact allergy to FMI in dermatitis patients referred for patch testing to a single tertiary centre over a 30-year period.
- To test the hypothesis that the prevalence of contact allergy to FMI in dermatitis patients has decreased in recent years due to implemented preventive initiatives.
- To investigate temporal changes in clinical characteristics and allergen exposure in dermatitis patients with contact allergy to FMI.

## 3.2 Study part 1b (manuscript II)

- To report an overall estimate of contact allergy to fragrance substances among dermatitis patients for the period 2010 to 2015.
- To report the prevalence and clinical relevance of contact allergy to the individual 26 fragrance allergens with mandatory labelling within the EU.
- To investigate breakdown testing of FMI and FMII in consecutive dermatitis patient tested concomitantly with both mixes and their single constituents.
- To investigate concomitant patch test reactions to FMI, FMII and the individual fragrances among the 26 EU-labelled fragrance allergens which are not present in FMI or FMII.

## 3.3 Study part 2 (manuscript III)

- To determine clinical relevance of both positive and doubtful patch test reactions to Lim-OOHs.
- To investigate the elicitation threshold in patients with either a positive or a doubtful patch test reaction to Lim-OOHs.
- To explore the dose-response relationship in subjects allergic to Lim-OOHs following both single patch test exposure as well as repeated daily exposure in a ROAT.

# 4. Methods

A detailed description of the material and applied methods used in the thesis are given in manuscript I-III. A summary is provided below, including aspects of the methods that are only briefly mentioned in the manuscripts.

## 4.1 Study part 1: Observational registry studies (manuscripts I and II)

### *4.1.1 The Danish national database for contact allergy*

For study part 1, manuscripts I and II are based on data extracted from The Danish national database for contact allergy (referred to as 'the database'). The database is administered and maintained by the Danish National Allergy Research Centre. Both the National Allergy Research Centre and the database were established in 2001 with the overall aim of monitoring the prevalence of allergy to chemical substances[108]. The database receives patch test data as well as data on demographic and clinical variables for dermatitis patients who are patch tested by members of the Danish Contact Dermatitis Group. The group is constituted of dermatologists in private practice and dermatology departments at the Danish university hospitals who perform patch testing as part of the diagnostic workup for referred dermatitis patients. The database also contains historical patch test data on dermatitis patients patch tested prior to 2001. Clinical characteristics for patch tested dermatitis patients, according to the most recent version of the MOAHLFAA index[109], have been registered in the database since the beginning of the new millennium. This index characterizes dermatitis patients with regards to sex, occupational relevance of dermatitis, a life time prevalence of atopic dermatitis, a point prevalence of hand dermatitis, leg dermatitis and facial dermatitis respectively, and the proportion of patients aged 40 years or above. The database also holds information on the relevance of registered positive patch test reactions, that is whether the presence of dermatitis in a patient can be related in time and anatomical localisation to an established exposure to the culprit contact allergen[62]. Additionally, the database holds information on consumer products, labelled to contain the contact allergen to which the individual is sensitized, if relevance of a positive patch test has been established through examination of consumer product declarations brought in by the patient.

For manuscript I, data were extracted from the database on contact allergy to FMI for unselected consecutive dermatitis referred for patch testing to the Department of Dermatology and Allergy, Herlev and Gentofte University Hospital between 1986 and 2015.
For manuscript II, data were extracted from the database on contact allergy to FMI, FMII, and the 26 EU-labelled fragrance contact allergens for unselected dermatitis patients referred for patch testing to the Department of Dermatology and Allergy, Herlev and Gentofte University Hospital between 2010 and 2015. Of note, data on patch testing with FMI between 2010 and 2015 is included in both manuscript I and II.

## 4.2 Study part 2: Clinical experimental study (manuscript III)

### *4.2.1 Repeated open application test (ROAT) study*

Manuscript III presents data from a double-blinded vehicle-controlled ROAT study with Lim-OOHs, simulating exposure to oxidized limonene in a fine fragrance. The study was carried out in collaboration with colleagues at the Department of Occupational Dermatology and the Department of Dermatology and Venereology, Sahlgrenska University Hospital, Gothenburg. Furthermore, colleagues at the Department of Chemistry and Molecular Biology at the University of Gothenburg, and the Department of Analytical Chemistry and Environmental Science at the University of Stockholm also contributed to the study with regards to the chemical analyses. The ROAT study included participants with a previous positive or doubtful patch test reaction to standard Lim-OOHs 0.3% in pet., who were identified among adult dermatitis patients patch tested between 2012 and 2017 at the Department of Occupational Dermatology, Sahlgrenska University Hospital or the Department of Dermatology and Allergy, Herlev and Gentofte University Hospital. Healthy controls in Denmark were recruited by a post on the website www.forsøgsperson.dk, while healthy controls in Sweden were recruited among office workers at the Sahlgrenska University Hospital.

In the ROAT, allergic and doubtful allergic subjects were exposed twice daily for up to 21 days to three doses of Lim-OOHs in ethanol/water (80:20) to simulate realistic exposure to oxidized limonene in a fine fragrance, as well as a vehicle control, see Figure 1. Healthy controls were only exposed to the highest dose of Lim-OOHs in the ROAT as well as a vehicle control. Prior to initiation of the ROAT, all participants were patch tested with standard Lim-OOHs 0.3% in pet. obtained from Chemotechnique Diagnostics (Vellinge, Sweden)[110]. This patch test preparation was previously known as "Oxidized limonene 3% in pet. (0.33% Lim-OOHs)"[111]. Allergic and doubtful allergic subjects were additionally patch tested with a dilution series of Lim-OOHs in the same ethanol/water vehicle as used in the ROAT. All test solutions, except the standard Lim-OOHs 0.3% in pet., were blinded to the investigators, which in Denmark was done with the assistance of the research nurse and the secretary at the National Allergy Research Centre.

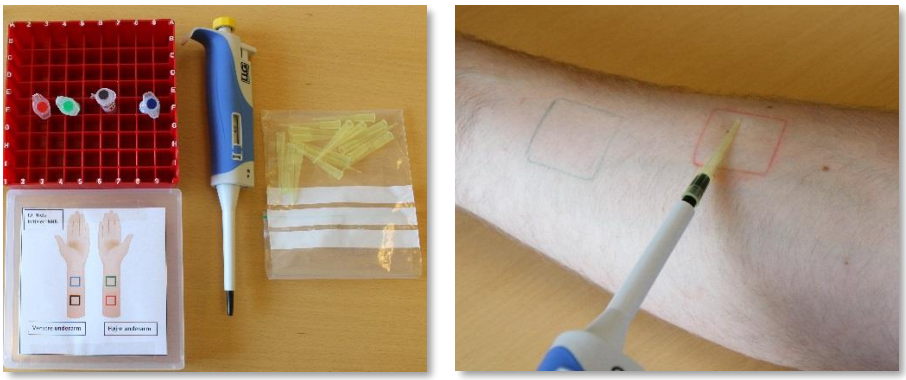
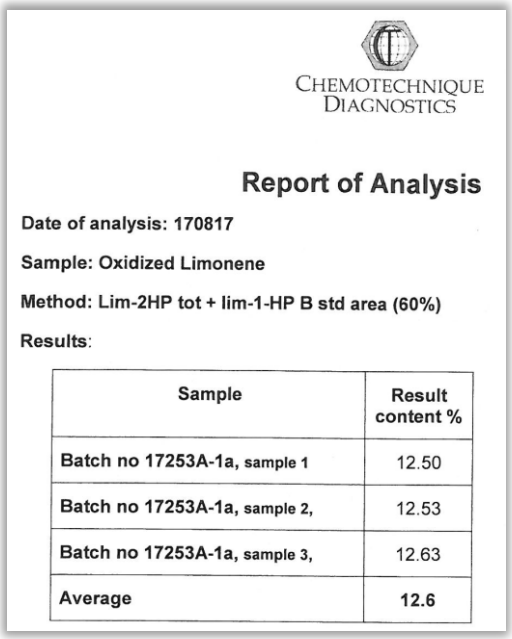


*Figure 1: For the ROAT study with Lim-OOHs, colour-coded blinded test solutions were provided on a weekly basis to participants. Twice daily for up to 21 days, 25 µl of each test solution were applied to colour-matched 3x3 cm$^2$ test areas on the volar forearms. Compliance was measured by weighing of containers before and after being provided to the participants.*

For the test solutions used in the ROAT and the dilution patch test series with Lim-OOHs, oxidized limonene with a documented content of 12.6% of the main sensitizing Lim-OOHs (limonene-1-hydroperoxide and limonene-2-hydroperoxide) was purchased from Chemotechnique Diagnostics and stored at -20 degrees Celsius under argon or nitrogen until test solutions were made. The oxidized limonene was prepared by Chemotechnique Diagnostics according to a standardized oxidation protocol, in which a sample of purified limonene was stirred for one hour, four times a day, and illuminated with a daylight lamp for 12 hours a day. This oxidation process was stopped after eight weeks (personal correspondence with Chemotechnique Diagnostics). The specification for the purchased oxidized limonene and its content of Lim-OOHs is shown in Figure 2.



CHEMOTECHNIQUE DIAGNOSTICS

**Report of Analysis**

**Date of analysis: 170817**

**Sample: Oxidized Limonene**

**Method: Lim-2HP tot + lim-1-HP B std area (60%)**

**Results**:

| Sample | Result content % |
|---|---|
| **Batch no 17253A-1a,** sample 1 | 12.50 |
| **Batch no 17253A-1a,** sample 2, | 12.53 |
| **Batch no 17253A-1a,** sample 3, | 12.63 |
| **Average** | **12.6** |

*Figure 2: Specification for the oxidized limonene, purchased for the ROAT and dilution patch test series, regarding content of the main sensitizing limonene hydroperoxides, limonene-1-hydroperoxide (lim-1-HP) and limonene-2-hydroperoxide (Lim-2HP).*

Prior to study start, stability analyses of Lim-OOHs in the ethanol/water vehicle, as well as possible *de novo* formation of Lim-OOHs in "pure" (unoxidized) limonene in the ethanol/water vehicle, were investigated by colleagues at the Department of Analytical Chemistry and Environmental Science at the University of Stockholm[112]. The "pure" limonene for these investigations were supplied by Chemotechnique Diagnostics from the same batch as used to produce the oxidized limonene. Based on the results of these analyses, of which the results are presented in manuscript III, test solutions for the patch test dilution series and the ROAT were prepared weekly to avoid changes in the applied concentrations of Lim-OOHs.

## 4.3 Ethical considerations

The observational registry studies (manuscripts I and II) were approved by the local Data Protection Agency at Herlev and Gentofte University Hospital (internal reference: HGH-2016-064), while registry studies in Denmark do not require permission from an ethics committee. The clinical experimental study (manuscript III) was approved by both the local Data Protection Agency (internal reference: HGH-2017-017) and the regional Ethics Committee (reference: H-16050154). A separate ethical permission was obtained for the Swedish part of the study. The clinical study was prospectively registered at www.clinicaltrials.gov (NCT03313232), and all aspects of the study involving human subjects adhered to the Declaration of Helsinki[113]. All participants in the clinical study were compensated financially according to the number of completed study visits (maximum 7 visits), with participants in Denmark receiving 500 DKK (approximately 70 EUR) per visit. All data in manuscripts I-III are presented anonymously.

## 4.4 Statistical considerations

Data management and statistical analyses were carried out in SAS® Enterprise Guide®, version 7.1 (SAS Institute Inc., Cary, NC, USA). Graphs and figures were made using GraphPad Prism version 7 (GraphPad software, La Jolla, California, USA). Where relevant, descriptive data and statistical analyses adhere to published guidelines on presentation of contact allergy data[114]. For hypothesis testing, two-sided *p*-values < 0.05 were deemed statistically significant. Prevalence estimates of contact allergy are presented with 95% confidence intervals (CI). Independent group comparisons were done using the $Chi^2$ test or Fisher's exact test for categorical variables, and Mann-Whitney U test for continuous variables. In manuscript I, sex-specific analyses of temporal trends were done using the Cochrane-Armitage trend test. In manuscript II, prevalence estimates for temporal trends for the investigated fragrance contact allergens were standardized with regards to sex and age below or above 40 years[115]. In manuscript III, the dose-response relationship in subjects with contact allergy to Lim-OOHs was investigated using probability unit (probit) analyses, assuming a logistic distribution for the modelled response frequencies[116].

### *4.4.1 Sample size calculation for study part 2 (clinical experimental study)*

Based on a previous ROAT with another oxidized fragrance substance (oxidized linalool containing sensitizing linalool hydroperoxides)[96], it was conservatively estimated that 50% of patients sensitized to Lim-OOHs would have a positive reaction to the ROAT test solution containing the highest dose of Lim-OOHs after three weeks of exposure. Expecting that no more than 5% of the healthy controls would have a positive ROAT, this would require a sample size of 12 participants in each group, aiming at 80% power and a risk of type 1 error of 5%[117].

## 5. Manuscripts and summary of study results

The main findings related to the objectives of this thesis are summarized below. The original manuscripts are included after each summary.

### 5.1 Trends in contact allergy to fragrance mix I in consecutive Danish patients with eczema from 1986 to 2015: a cross-sectional study (manuscript I):

- Overall, contact allergy to FMI was diagnosed in 7.8% of consecutive dermatitis patients referred for patch testing between 1986 and 2015.
- In female dermatitis patients, a significant increase in the prevalence of contact allergy to FMI was observed across three decades.
- For the period 2011-2015, 10.4% of female dermatitis patients and 7.3% of male dermatitis patients were diagnosed with contact allergy to FMI, with both estimates being significantly higher than for the previous 5-year period.
- Contact allergy to FMI was of clinical relevance in 78.2% of patients diagnosed between 2006 and 2015, with no temporal changes between 2006-2010 and 2011-2015.
- Between 2011 and 2015 did 36.1% of patients sensitized to FMI suffer from facial dermatitis which was an increase ($p = 0.05$) compared to the previous 5-year period (28.6%).
- The proportion of patients sensitized to FMI with a life-time prevalence of atopic dermatitis did not change between 2006-2010 (20.3%) and 2011-2015 (20.7%).
- Exposure to one or more of the fragrance contact allergens present in FMI was through the use of cosmetic products in 95.2% of patients with a current clinical relevance of their positive patch test to FMI.
- Shower products and lotions/creams were the cosmetic products most often causing allergic contact dermatitis in patients sensitized to FMI.
- Cosmetic styling products (hair styling, make-up, and lip balm) causing allergic contact dermatitis in patients sensitized to FMI increased from being ranked as the fifth most common product category in 2006-2010, to the third most common product category in 2011-2015.

BJD
British Journal of Dermatology

# Trends in contact allergy to fragrance mix I in consecutive Danish patients with eczema from 1986 to 2015: a cross-sectional study*

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## Summary

*Background* For more than 30 years, fragrance mix I (FMI) has been the most important screening marker for fragrance contact allergy. Meanwhile, governmental and corporate initiatives have been implemented, aimed at reducing sensitization to fragrance allergens, including the single constituents of FMI.
*Objectives* To examine trends in contact allergy to FMI from 1986 to 2015 in patients with dermatitis, and to test the hypothesis that sensitization to the fragrance screening marker has decreased within recent years.
*Methods* This was a cross-sectional registry study on patch test results to FMI among consecutively tested patients with dermatitis from a single university clinic across three 10-year periods. From 2006 to 2015, data on eczema location according to the MOAHLFA index (male; occupation; atopic dermatitis; hand; leg; face; age ≥ 40 years), clinical relevance of sensitization, and cosmetic exposures were available.
*Results* Of 24 168 patients, 7·8% (95% confidence interval 7·4–8·1) were sensitized to FMI. For women, a significant trend (P = 0·004) was observed for an increase in sensitization to FMI across the three decades. From 2011 to 2015, the prevalence of contact allergy to FMI increased significantly for women (8·0% vs. 10·4%, P = 0·002) and men (4·4% vs. 7·3%, P = 0·002) compared with the previous 5-year period. From 2006 to 2015, clinical relevance was established in 78·2% of FMI-positive patients with no differences over time. An increase (28·6% vs. 36·1%, P = 0·05) in FMI-positive patients suffering from facial dermatitis was observed for the period 2011 to 2015 compared with 2006 to 2010.
*Conclusions* The prevalence of contact allergy to FMI has been increasing in recent years. There was no demonstrable effect of previous preventive initiatives.

### What's already known about this topic?

- Fragrance substances are one of the main causes of contact allergy.
- Patch testing with fragrance mix I (FMI) was introduced more than 30 years ago.
- Preventive initiatives to reduce sensitization to established fragrance allergens have been implemented within recent years.

### What does this study add?

- In female patients with dermatitis, a trend for an increase in FMI sensitization was observed across three decades.
- Within the last decade, FMI sensitization has increased for both male and female patients with dermatitis.
- Among FMI-sensitized patients, the prevalence of facial eczema has increased within recent years.

Fragrance substances are one of the main causes of contact allergy, both in the general European population and among patients with dermatitis.[1,2] Diagnosing fragrance allergy is complicated by the vast amount of known fragrance contact allergens,[3] and in an attempt to solve these impractical issues, patch testing with fragrance mixtures was introduced more than 30 years ago.[4] Since its inception in 1979, fragrance mix I (FMI) has been the most important screening marker for contact allergy to fragrances. Consisting of seven fragrance chemicals (amyl cinnamal, cinnamyl alcohol, cinnamal, eugenol, geraniol, hydroxycitronellal and isoeugenol) and the natural extract *Evernia prunastri* (oakmoss absolute), FMI has remained unchanged in composition since 1984, when the concentration of the individual constituents was reduced from 2% to 1%, and the emulsifier sorbitan sesquioleate (SSO) was added to the mix.[5] Previous studies in European patch test populations have established higher age and female sex as risk factors for sensitization to FMI.[6,7]

Since the first fragrance symposium was held during the 1996 Jadassohn Centenary Congress in London,[8] several initiatives to prevent or reduce the consequences of fragrance allergy in consumers have been implemented. In 1999, the predecessor to the Scientific Committee on Consumer Safety (SCCS) of the European Commission identified 26 well-established fragrance allergens, including all single constituents of FMI, which by 2005 were mandatory to label if present in cosmetic products.[9] In 2008, the Quantitative Risk Assessment (QRA) was introduced by industry as a predictive model for establishing safe use concentrations of fragrance allergens in consumer products, in order to prevent sensitization from exposure to these.[10] Most recently, the SCCS opinion was updated in 2012 to include a list of 20 fragrance substances, including all single constituents of FMI except amyl cinnamal, which were reported to be of special concern due to a high number of published cases of contact allergy.[3]

For the current study, we report the results of FMI patch testing consecutive patients with dermatitis from 1986 to 2015. We hypothesized that the prevalence of contact allergy to FMI among dermatitis patients is decreasing within recent years in the context of the abovementioned initiatives.

## Materials and methods

### Study design and data collection

This was a cross-sectional registry study on data obtained from the clinical database on contact allergy at the Department of Dermatology and Allergy, Copenhagen University Hospital Herlev-Gentofte, Denmark.[11] Briefly, patients suspected of suffering from allergic contact dermatitis are referred from general practitioners, specialists in dermatovenereology and other hospital departments for patch testing at our department. Since 1979, all patch test results have been registered electronically in our clinical database. For the current study, we included available patch test results for FMI from unselected consecutive dermatitis patients, irrespective of age, who were patch tested from January 1986 to December 2015.

### Patch testing procedure

Throughout the study period, FMI as part of the European baseline series of contact allergens from Trolab® was provided by Almirall Hermal GmbH (Reinbek, Germany). Patch testing was performed using 8-mm Finn Chambers® (SmartPractice, Phoenix, AZ, USA) applied on the upper back for 48 h with Scanpor® tape (Norgesplaster, Vennesla, Norway). Readings were done on day 2, day 3 or 4, and day 7 throughout the entire study period, with maximum reactions presented here. Grading of positive allergic reactions was done according to international guidelines, which retrospectively have been consistent with the current guidelines published by the European Society of Contact Dermatitis (ESCD).[12–14] For a patch test reaction to be considered positive, homogeneous infiltration and erythema of the entire test area was required for a weak positive reaction (+), with additional vesicles defining a strong positive (++) and coalescing vesicles an extreme positive (+++) reaction. An irritant reaction, doubtful reaction or negative reading was interpreted as a negative (nonallergic) response. For patients patch tested several times with FMI during the 30-year study period, patch test results were included until the first positive result was registered. If undergoing the patch test procedure again at a later time point, patients are as a standard not tested with the allergens to which they are proven sensitized. Results to the particular allergen are then registered in the database as 'Not tested – sensitized'.

### Covariates

Basic demographic characteristics in terms of sex and age at time of patch testing were available for all patients. For patients tested between 2006 and 2015, the following covariates were additionally available: proportions of clinical characteristics, according to the MOAHLFA index (male; occupation; atopic dermatitis; hand; leg; face; age $\geq$ 40 years),[15] were assessed in terms of eczema location at the time of patch testing, as well as having a history of atopic dermatitis. Assessment of clinical relevance of a positive patch test reaction to FMI (i.e. the presence of dermatitis related temporally and anatomically to an established exposure of FMI fragrance constituents) was done according to guidelines.[14] If current relevance of contact allergy to FMI was established through examination of declarations of consumer products, these were additionally registered and included in the study. For presentation of data, cosmetic exposures were grouped into six major categories. Covariates along with final patch test results were registered by the treating dermatologist at the day of the final assessment of the patient's patch test.

### Statistical analysis

Data management and statistical analyses were performed with SAS® Enterprise Guide® software, version 7.1 (SAS Institute Inc., Cary, NC, U.S.A.), following guidelines for presentation of contact allergy data.[16] Graphing was done using

GraphPad Prism version 7.02 (GraphPad software, La Jolla, CA, USA). Prevalences of contact allergy were calculated as proportions of patients with a positive patch test of all patients tested during a specified time period. Patch test results on individuals not tested due to known sensitivity or not tested for other unknown reasons were not included in the analyses. Overall sex-specific trends in contact allergy to FMI were assessed by the Cochran–Armitage trend test, dividing the study population into three 10-year periods: 1986–1995, 1996–2005 and 2006–2015. Contact allergy to FMI was assessed in more detail for the period 2006–2015 as more covariates were available to characterize patients with a positive patch test. In order to do so, we compared patch test data and clinical characteristics for the period 2006–2010 to 2011–2015. For subgroup analyses of contact allergy to FMI, patients were stratified by sex and according to age above 40 years, which is routinely done to describe patch test populations.[15] Comparisons of proportions in the two 5-year periods were calculated with the $\chi^2$-test. For sensitivity analyses, we examined the prevalence of contact allergy to FMI within recent years, excluding SSO-positive patients from 2010 (when consecutive patch testing with the emulsifier was initiated) and onward. It has recently been reported that SSO added to FMI can cause false positive reactions to the mix in patients sensitized to the emulsifier itself.[17] Two-sided P-values $< 0{\cdot}05$ were considered to be statistically significant.

### Ethical approval

The study was approved by the Danish Data Protection Agency (reference: 2012-58-0004, internal reference: HGH-2016-064, I-Suite number: 04363). In Denmark, registry studies do not require approval from an ethics committee. The study is reported in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) recommendations.[18] All data were extracted from the database in June 2016 and handled anonymously.

## Results

During the study period, 24 399 consecutive patch test results to FMI were available. These originated from a total of 22 550 patients, corresponding to 7% of patients being patch tested more than once at separate time points during the 30-year study period: 1356 patients were tested twice, 180 patients were tested three times, 36 patients were tested four times, five patients were tested five times, and one patient was tested six times. During the study period, 70 (0·3%) patients were not tested with FMI for unknown reasons, while 161 (0·7%) were not tested due to known sensitivity from previous patch testing, resulting in a study population of 24 168 consecutive patch test results to FMI. Demographic and clinical characteristics for the study population, stratified into 10-year periods, are shown in Table 1.

**Table 1** Age and sex distributions of the total study population (n = 24 168) stratified into 10-year periods, and clinical characteristics, according to the MOAHLFA index[15] (male; occupation; atopic dermatitis; hand; leg; face; age ≥ 40 years), for patients patch tested from 2006 to 2015

| | 1986–1995 (n = 7129) | 1996–2005 (n = 8369) | 2006–2015 (n = 8670) |
|---|---|---|---|
| Age in years, mean ± SD | 48·2 ± 19·4 | 48·8 ± 18·1 | 47·0 ± 17·9 |
| Age ≤ 40 years | 2730 (38·3) | 2936 (35·1) | 3168 (36·5) |
| Age > 40 years | 4399 (61·7) | 5433 (64·9) | 5502 (63·5) |
| Women | 4498 (63·1) | 5438 (65·0) | 5914 (68·2) |
| Men | 2631 (36·9) | 2931 (35·0) | 2756 (31·8) |
| Atopic dermatitis | Not available | Not available | 1725 (19·9) |
| Hand eczema | Not available | Not available | 3419 (39·4) |
| Leg eczema | Not available | Not available | 221 (2·55) |
| Facial eczema | Not available | Not available | 2277 (26·3) |

Data are presented as n (%) unless otherwise stated. [Corrections added after initial online publication on 24 February, 2017: The values of Atopic dermatitis, Hand eczema, Leg eczema and Facial eczema were changed.]

### Overall trends from 1986 to 2015

During the 30-year study period, 1879 patients [7·8%, 95% confidence interval (CI) 7·4–8·1] were diagnosed with contact allergy to FMI. Between 583 and 1117 consecutive patients with dermatitis were patch tested each year. The lowest prevalence of positive reactions was observed in 2003 (4·7%, 95% CI 3·1–6·2) and the highest prevalence observed in 2011 with 10·6% (95% CI 8·6–12·6%) positive reactions (Table S1 lists detailed yearly patch test results to FMI; see Supporting Information). Overall and sex-specific trends in contact allergy to FMI are shown in Figure 1, comparing data from the three 10-year periods. For women, a significant trend (P = 0·004) was observed, increasing from 7·8% (95% CI 7·0–8·6) in 1986–1995, to 9·0% (95% CI 8·2–9·7) and 9·4% (95% CI 8·7–10·2) positive reactions, respectively, in the subsequent periods. For men, no overall trend (P = 0·53) for an increase in the prevalence of contact allergy to FMI across the three decades was observed, with 5.6% (95% CI 4.8–6.6), 5.9% (95% CI 5.1–6.8) and 6.0% (95% CI 5.2–7.0%) positive reactions, respectively.

#### 2006–2010 vs. 2011–2015

Comparing data from the two 5-year periods, we observed an increase in the frequency of contact allergy to FMI for both sexes. In women, contact allergy to FMI increased significantly (P = 0·002) from 190 of 2387 (8·0%, 95% CI 6·9–9·1) to 367 of 3527 (10·4%, 95% CI 9·4–11·4). An increase was observed for both age groups of women, but remained significant (P = 0·008) only in women > 40 years of age (Table 2). A similar pattern was observed for men, with a significant (P = 0·002) increase from 51 of 1169 (4·4%, 95% CI 3·2–5·6) positive reactions between 2006 and 2010 to 115 of

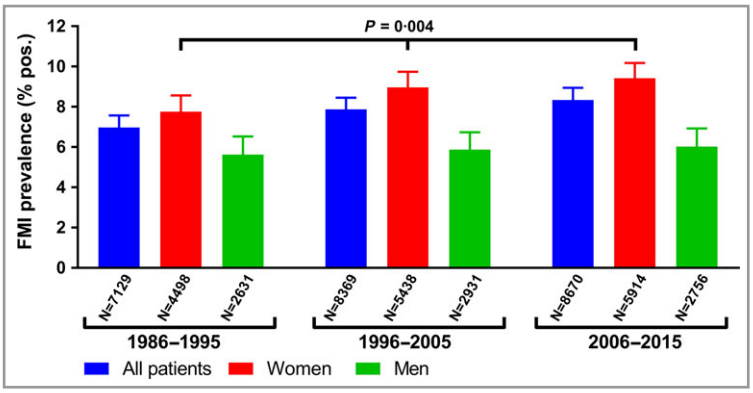


**Fig 1.** Overall trends in contact allergy to fragrance mix I over three decades, stratified by sex, see text for additional details.

**Table 2** Prevalences of contact allergy to fragrance mix I (FMI), comparing the 5-year periods 2006–2010 and 2011–2015, stratified by gender and age groups

| Subgroups of FMI-positive patients | 2006–2010 n positive/n tested (%) | 2011–2015 n positive/n tested (%) | FMI positive 2006–2010 vs. 2011–2015, P-value |
|---|---|---|---|
| Women ≤ 40 years | 49/935 (5·2) | 87/1301 (6·7) | 0·16 |
| Women > 40 years | 141/1452 (9·7) | 280/2226 (12·6) | 0·008* |
| Men ≤ 40 years | 10/386 (2·6) | 26/546 (4·8) | 0·09 |
| Men > 40 years | 41/783 (5·2) | 89/1041 (8·5) | 0·007* |

*P < 0·05 ($\chi^2$-test).

**Table 3** Proportions of clinical characteristics and established relevance for patients with a positive patch test to fragrance mix I (FMI) comparing 2006–2010 and 2011–2015

| | FMI positive 2006–2010 (n = 241) | FMI positive 2011–2015 (n = 482) | FMI positive 2006–2010 vs. 2011–2015, P-value |
|---|---|---|---|
| Atopic dermatitis | 49 (20·3) | 100 (20·7) | 0·89 |
| Eczema location | | | |
| Hand eczema | 91 (37·8) | 198 (41·1) | 0·39 |
| Leg eczema | 6 (2·49) | 15 (3·11) | 0·64 |
| Facial eczema | 69 (28·6) | 174 (36·1) | 0·05 |
| Patch test relevance | | | |
| Current relevance | 165 (68·5) | 311 (64·5) | 0·29 |
| Past relevance | 76 (31·5) | 167 (34·6) | 0·40 |
| Current and/or past relevance | 186 (77·2) | 380 (78·8) | 0·61 |

Data are presented as n (%).

1587 (7·3%, 95% CI 5·9–8·6) during the subsequent 5 years. Stratifying by age showed an increase in both subgroups of men, which remained significant (P = 0·007) in men > 40 years of age.

In all patients sensitized to FMI, an increase (P = 0·05) was seen in the frequency of patients with facial eczema from 28·6% to 36·1% comparing the two 5-year periods (Table 3). Among FMI-positive patients, no differences were observed in the frequencies of hand (37·8% vs. 41·1%, P = 0·39) and leg (2·5% vs. 3·1%, P = 0·64) eczema, as well as no differences in having a history of atopic dermatitis (20·3% vs. 20·7%, P = 0·89). Of the 723 patients with a positive patch test reaction to FMI between 2006 and 2015, the majority (78·2%) were of clinical relevance, with comparable results between the two 5-year periods (Table 3). Most reactions were of current clinical relevance, decreasing insignificantly (P = 0·29) from 68·5% to 64·5%.

Exposure data were available for 463 of 476 patients with an established current relevance of their positive patch test to FMI, with a total of 871 consumer products registered. Of these, 829 (95·2%) were cosmetic products, with 56·0% categorized as stay-on cosmetic products, and 44·0% as wash-off products. Further classification besides either stay-on or wash off was not available for the majority (55·9%) of the cosmetic products (Table 4). Of the specified cosmetic exposures, comparing data from 2006–2010 and 2011–2015, shower products (42·9% and 39·8%, respectively) and lotions/creams (34·8% and 29·1%, respectively) were the top ranking product categories. Cosmetic styling products increased from 6·3% and ranked as the fifth largest product category in 2006–2010 to 15·0% and ranked as the third largest category in 2011–2015. Deodorants, ranked third with 9·8% in 2006–2010, were ranked as fourth with 11·0% in 2011–2015.

In sensitivity analyses, we identified 11 patients who had a positive patch test reaction to SSO between 2010 and 2015. Of these, eight patients had a concomitant positive patch test to FMI. Excluding SSO-positive patients from the analyses did not change the observed increase in contact allergy to FMI among female patients with dermatitis comparing data from 2006–2010 and 2011–2015. Among male patients with dermatitis, the increase in FMI sensitization was

**Table 4** Cosmetic exposures to fragrance mix I allergens causing allergic contact dermatitis in sensitized patients

| Cosmetic products | 2006–2010 (n = 227) | 2011–2015 (n = 602) |
|---|---|---|
| Unspecified cosmetic exposures | 115 (50·7) | 348 (57·8) |
| Specified cosmetic exposures | 112 (49·3) | 254 (42·2) |
| Product categories, absolute numbers and percentages of the total number of specified cosmetic exposures | | |
| Shower products (shampoo, conditioner, liquid soap, shaving cream, make-up remover) | 48 (42·9) | 101 (39·8) |
| Lotion and cream (lotion, cream, make-up cream, SPF lotion) | 39 (34·8) | 74 (29·1) |
| Cosmetic styling (hair styling, make-up, lip balm) | 7 (6·3) | 38 (15·0) |
| Deodorants (spray, roll-on, stick) | 11 (9·8) | 28 (11·0) |
| Hydro-alcoholics (aftershave, fine fragrance) | 7 (6·3) | 11 (4·3) |
| Oral care (toothpaste) | 0 | 2 (0·79) |

Data are presented as n (%). SPF, sun protection factor.

attenuated but remained significant ($P = 0·01$), increasing from 4·4% (51 of 1169) to 6·9% (109 of 1580) positive reactions (Table S2 lists detailed results; see Supporting Information).

## Discussion

The current study investigated trends in contact allergy to FMI among consecutively patch tested patients with dermatitis in a single university clinic from 1986 to 2015. We hypothesized that the prevalence of contact allergy to FMI would decrease within recent years; however, this was not confirmed. Among all female patients with eczema, we found a significantly increased trend across the three decades in sensitization to FMI. Assessing the development in contact allergy to FMI within the last decade in more detail, we observed a significant increase in sensitization among female patients to a prevalence of 10·4% from 2011 to 2015. Similarly, a significant increase was observed in male patients to a prevalence of 7·3%. Stratifying by age revealed both absolute and relative increases in FMI sensitization among all subgroups, although the observed increases only remained significant in both female and male patients with eczema > 40 years of age.

The findings of the current study indicate a continued, and possibly increasing, exposure to well-established fragrance allergens, used in concentrations in consumer products causing contact allergy to FMI among patients with eczema. The International Fragrance Association (IFRA) has developed standards for quantitative limits on use concentrations of fragrance chemicals in consumer products, including standards for all single constituents of FMI (http://www.ifraorg.org/en-us/standards). These IFRA standards have been based on the industry-promoted and entirely theoretical QRA model, aimed at preventing dermal sensitization, since its introduction in 2008.[10] We find no indications of any decrease in sensitization to FMI within recent years, as would otherwise have been expected had the QRA model been effective. The validity of the current version of the QRA has previously been questioned, as well as concerns raised that the model fails to assess aggregate exposures to fragrance chemicals,[19] which occurs commonly.[20] The fragrance industry has recently proposed several changes to the safety factors applied in the QRA, which are used to calculate acceptable exposure levels to fragrance allergens in consumer products.[21] However, the resulting proposed acceptable exposure levels seem very similar to the ones derived from the original QRA and the issue of aggregate exposure is still not addressed.

In the literature, Nardelli *et al.* have published data on FMI sensitization from a single university contact allergy unit in Belgium on 10 128 consecutive patients with dermatitis tested from 1990 to 2005.[22] A peak prevalence of FMI sensitization was seen in 1999, followed by a steady decrease to 2005. In their most recent update of patch test results, Nardelli *et al.* reported a cross-sectional prevalence of only 9·6% positive reactions to FMI from 1990 to 2011.[23] Recently, data from the Information Network of Departments of Dermatology were published on more than 130 000 patients tested with FMI from 1999 to 2012 in several dermatology clinics in Germany, Switzerland and Austria.[7] Overall, a total of 8·7% of patients were diagnosed with contact allergy to FMI. Stratifying by age groups and sex revealed significant trends for a decrease in sensitization to FMI from 1999 to 2006, followed by a significant increase from 2007 to 2012 for all strata examined.

For the current study we did not include patch test results on breakdown testing with the eight single constituents of FMI, as we have only tested consecutive patients with these since July 2009. Results on patch testing consecutive patients with the fragrance screening markers of the European baseline series of allergens as well as the 26 European Union (EU)-labelled fragrance allergens will be reported in a future publication. It is well known that breakdown testing with the eight single constituents comprising FMI in standard concentrations at 1% in petrolatum gives negative results in a high proportion of FMI-positive patients.[5,23–26] Mann *et al.* have recently shown that testing the single constituents of FMI (except cinnamal) at 2% concentrations could identify more cases of fragrance-allergic patients.[27] As mentioned, it has recently been reported that the emulsifier SSO added to FMI can cause false positive reactions to the mix in patients sensitized to emulsifier.[17] Excluding SSO-positive patients from 2010 onwards in the current study did not affect the conclusions on the observed increase in FMI sensitization within recent years. SSO sensitivity is rare in our patch test population, and we have previously shown that from 2010 to 2014 only 1·4% of FMI-positive patients had a concomitant positive reaction to the emulsifier.[28]

To our knowledge, the current study assesses the longest period of FMI patch testing consecutive patients with

dermatitis. We demonstrated the same overall pattern in sensitization to FMI for the earlier years investigated as previously reported.[5,6] Compared with the data reported by Thyssen *et al.*,[6] we included patch test results for patients tested multiple times during the study period, censoring patients from further analyses after their first positive patch test was recorded. This was done in a pragmatic approach to allow for changes in exposure in the same individual over time to be accounted for. Furthermore, we included data on the clinical relevance of a positive patch test reaction to FMI within the last 10 years examined. Within this period, the majority of patients had a clinical relevance of their contact allergy to FMI, and we did not observe any differences in this proportion over time. Hence, the observed increase in contact allergy to FMI among dermatitis patients is real, and not just a coincident finding driven by patch testing more patients within recent years. It is also a strength of our study that health care in general, including referral for patch testing, is free of charge in Denmark, which minimizes any risk of patient selection based on financial incentives. In the current study we did not observe any increase in the proportion of FMI-positive patients with a history of atopic dermatitis. Newer studies have otherwise indicated a possible association between FMI sensitization and atopic dermatitis,[7,29] which could potentially confound the observed increase in contact allergy to FMI. However, we did observe an increase in the proportion of FMI-positive patients suffering from facial eczema. This finding was extended to an observed increase in cosmetic styling products, including make-up and hair styling products, as a relevant exposure to FMI fragrance ingredients. We identified shower products and lotions/creams as the major exposure categories, which is in line with previous reported results from our patch test population in patients tested with all 26 EU-labelled fragrance ingredients.[30]

In summary, we found a significant trend for an increase in the prevalence of contact allergy to the FMI among female patients with dermatitis over a 30-year period. Within the last decade, a significant increase was observed, irrespective of sex, indicating continued exposure to well-established fragrance allergens causing sensitization to FMI. Improved regulation aimed at reducing the use concentrations of known fragrance allergens in consumer products seems warranted.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Table S1.** Yearly prevalences of contact allergy to fragrance mix I (FMI) from 1986 to 2015.

**Table S2.** Prevalence of contact allergy to fragrance mix I (FMI) comparing the 5-year periods 2006–2010 and 2011–2015.

## Supplementary tables

**Supplementary table 1: Yearly prevalences of contact allergy to fragrance mix I (FMI) from 1986 to 2015 at the Department of Dermatology and Allergy, Herlev-Gentofte Hospital, Denmark.**

| Year | Tested FMI (n) | FMI positive (n) | FMI positive (%), 95% CI | Not tested due to known sensitivity (n) | Not tested for unknown reasons (n) |
|---|---|---|---|---|---|
| **1986** | 710 | 44 | 6.2 (4.5-8.2) | 0 | 0 |
| **1987** | 583 | 39 | 6.7 (4.8-9.0) | 0 | 0 |
| **1988** | 645 | 49 | 7.6 (5.5-9.7) | 0 | 1 |
| **1989** | 626 | 34 | 5.4 (3.8-7.5) | 0 | 1 |
| **1990** | 674 | 44 | 6.5 (4.6-8.5) | 0 | 0 |
| **1991** | 700 | 46 | 6.6 (4.7-8.5) | 0 | 0 |
| **1992** | 777 | 43 | 5.5 (3.9-7.2) | 0 | 3 |
| **1993** | 876 | 76 | 8.7 (6.7-10.6) | 0 | 1 |
| **1994** | 833 | 76 | 9.1 (7.1-11.1) | 0 | 1 |
| **1995** | 705 | 46 | 6.5 (4.6-8.4) | 0 | 6 |
| **1996** | 779 | 62 | 8.0 (6.0-9.9) | 0 | 5 |
| **1997** | 788 | 74 | 9.4 (7.3-11.5) | 0 | 7 |
| **1998** | 851 | 88 | 10.3 (8.2-12.5) | 0 | 3 |
| **1999** | 926 | 90 | 9.7 (7.8-11.7) | 0 | 11 |
| **2000** | 952 | 91 | 9.6 (7.6-11.5) | 8 | 4 |
| **2001** | 865 | 51 | 5.9 (4.3-7.5) | 16 | 1 |
| **2002** | 909 | 64 | 7.0 (5.3-8.8) | 11 | 0 |
| **2003** | 772 | 36 | 4.7 (3.1-6.2) | 11 | 0 |
| **2004** | 743 | 54 | 7.3 (5.3-9.2) | 15 | 0 |
| **2005** | 784 | 49 | 6.3 (4.5-8.0) | 11 | 1 |
| **2006** | 687 | 44 | 6.4 (4.5-8.3) | 16 | 0 |
| **2007** | 664 | 40 | 6.0 (4.1-7.9) | 8 | 0 |
| **2008** | 623 | 42 | 6.7 (4.7-8.8) | 5 | 16 |
| **2009** | 752 | 47 | 6.3 (4.5-8.1) | 7 | 2 |
| **2010** | 830 | 68 | 8.2 (6.3-10.1) | 7 | 1 |
| **2011** | 962 | 102 | 10.6 (8.6-12.6) | 8 | 2 |
| **2012** | 1004 | 101 | 10.1 (8.2-12.0) | 10 | 1 |
| **2013** | 1012 | 98 | 9.7 (7.8-11.6) | 10 | 1 |
| **2014** | 1117 | 88 | 7.9 (6.3-9.5) | 8 | 2 |
| **2015** | 1019 | 93 | 9.1 (7.3-10.9) | 10 | 0 |

**Supplementary table 2: Prevalence of contact allergy to fragrance mix I (FMI) comparing the 5-year periods 2006-2010 and 2011-2015, excluding patients with a positive patch test to SSO (n=11) from 2010 and forward.**

| Subgroups of FMI positive patients | 2006-2010 $n_{positive}/n_{tested}$ (%) | 2011-2015 $n_{positive}/n_{tested}$ (%) | FMI positive 2006-2010 vs 2011-2015, p-value |
|---|---|---|---|
| **Women ≤40 years** | 49/935 (5.2) | 86/1299 (6.6) | 0.18 |
| **Women > 40 years** | 141/1452 (9.7) | 279/2225 (12.5) | 0.008* |
| **Men ≤40 years** | 10/386 (2.6) | 25/545 (4.6) | 0.11 |
| **Men > 40 years** | 41/783 (5.2) | 84/1035 (8.1) | 0.02* |

*p<0.05 (Chi-square test)

### 5.2 Non-mix fragrances are top sensitizers in consecutive dermatitis patients – a cross-sectional study of the 26 EU-labelled fragrance allergens (manuscript II):

- Contact allergy to at least one of the investigated fragrance substances was found in 15.7% of consecutively patch tested dermatitis patients between 2010 and 2015.
- Dermatitis patients with contact allergy to fragrance substances were significantly older, more likely to be female, and had higher frequencies of leg and facial dermatitis compared to patients with no contact allergy to fragrance substances.
- Among the 26 EU-labelled fragrance contact allergens, the highest prevalence estimates of contact allergy were observed for linalool hydroperoxides (Lin-OOHs) (3.9%), *Evernia furfuracea* (tree moss) (3.0%), Lim-OOHs (2.5%), and HICC (2.1%).
- High proportions of doubtful patch test reactions were observed for Lin-OOHs (20.9%), FMI (15.4%), and Lim-OOHs (13.7%).
- Among the 26 EU-labelled fragrance contact allergens with at least 10 positive patch test reactions, clinical relevance was established in 59.4% to 83.1% of dermatitis patients.
- Among patients sensitized to FMI did 32.7% have a concomitant positive patch to at least one FMI constituent. Among patients sensitized to FMII did 57.0% have a concomitant positive patch test to at least one FMII constituent. This difference was statistically significant ($p < 0.0001$).
- Only 3.2% of patients with a negative patch test to FMI had a concomitant positive patch test to at least one FMI constituent, while 12.4% of FMII negative patients had a concomitant positive patch test to at least one FMII constituent. The difference was statistically significant ($p = 0.0008$).
- Concomitant positive patch test reactions to FMI and/or FMII were observed in 31.6% of patients sensitized to Lin-OOHs, 50.3% of patients sensitized to *Evernia furfuracea*, and 30.5% of patients sensitized to Lim-OOHs, respectively.

# Non-mix fragrances are top sensitizers in consecutive dermatitis patients – a cross-sectional study of the 26 EU-labelled fragrance allergens

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## Summary

**Background.** For cosmetics, it is mandatory to label 26 fragrance substances, including all constituents of fragrance mix I (FM I) and fragrance mix II (FM II). Earlier reports have not included oxidized *R*-limonene [hydroperoxides of *R*-limonene (Lim-OOH)] and oxidized linalool [hydroperoxides of linalool (Lin-OOH)], and breakdown testing of FM I and FM II has mainly been performed in selected, mix-positive patients.

**Objectives.** To report the prevalence of sensitization to the 26 fragrances, and to assess concomitant reactivity to FM I and/or FM II.

**Methods.** A cross-sectional study on consecutive dermatitis patients patch tested with the 26 fragrances and the European baseline series from 2010 to 2015 at a single university clinic was performed.

**Results.** Of 6004 patients, 940 (15.7%, 95%CI: 14.7–16.6%) were fragrance-sensitized. Regarding the single fragrances, most patients were sensitized to Lin-OOH (3.9%), *Evernia furfuracea* (3.0%), Lim-OOH (2.5%), and hydroxyisohexyl 3-cyclohexene carboxaldehyde (2.1%). Significantly fewer patients were 'FM I-positive and constituent-positive' than 'FM II-positive and constituent-positive' (32.7% versus 57.0%, $p < 0.0001$). Additionally, significantly more patients were 'FM II-negative but constituent-positive' than 'FM I-negative but constituent-positive' (12.4% versus 3.2%, $p = 0.0008$).

**Conclusions.** Non-mix fragrances are the most important single fragrance allergens among consecutive patients. The test concentration of the single FM I constituents should be increased when possible.

**Key words:** 26 fragrances; clinical relevance; concomitant reactivity; contact allergy; fragrance mix; non-mix fragrances; oxidized limonene; oxidized linalool.

Fragrance substances are recognized as some of the most common causes of contact allergy, both in the general population and among dermatitis patients (1–3). It has previously been shown that fragrance allergy causes a decreased quality of life, especially among younger female dermatitis patients and in patients sensitized to multiple fragrance allergens (4). We have recently shown that contact allergy to fragrance mix I (FM I), the most important screening marker for fragrance allergy, has been increasing among dermatitis patients within recent years, irrespective of sex and age (5).

Since 2005, it has been mandatory to label 26 well-established fragrance allergens within the EU if they are present at ≥10 ppm in leave-on cosmetic products,

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and at ≥100 ppm in rinse-off cosmetic products or household detergents (6). Fourteen of these 26 fragrance allergens constitute the single fragrance ingredients present in FM I and fragrance mix II (FM II), both of which are present in the current European baseline series of contact allergens (7). FM I 8% pet. contains seven fragrance chemicals (amyl cinnamal, cinnamyl alcohol, cinnamal, eugenol, geraniol, hydroxycitronellal, and isoeugenol), and the natural extract *Evernia prunastri*. In addition, FM I 8% pet. contains the emulsifier sorbitan sesquioleate (SSO), which can also cause contact allergy, at 5% (8). FM II 14% pet. was introduced in 2005, and consists of six fragrance chemicals: citronellol 0.5%, citral 1.0%, coumarin 2.5%, hydroxyisohexyl 3-cyclohexene carboxaldehyde (HICC) (Lyral®) 2.5%, farnesol 2.5%, and hexyl cinnamal 5.0% (9, 10). In contrast to FM I, for which the concentrations of single constituents for breakdown testing vary between manufacturers, breakdown patch testing with the single constituents of FM II uses double the concentrations of those found in the mix. Previous investigations into breakdown testing with the single constituents of FM I and FM II have mainly been performed in patients with either a positive patch test reaction to the respective mix or in patients with fragrance allergy (11–16).

Of the 26 fragrances not present in either FM I or FM II, that is, the 'non-mix' fragrances, the fragrance terpenes linalool and *R*-limonene are among the most extensively used fragrance substances in scented consumer products such as personal care products, household detergents, and hand cleansing agents (17). It has been established that both fragrance terpenes are prehaptens, with oxidation of these resulting in the formation of specific allergenic hydroperoxides (18). Recent multicentre trials have shown that contact allergy to oxidized *R*-limonene and oxidized linalool, with stable and standardized concentrations of the main allergenic hydroperoxides, is common (19–22). Previous reports on patch testing of consecutive dermatitis patients with the 26 EU-labelled fragrances have not included results on the oxidized forms of *R*-limonene and linalool (23–27).

The aim of this study was to assess the prevalence of contact allergy to the 26 fragrance allergens from 2010 to 2015, including results for oxidized *R*-limonene and oxidized linalool from 2012 to 2015. In addition, results concerning concomitant reactions with FM I and/or FM II were analysed.

## Materials and Methods

Data for this cross-sectional registry study were obtained from the clinical database on contact allergy at the Department of Dermatology and Allergy, Copenhagen University Hospital Herlev-Gentofte, Denmark (28). We included available patch test results for FM I, FM II and 25 of the 26 EU-labelled fragrances from consecutive dermatitis patients who were patch tested with our baseline series between January 2010 and December 2015, irrespective of age. Patients had not been tested with methyl 2-octynoate, which may cause active sensitization (29). For *R*-limonene and linalool, we report patch test results from January 2012, when consecutive patch testing with the oxidized forms of these fragrance allergens was implemented, replacing patch testing with the unoxidized fragrance terpenes. For the current investigation, we did not include patch test results for balsam of Peru (*Myroxylon pereirae*) and colophonium from the European baseline series, owing to the wide variation in the importance of these allergens as screening markers for fragrance allergy (30).

In addition to the European baseline series, baseline patch testing at our department was performed with our fragrance series from Trolab®, provided during the study period by Almirall Hermal (Reinbek, Germany), consisting of the 25 fragrance ingredients and SSO 20% pet. The oxidized forms of linalool and *R*-limonene, with standardized and stable concentrations of hydroperoxides of limonene (Lim-OOH) 0.3% pet. and hydroperoxides of linalool (Lin-OOH) 1% pet., respectively, were supplied by Chemotechnique Diagnostics (Vellinge, Sweden). Between 45.4–53.1% of patients aged <18 years (n = 262) were not tested with the single constituents of our fragrance series, but only with FM I, FM II, and HICC, owing to the limited space on their back. Patch testing was performed with Finn Chambers® (8 mm; SmartPractice, Phoenix, AZ, USA) applied on the upper back for 48 h with Scanpor® tape (Norgesplaster, Vennesla, Norway). Patch test readings were performed on day (D) 2, D3 or D4, and D7, and the maximum reactions are presented here. Grading of positive allergic reactions as weak (+), strong (++), and extreme (+++), and the scoring of doubtful (?+) and irritant reactions (IRs), were performed according to international guidelines, which, retrospectively, are compliant with the current criteria implemented by the ESCD in 2015 (7, 31). In the assessment of concomitant reactivity to FM I and FM II and their single constituents, patch test reactions to the respective mix were grouped as either positive, doubtful (?+), or negative (including IRs).

The clinical characteristics of patch tested patients were available according to the MOAHLFA index, describing the proportion of patients with regard to sex, occupational relevance of dermatitis, a lifetime prevalence of atopic dermatitis assessed by the treating dermatologist,

**Table 1.** Demographic and clinical characteristics according to MOAHLFA for all patients tested, fragrance-sensitized patients, and patients with no positive patch test reactions to the investigated fragrance allergens

| | All patients N = 6004 | Fragrance-positive* n = 940 | Fragrance-negative n = 5064 | Fragrance-positive versus fragrance-negative, *p*-value† |
|---|---|---|---|---|
| Age (years), mean (SD) | 47.1 (18.1) | 50.5 (16.3) | 46.5 (18.3) | <0.0001 |
| Age ≥40 years, n (%) | 3900 (65.0) | 698 (74.3) | 3202 (63.2) | <0.0001 |
| Male, n (%) | 1865 (31.1) | 245 (26.1) | 1620 (32.0) | 0.0003 |
| Occupational dermatitis, n (%) | 1205 (20.1) | 174 (18.5) | 1031 (20.4) | 0.19 |
| Atopic dermatitis, n (%) | 1250 (20.8) | 184 (19.6) | 1066 (21.1) | 0.31 |
| Hand dermatitis, n (%) | 2301 (38.3) | 376 (40.0) | 1925 (38.0) | 0.25 |
| Leg dermatitis, n (%) | 102 (1.70) | 28 (2.98) | 74 (1.46) | 0.0009 |
| Face dermatitis, n (%) | 1653 (27.5) | 321 (34.2) | 1332 (26.3) | <0.0001 |

SD, standard deviation.
*Contact allergy to at least one of the investigated fragrance allergens (excluding the emulsifier sorbitan sesquioleate).
†$Chi^2$ test for categorical variables and Mann–Whitney *U*-test for continuous variables.

a point prevalence of hand, leg and facial dermatitis, and the proportion of patients aged >40 years (32). In patients with a positive patch test reaction to any of the investigated fragrance allergens, the clinical relevance of sensitization (i.e. the presence of dermatitis related temporally and anatomically to an established exposure to the relevant allergen) was assessed according to current guidelines (7). The current clinical relevance of a positive patch test reaction was mainly established through the patient's medical history or examination of declarations on relevant consumer products, and more rarely through patch or use tests with relevant products or chemical analyses of these.

Data management and statistical analyses were performed with SAS® Enterprise Guide®, version 7.1 (SAS Institute, Cary, NC, USA), following guidelines for the presentation of contact allergy data (33). Comparisons of patient subgroups were performed with the $chi^2$ test for categorical variables, and with the Mann–Whitney *U*-test for continuous variables. Figures were prepared with GRAPHPAD PRISM version 7.02 (GraphPad Software, La Jolla, CA, USA). For temporal trends, sensitization prevalences were standardized according to sex and age over/under 40 years (34). Patch test results for individuals not tested because of known sensitivity or not tested for other unknown reasons were treated as missing, and excluded from analyses. Two-sided *p*-values of <0.05 were considered to be statistically significant. All data were extracted from the database in June 2016 and handled anonymously.

## Results

During the study period, 6058 dermatitis patients were patch tested with the investigated fragrance allergens. Among these, 6004 patch test results (99.1%) were obtained for consecutive unselected patients, constituting the study population. A total of 940 (15.7%, 95%CI: 14.7–16.6%) patients were sensitized to at least one of the investigated fragrance allergens (i.e. not including the emulsifier SSO). Demographic and clinical characteristics according to MOAHLFA for the total study population, fragrance-sensitized patients and patients with no positive patch test reactions to any of the investigated fragrance allergens are shown in Table 1. Fragrance-sensitized patients were significantly older, more likely to be female, and significantly more often suffered from leg and face dermatitis. The age range for the total study population was 3–96 years, and that for fragrance-sensitized patients was 5–91 years.

Patch test reactions to the investigated allergens, including the patch test concentrations used, are shown in Table 2 in descending order by prevalence of sensitization. For the individual fragrances, the highest prevalences of contact allergy were observed for Lin-OOH (3.9%, 95%CI: 3.2–4.5%), *Evernia furfuracea* (3.0%, 95%CI: 2.6–3.5%), Lim-OOH (2.5%, 95%CI: 2.0–3.0%), and HICC (2.1%, 95%CI: 1.7–2.5%). High proportions of doubtful patch test reactions were seen especially for Lin-OOH (20.9%), FM I (15.4%), and Lim-OOH (13.7%), and to a lesser extent for FM II (8.9%), as compared with the remaining allergens. Benzyl benzoate 1% pet. was the only fragrance allergen with no positive patch test reactions during the study period. The established clinical relevance in patients with positive patch test reactions to the investigated fragrance allergens is shown in Table 3. For the fragrance mixtures, current and/or past relevance was established in 79.5% of FM I-sensitized and 82.4% of FM II-sensitized patients, respectively, with the majority of these being of current clinical relevance. For the 26 fragrance allergens with an absolute number of at least 10 positive patch test reactions during the study period,

**Table 2.** Consecutive patch test results for fragrance mix I (FM I), fragrance mix II (FM II) and 25 EU-labelled fragrance substances from 2010 to 2015, N = 6004

| | | n (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Allergen (all in pet.) | % positive (95%CI)[a] | + | ++ | +++ | ?+ | IR | Negative | NT-S | NT |
| Fragrance mix I 8% | 9.3 (8.5–10.0) | 255 (4.3) | 277 (4.6) | 18 (0.30) | 925 (15.4) | 120 (2.0) | 4349 (72.4) | 53 (0.88) | 7 (0.12) |
| Fragrance mix II 14% | 4.4 (3.9–4.9) | 103 (1.7) | 155 (2.6) | 4 (0.07) | 535 (8.9) | 72 (1.2) | 5096 (84.9) | 21 (0.35) | 18 (0.30) |
| Hydroperoxides of linalool 1%[b] | 3.9 (3.2–4.5) | 101 (2.4) | 52 (1.2) | 2 (0.05) | 878 (20.9) | 301 (7.2) | 2697 (64.3) | 3 (0.07) | 160 (3.8) |
| *Evernia furfuracea* 1% | 3.0 (2.6–3.5) | 79 (1.3) | 95 (1.6) | 3 (0.05) | 141 (2.4) | 18 (0.30) | 5509 (91.8) | 8 (0.13) | 151 (2.5) |
| Hydroperoxides of limonene 0.3%[b] | 2.5 (2.0–3.0) | 64 (1.5) | 34 (0.8) | 4 (0.10) | 574 (13.7) | 245 (5.8) | 3133 (74.7) | 3 (0.07) | 137 (3.3) |
| Hydroxyisohexyl 3-cyclohexene carboxaldehyde 5%[c] | 2.1 (1.7–2.5) | 28 (0.47) | 89 (1.5) | 7 (0.12) | 74 (1.2) | 5 (0.08) | 5772 (96.1) | 13 (0.22) | 16 (0.27) |
| Cinnamal 1%[d] | 1.4 (1.1–1.7) | 36 (0.60) | 45 (0.75) | 1 (0.02) | 71 (1.2) | 12 (0.20) | 5683 (94.7) | 5 (0.08) | 151 (2.5) |
| *Evernia prunastri* 1%[d] | 1.3 (1.0–1.6) | 30 (0.50) | 45 (0.75) | 2 (0.03) | 107 (1.8) | 5 (0.08) | 5655 (94.2) | 11 (0.18) | 149 (2.5) |
| Isoeugenol 1%[d] | 1.1 (0.82–1.4) | 22 (0.37) | 41 (0.68) | 1 (0.02) | 73 (1.2) | 7 (0.12) | 5706 (95.0) | 4 (0.07) | 150 (2.5) |
| Hydroxycitronellal 1%[d] | 0.92 (0.67–1.2) | 28 (0.47) | 25 (0.42) | 1 (0.02) | 66 (1.1) | 4 (0.07) | 5725 (95.4) | 8 (0.13) | 147 (2.5) |
| Farnesol 5%[c] | 0.82 (0.58–1.1) | 28 (0.47) | 20 (0.33) | – | 129 (2.2) | 19 (0.32) | 5654 (94.2) | 3 (0.05) | 151 (2.5) |
| Cinnamyl alcohol 1%[d] | 0.64 (0.42–0.85) | 22 (0.37) | 15 (0.25) | – | 81 (1.4) | 9 (0.15) | 5685 (94.7) | 1 (0.02) | 191 (3.2) |
| Hexyl cinnamal 10%[c] | 0.45 (0.27–0.63) | 15 (0.25) | 11 (0.18) | – | 55 (0.92) | 4 (0.07) | 5694 (94.8) | 1 (0.02) | 224 (3.7) |
| Citral 2%[c] | 0.39 (0.22–0.56) | 15 (0.25) | 7 (0.12) | 1 (0.02) | 68 (1.1) | 18 (0.30) | 5724 (95.3) | 3 (0.05) | 168 (2.8) |
| Eugenol 1%[d] | 0.36 (0.20–0.52) | 12 (0.20) | 8 (0.13) | 1 (0.02) | 59 (0.98) | 7 (0.12) | 5764 (96.0) | 1 (0.02) | 152 (2.5) |
| Butylphenyl methylpropional 10% | 0.33 (0.17–0.48) | 10 (0.17) | 8 (0.13) | 1 (0.02) | 33 (0.55) | 4 (0.07) | 5759 (95.9) | – | 189 (3.2) |
| Geraniol 1%[d] | 0.26 (0.12–0.40) | 13 (0.22) | 2 (0.03) | – | 140 (2.3) | 16 (0.27) | 5644 (94.0) | – | 189 (3.2) |
| Sorbitan sesquioleate 20%[d] | 0.19 (0.07–0.31) | 6 (0.10) | 5 (0.08) | – | 62 (1.0) | 21 (0.35) | 5778 (96.2) | 1 (0.02) | 131 (2.2) |
| Amyl cinnamyl alcohol 1% | 0.14 (0.03–0.24) | 4 (0.07) | 4 (0.07) | – | 35 (0.58) | 4 (0.07) | 5747 (95.7) | – | 210 (3.5) |
| Coumarin 5%[c] | 0.14 (0.03–0.24) | 3 (0.05) | 5 (0.08) | – | 36 (0.60) | 6 (0.10) | 5762 (96.0) | 2 (0.03) | 190 (3.2) |
| Amyl cinnamal 1%[d] | 0.14 (0.03–0.24) | 5 (0.08) | 3 (0.05) | – | 76 (1.3) | 4 (0.07) | 5726 (95.4) | – | 190 (3.2) |
| Benzyl alcohol 1% | 0.09 (0.03–0.20) | 3 (0.05) | 2 (0.03) | – | 17 (0.28) | 2 (0.03) | 5790 (96.4) | – | 190 (3.2) |
| Citronellol 1%[c] | 0.07 (0.02–0.18) | 3 (0.05) | 1 (0.02) | – | 66 (1.1) | 11 (0.18) | 5732 (95.5) | – | 191 (3.2) |
| Anise alcohol 1% | 0.03 (0–0.12) | – | 2 (0.03) | – | 16 (0.27) | 2 (0.03) | 5790 (96.4) | – | 194 (3.2) |
| α-Isomethylionone 1% | 0.03 (0–0.12) | 2 (0.03) | – | – | 16 (0.27) | 4 (0.07) | 5790 (96.4) | – | 192 (3.2) |
| Benzyl cinnamate 5% | 0.02 (0–0.1) | 1 (0.02) | – | – | 21 (0.35) | 3 (0.05) | 5788 (96.4) | – | 191 (3.2) |
| Benzyl salicylate 1% | 0.02 (0–0.1) | 1 (0.02) | – | – | 30 (0.50) | 3 (0.05) | 5781 (96.3) | – | 189 (3.2) |
| Benzyl benzoate 1% | – | – | – | – | 38 (0.63) | 10 (0.17) | 5765 (96.0) | – | 191 (3.2) |

?+, doubful reaction, IR, irritant reaction; NT, not tested; NT-S, not tested – known sensitized.
[a]NT and NT-S were treated as missing.
[b]Implemented in our fragrance series in 2012 (N = 4194).
[c]Constituent of FM II.
[d]Constituent of FM I.

clinical relevance was established in 59.4% (isoeugenol) to 83.1% (HICC).

### FM I and single constituents

Of the total study population, 5772 (96.1%) were tested concomitantly with FM I and all of its single constituents, including the emulsifier SSO. Concomitant reactivities to FM I and its constituents were assessed among these, excluding patients (n = 10) sensitized to SSO 20% pet. Among 529 patients with contact allergy to FM I, 173 (32.7%) had a concomitant positive patch test reaction to at least one of the single fragrance constituents of the mix. The distribution of patch test reactions to FM I among constituent-positive patients is shown in Table 4. In total, 173 of 188 patients (92.0%) with one or more positive patch test reactions to the single constituents of FM I had a concomitant positive patch test reaction to the mix. An additional 9 (4.8%) patients had a doubtful patch test reaction to FM I, and only 6 (3.2%) constituent-positive patients had a concomitant negative patch test result with FM I. Figure 1 shows age- and sex standardized yearly prevalence rates of contact allergy to FM I and its single constituents across the study period.

**Table 3.** Proportions of clinical relevance in patients sensitized to the investigated allergens

| Allergen-positive | Current (%) | Past (%) | Current and/or past (%) |
|---|---|---|---|
| Fragrance mix I 8% (n = 550) | 65.8 | 33.5 | 79.5 |
| Fragrance mix II 14% (n = 262) | 67.9 | 35.1 | 82.4 |
| Hydroperoxides of linalool 1% (n = 155) | 63.2 | 26.5 | 76.1 |
| *Evernia furfuracea* 1% (n = 177) | 45.2 | 28.8 | 62.7 |
| Hydroperoxides of limonene 0.3% (n = 102) | 63.7 | 24.5 | 75.5 |
| Hydroxyisohexyl 3-cyclohexene carboxaldehyde 5% (n = 124) | 66.9 | 37.9 | 83.1 |
| Cinnamal 1% (n = 82) | 53.6 | 31.7 | 68.3 |
| *Evernia prunastri* 1% (n = 77) | 58.4 | 29.9 | 71.4 |
| Isoeugenol 1% (n = 64) | 39.1 | 31.3 | 59.4 |
| Hydroxycitronellal 1% (n = 54) | 50.0 | 40.7 | 70.4 |
| Farnesol 5% (n = 48) | 54.2 | 29.2 | 68.8 |
| Cinnamyl alcohol 1% (n = 37) | 59.5 | 35.1 | 73.0 |
| Hexyl cinnamal 10% (n = 26) | 61.5 | 30.8 | 76.9 |
| Citral 2% (n = 23) | 60.9 | 34.8 | 73.9 |
| Eugenol 1% (n = 21) | 38.1 | 33.3 | 66.7 |
| Butylphenyl methylpropional 10% (n = 19) | 68.4 | 10.5 | 73.7 |
| Geraniol 1% (n = 15) | 53.3 | 40.0 | 73.3 |
| Sorbitan sesquioleate 20% (n = 11) | 36.4 | 9.1 | 45.5 |
| Amyl cinnamyl alcohol 1% (n = 8) | 50.0 | 37.5 | 87.5 |
| Coumarin 5% (n = 8) | 75.0 | 62.5 | 87.5 |
| Amyl cinnamal 1%(n = 8) | 50.0 | 37.5 | 87.5 |
| Benzyl alcohol 1% (n = 5) | 40.0 | 40.0 | 60.0 |
| Citronellol 1% (n = 4) | 75.0 | 50.0 | 75.0 |
| Anise alcohol 1% (n = 2) | 50.0 | 50.0 | 100 |
| $\alpha$-Isomethylionone 1% (n = 2) | 50.0 | 50.0 | 100 |
| Benzyl cinnamate 5% (n = 1) | 100 | 0 | 100 |
| Benzyl salicylate 1% (n = 1) | 0 | 0 | 0 |

In FM I-sensitized patients, stratifying on the strength of patch test reactions to FM I showed a stepwise increase in the frequency of patients with at least one positive patch test reaction to any of the single constituents: of 244 patients with a + reaction to FM I, 36 (14.8%) reacted to at least one of the constituents, increasing to 122 of 267 (45.7%) with a ++ reaction and 15 of 18 (83.3%) with a +++ reaction to FM I, respectively (Cochrane–Armitage trend test, $p < 0.0001$). Stratification of FM I-positive patients on MOAHLFA index variables and established clinical relevance of sensitization did not show any differences with regard to the proportion of patients with a concomitant positive patch test reaction to at least one single constituent (Table 5).

### FM II and single constituents

A total of 5735 patients (95.5%) were tested concomitantly with FM II and all of its single constituents, and were assessed for further analyses. Of 256 FM II-positive patients, 146 (57.0%) had a positive reaction to one or more of the single constituents during the study period, which was a significantly higher proportion than observed for FM I ($chi^2$ test, $p < 0.0001$). Concomitant reactivity to FM II in patients sensitized to the single constituents of the mix is shown in Table 4. A total of 146 of the 194 patients (75.3%) with a positive reaction to at least one constituent of FM II had a concomitant positive patch test reaction to the mix. Among constituent-positive patients, 24 (12.4%) had a doubtful concomitant patch test reaction to FM II, which was the same as the proportion of constituent-positive patients with a concomitant negative patch test result with FM II. The observed proportion of 'constituent-positive and mix-negative' patients was significantly higher than the observed proportion for FM I ($chi^2$ test, $p = 0.0008$). Regarding the single fragrance allergens, the highest proportion of concomitant negative patch test results with FM II was observed among farnesol-sensitized patients; 14 of 46 patients (30.4%) had negative test results with the mix. Figure 2 summarizes age- and sex-standardized yearly sensitization prevalences of contact allergy to FM II and its single constituents among patients tested concomitantly with these.

As for FM I, after stratification on patch test reactivity to the mix, a significant trend was observed for the proportion of FM II-positive patients with at least one positive patch test reaction to a single constituent: of patients with a +, ++ and +++ reaction to FM II, 33 of 101 (32.7%), 109 of 151 (72.2%) and 4 of 4 (100%), respectively, had one or more positive reactions to the single mix constituents (Cochrane–Armitage exact trend test, $p < 0.0001$). Similarly to what was found for FM I, no differences were observed for the proportion of FM II-positive patients with a concomitant reaction to at least one constituent when stratifying for clinical characteristics and established clinical relevance (Table 5).

### Non-mix fragrance allergens and concomitant reactivity to FM I and FM II

During the study period, a total of 5940 patients (98.9%) were tested concomitantly with at least FM I and FM II. For the four non-mix fragrance substances with at least 10 positive patch test reactions, the proportion of concomitant positive patch test reactions to either FM I or FM II (Table 6) ranged from 30.0% (Lim-OOH) to 73.7% (butylphenyl methylpropional/Lilial®). Figure 3 shows age- and sex standardized frequencies of sensitization by test year to the non-mix fragrance substances with at least 10 positive patch test reactions during the total study period. The number of patients patch tested

**Table 4.** Distribution of patch test reactivity to fragrance mix I (FM I) and fragrance mix II (FM II) in patients with a positive patch test reaction to the respective single constituents of the mix

| FM I single constituent-positive | FM I-positive, n (%) | FM I-doubtful, n (%) | FM I-negative, n (%) |
|---|---|---|---|
| Cinnamal, n = 74 | 69 (93.2) | 4 (5.4) | 1 (1.4) |
| *Evernia prunastri*, n = 70 | 67 (95.7) | 1 (1.4) | 2 (2.9) |
| Isoeugenol, n = 55 | 50 (90.9) | 5 (9.1) | 0 |
| Hydroxycitronellal, n = 44 | 42 (95.5) | 1 (2.3) | 1 (2.3) |
| Cinnamyl alcohol, n = 34 | 33 (97.1) | 1 (2.9) | 0 |
| Eugenol, n = 17 | 16 (94.1) | 0 | 1 (5.9) |
| Geraniol, n = 13 | 9 (69.2) | 2 (15.4) | 2 (15.4) |
| Amyl cinnamal, n = 8 | 6 (75.0) | 1 (12.5) | 1 (12.5) |
| *At least one FM I constituent, n = 188* | *173 (92.0)* | *9 (4.8)* | *6 (3.2)* |
| **FM II single constituent-positive** | **FM II-positive, n (%)** | **FM II-doubtful, n (%)** | **FM II-negative, n (%)** |
| HICC, n = 120 | 110 (91.7) | 8 (6.7) | 2 (1.7) |
| Farnesol, n = 46 | 23 (50.0) | 9 (19.6) | 14 (30.4) |
| Hexyl cinnamal, n = 26 | 19 (73.1) | 3 (11.5) | 4 (15.4) |
| Citral, n = 21 | 15 (71.4) | 2 (9.5) | 4 (19.1) |
| Coumarin, n = 7 | 4 (57.1) | 2 (28.6) | 1 (14.3) |
| Citronellol, n = 4 | 4 (100) | 0 | 0 |
| *At least one FM II constituent, n = 194* | *146 (75.3)* | *24 (12.4)* | *24 (12.4)* |

HICC, hydroxyisohexyl 3-cyclohexene carboxaldehyde.

with the single non-mix fragrances varied slightly for each year.

## Discussion

The current study investigated sensitization in recent years to 25 of the 26 fragrance allergens with mandatory labelling in cosmetics and household detergents among consecutively patch tested dermatitis patients from a single university clinic. During the 6-year study period, 15.7% of patients had a positive patch test reaction to FM I, FM II, or one of the 25 fragrance substances. Of the individual fragrance allergens, Lin-OOH showed the highest prevalences of sensitization, with 3.9% positive patch test reactions, followed by *E. furfuracea* (3.0%), Lim-OOH (2.5%), and HICC (2.1%). Among FM I-positive patients, 32.7% had a concomitant positive patch test reaction to at least one mix constituent, and 3.2% of the patients with a positive patch test reaction to a FM I constituent had a concomitant negative patch test result to the mix. Among FM II-positive patients, 57.0% had a concomitant positive patch test reaction to at least one mix constituent, and 12.4% of patients sensitized to at least one FM II constituent had a concomitant negative patch test result to the mix. Both the proportion of 'mix-positive and constituent-positive' patients and the proportion of 'mix-negative and constituent-positive' patients were significantly higher for FM II than for FM I.

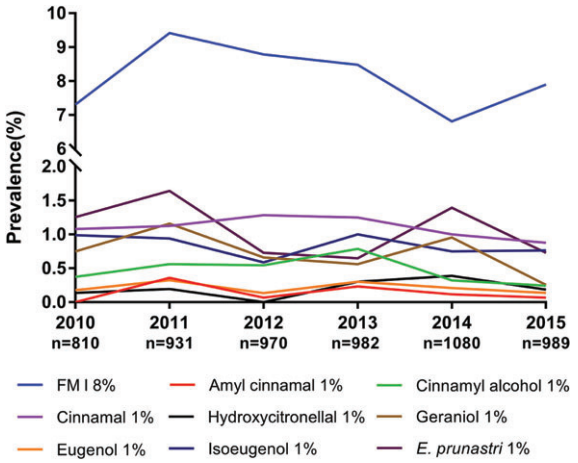


**Fig. 1.** Age- and sex standardized yearly prevalences of contact allergy to fragrance mix I (FM I) and its single constituents, in patients (n = 5762) tested concomitantly with these (excluding 10 patients sensitized to the emulsifier sorbitan sesquioleate).

Unlike previous studies reporting on consecutive patch testing with the 26 fragrance allergens (23–27), we included patch test results for Lin-OOH and Lim-OOH from 2012 onwards, when consecutive patch testing with the oxidized terpenes was implemented at our department. Beyond the oxidized terpenes, the ranking of the single constituents most often causing contact allergy observed in the current study was comparable to

**Table 5.** Associations (odds ratios) for subgroups of fragrance mix I (FM I)-positive (n = 529) and fragrance mix II (FM II)-positive (n = 256) patients with regard to a concomitant positive patch test reaction to at least one constituent of the respective mix

| | Odds ratio (95%CI) | |
|---|---|---|
| Subgroups of FM I/FM II-positive patients | FM I constituent-positive | FM II constituent-positive |
| Male | 1.28 (0.83–1.97) | 1.15 (0.63–2.09) |
| Occupational relevance (reference: no occupational relevance) | 1.22 (0.77–1.93) | 1.45 (0.78–2.72) |
| Atopic dermatitis | 1.17 (0.75–1.84) | 0.79 (0.44–1.42) |
| Hand eczema (reference: no hand eczema) | 1.07 (0.74–1.56) | 1.00 (0.61–1.64) |
| Leg eczema (reference.: no leg eczema) | 1.29 (0.41–4.02) | 0.74 (0.21–2.64) |
| Face eczema (reference: no face eczema) | 1.11 (0.76–1.62) | 0.81 (0.48–1.36) |
| Aged ≥40 years | 1.22 (0.78–1.90) | 0.74 (0.42–1.31) |
| Established current relevance (reference: unknown) | 0.97 (0.66–1.43) | 1.26 (0.74–2.14) |
| Established past relevance (reference: unknown) | 1.07 (0.73–1.57) | 1.45 (0.86–2.46) |
| Established current and/or past relevance (reference: unknown) | 1.04 (0.65–1.66) | 1.87 (0.96–3.61) |

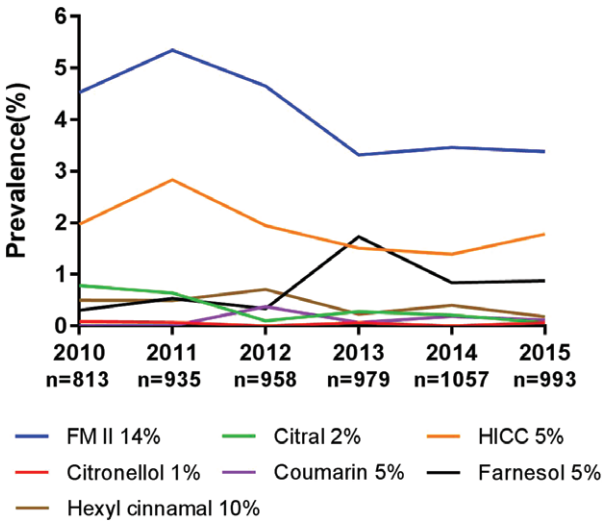


**Fig. 2.** Age- and sex standardized yearly prevalences of contact allergy to fragrance mix II (FM II) and its single constituents, in patients (n = 5735) tested concomitantly with these. HICC, hydroxyisohexyl 3-cyclohexene carboxaldehyde.

**Table 6.** Concomitant reactivity to non-mix fragrance substances and fragrance mix I (FM I) and fragrance mix II (FM II)

| | Concomitant positive reactions | | |
|---|---|---|---|
| Allergen-positive | FM I (%) | FM II (%) | FM I and/or FM II (%) |
| Hydroperoxides of linalool (n = 152) | 25.7 | 15.1 | 31.6 |
| *Evernia furfuracea* (n = 169) | 47.3 | 18.9 | 50.3 |
| Hydroperoxides of limonene (n = 100) | 26.0 | 13.0 | 30.0 |
| Butylphenyl methylpropional/ Lilial® (n = 19) | 63.2 | 73.7 | 73.7 |
| Amyl cinnamyl alcohol (n = 8) | 62.5 | 62.5 | 62.5 |
| Benzyl alcohol (n = 5) | 40.0 | 40.0 | 40.0 |
| Anise alcohol (n = 2) | 100 | 100 | 100 |
| α-Isomethylionone (n = 1) | 0 | 0 | 0 |
| Benzyl cinnamate (n = 1) | 0 | 0 | 0 |
| Benzyl salicylate (n = 1) | 100 | 100 | 100 |

that reported by Heisterberg et al. from the same patch test population between 2008 and 2010 (24, 25). The prevalences of sensitization to the natural extracts *E. furfuracea* (4.1% versus 3.0%) and *E. prunastri* (2.5% versus 1.3%) were lower in the current study than in the period 2008–2010. It is of note that the 2-year study period from 2008 to 2010 included a 6-month interval during which patch testing with the 26 fragrances was only performed in patients suspected of having fragrance allergy, which could potentially cause an increase in the observed proportion of positive patch test reactions (24). The differences in sensitization to the two natural extracts could also be an indication of an actual reduced frequency of sensitization among tested patients. Between 1997 and 2007, a large reduction was reported in the total tonnage of both processed treemoss (*E. furfuracea*) and oakmoss (*E. prunastri*) used by the cosmetics industry, which could indicate a general decrease in exposure (35). In the current study, we did observe a decrease in sensitization to *E. furfuracea* from 2010 to 2015, whereas changes in sensitization to *E. prunastri* across patch test years were more heterogeneous. Depending on the processing of the natural extracts, both of these can contain the potent contact sensitizers atranol and chloroatranol (35, 36). The Scientific Committee on Consumer Products, advising the European Commission, has stated that both atranol and chloroatranol should not be present in cosmetic products, owing to their high sensitizing potency (37). Commercial attempts have been made to reduce the content of atranol and chloroatranol in oakmoss absolute for its continued use in scented consumer products; however, manufacturers can only guarantee <100 ppm in the subsequent product (38). In the current study, 96% of consecutive dermatitis

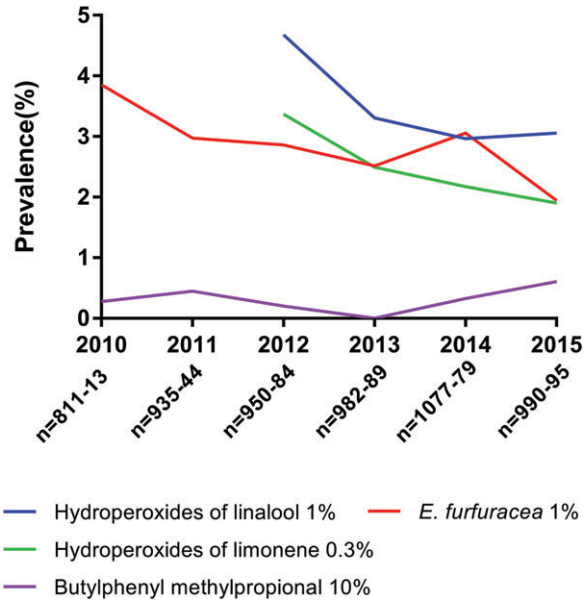


**Fig. 3**. Age- and sex standardized yearly prevalences of contact allergy to non-mix fragrance substances (only fragrances with at least 10 sensitized individuals during the study period are shown, for improved clarity).

patients sensitized to *E. prunastri* had a concomitant positive patch test reaction to FM I, and would hence have been diagnosed with fragrance allergy if they had been patch tested with only the European baseline series. For *E. furfuracea*, however, only 50% of sensitized patients had a concomitant positive patch test reaction to FM I and/or FM II. It has previously been established that at least two subgroups of *E. furfuracea*-sensitized patients can be defined: one is sensitized to common constituents found in both treemoss and oakmoss, such as atranol and chloroatranol, and the other is sensitized to (contaminating) resin acids, as indicated by a concomitant positive patch test reaction to colophonium (39). As we did not include results on contact allergy to colophonium in the current study, we do not know the additional proportion of patients sensitized to *E. furfuracea* who would have been otherwise detected as having a possible fragrance allergy on the basis of colophonium sensitization.

It was expected that both oxidized linalool and oxidized *R*-limonene would be among the single fragrance allergens with the highest sensitization prevalences in the current study, on the basis of previous clinical investigations reporting high frequencies of contact allergy to these (19–22). In the current study, a high proportion of patients had a doubtful patch test reaction to Lin-OOH (20.9%) and, to a lesser extent, to Lim-OOH (13.7%). In the previous multicentre trials on the oxidized terpenes, using the same patch test concentrations as in the current study, the frequencies of doubtful patch test reactions varied from none to 24.5% for Lim-OOH and none to 36.2% for Lin-OOH. These variations were attributed to differences in scoring practice between participating departments (20, 21). In a previous dose–response study on oxidized linalool (40), 62% of patients with a doubtful patch test reaction to a lower dose of the oxidized terpene had a weak positive patch test reaction when tested concomitantly with a twofold increased patch test concentration. This could indicate that at least some of the observed doubtful patch test reactions to Lin-OOH and Lim-OOH in the current study do represent contact allergy to the oxidized fragrance terpenes. Further investigations in this patient subgroup could be a future research area of interest. For both oxidized terpenes, the highest prevalence of sensitization was observed in 2012 following their introduction into our baseline patch test series, with a close to parallel decline in sensitization frequencies in the following years. However, both fragrance allergens, which are ubiquitously found in scented consumer products, have remained well above the proposed lower limit of 0.5–1% positive patch test reactions for inclusion in baseline testing of consecutive dermatitis patients (7).

The current study showed several interesting aspects with regard to breakdown testing of FM I and FM II in consecutive patients. We were able to reproduce the findings reported by Geier et al. (15) in selected FM I-positive patients, showing a significant trend in the proportion of FM I-positive and FM II-positive patients with a concomitant positive patch test reaction to at least one mix constituent, after stratification on the strength of reaction to the relevant mix. Additionally, we showed that clinical characteristics, such as age, sex, and a history of atopic dermatitis, as well as established clinical relevance of contact allergy to the relevant mix, was not related to the proportion of mix-positive patients reacting to at least one single mix constituent.

Approximately one-third of FM I-positive patients had a concomitant positive patch test reaction to at least one mix constituent. In previous investigations on aimed breakdown testing in patients with an established contact allergy to FM I, the proportion of patients sensitized to at least one mix constituent has varied substantially, from 55% to 84% (11, 12, 14–16). However, in all of these investigations, some degree of bias in selecting FM I-positive patients for breakdown testing was present. Mann et al. (26) reported on 1951 consecutive eczema patients who were patch tested with the 26 fragrance allergens from 2011 to 2012 as a supplement to the baseline series. The single constituents of FM I were all

(except for cinnamal) tested 2% pet. Among 124 FM I-positive patients, 54% had one or more concomitant positive patch test reactions to the single constituents. Additionally, 42% of patients with a positive patch test reaction to at least one FM I constituent did not have a concomitant positive patch test reaction to FM I, as compared with only 8% in the current study. Unfortunately, Mann et al. (26) did not report on the separate frequencies of irritant patch test reactions observed when patch testing was performed with the higher concentrations of the single FM I constituents. In the current study, none of the single FM I constituents tested at 1% could explain the observed variation in FM I sensitization across patch test years. We have recently published the results on contact allergy to FM I over time observed in the current study as part of a larger investigation on trends in FM I sensitization from 1986 to 2015 (5).

For FM II, the concentrations used for testing with the single constituents are twice as high as those in the mix. As compared with FM I, we observed a significantly increased proportion of both FM-II positive patients with a positive breakdown test result and a significantly increased proportion of FM-II constituent-positive patients with a concomitant negative reaction to the mix. In the current study, 57% of FM II-positive patients had a concomitant positive patch test reaction to at least one constituent. In selected FM II-positive patients, positive breakdown testing results have previously been reported in 64–72% of patients (10, 15). Interestingly, Mann et al. (26) reported that only 34% of 64 consecutive FM II-positive patients had a concomitant positive patch test reaction to one or more constituents. Regarding the differences to the current study, possible explanations could be differences in patch test reading practice (inclusion of D7 readings in the current study) or differences in exposures. In the current study, 12.4% of constituent-positive patients had a concomitant negative patch test result to FM II; however Mann et al. (26) did not differentiate between doubtful and negative (including irritant) patch test reactions in their report.

HICC remains the most important fragrance allergen present in FM II. The observed prevalence of HICC sensitization from 2010 to 2015 is comparable to the prevalence of 2.3% reported by Heisterberg et al. (24, 25) from 2008 to 2010 in the same patch test population. In 2012, the Scientific Committee on Consumer Safety concluded, in an extensive opinion on fragrance allergens in cosmetic products, that HICC should not be used in consumer products, because of an exceptionally high number of documented cases of contact allergy to this synthetic compound (17). We observed an age- and sex adjusted decrease in the frequency of contact allergy to HICC from 2.8% in 2011 to 1.4% in 2014; however, this was followed by an increase to 1.8% in 2015. Whether these changes in sensitization frequencies reflect a decrease in consumer exposure to HICC in recent years is unknown, and continued epidemiological surveillance of HICC sensitization in the coming years is of high interest. From the current study on consecutive patch tested dermatitis patients, it is, however, evident that sensitization to FM II is largely dependent on sensitization to HICC, with temporal trends in contact allergy to these paralleling each other closely.

In summary, several of the 26 fragrance allergens with mandatory labelling within the EU fulfil the criteria for inclusion in baseline patch testing of consecutive dermatitis patients, on the basis of high relative frequencies of sensitization. The single fragrances most often causing contact allergy in the current study were oxidized linalool and *R*-limonene, and the natural extract *E. furfuracea*. For these non-mix fragrance substances, only 30–50% of sensitized individuals are detected as fragrance-allergic when patch testing is performed with FM I and FM II. With regard to patch testing with the single constituents of FM I, results from the current and previous investigations seem to favour the use of higher patch test concentrations (except for cinnamal), as with FM II and its single constituents, in order to improve the diagnosis of fragrance contact allergy among dermatitis patients.

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### 5.3 Allergic contact dermatitis to hydroperoxides of limonene and dose-response relationship – a repeated open application test (ROAT) study (manuscript III):

- Among 11 subjects with a positive patch test to Lim-OOHs 0.3% pet. did 11 (100%), 7 (64%) and 3 (27%) react in the ROAT to the applied doses of Lim-OOHs of 3.0 µg/cm$^2$ (1260 ppm), 0.99 µg/cm$^2$ (420 ppm), and 0.33 µg/cm$^2$ (140 ppm), respectively. None of the 17 healthy controls exposed to the highest dose showed any reactions, and the difference in reactivity was statistically significant ($p < 0.0001$). Positive ROAT reactions are illustrated below in Figure 3.
- In 13 subjects with a doubtful patch test to Lim-OOHs did 2 (15%) react to the highest dose of Lim-OOHs in the ROAT ($p$=0.36 compared to the healthy controls). One doubtful allergic subject also reacted to both the middle and lowest dose of Lim-OOHs.
- Following single exposure to Lim-OOHs in a dilution patch test series, the minimal eliciting concentration (MEC) in allergic subjects was 24 ppm. In doubtful allergic subjects the MEC for Lim-OOHs was 73 ppm.
- The dose-response relationship in subjects with a positive patch test to Lim-OOHs 0.3% in pet., following both single patch test exposure as well as repeated exposure in the ROAT, resembles that of other well-established fragrance contact allergens.
- The estimated dose that will elicit allergic contact dermatitis in 10% of sensitized individuals following exposure in the ROAT was 0.20 µg Lim-OOHs/cm$^2$ per application, corresponding to an estimated concentration of Lim-OOHs in the simulated fine fragrance of 85 ppm.
- The estimated doses per application of Lim-OOHs that will elicit a reaction in 50% and 75% of allergic subjects were significantly lower following repeated exposure in the ROAT compared to single patch test exposure.

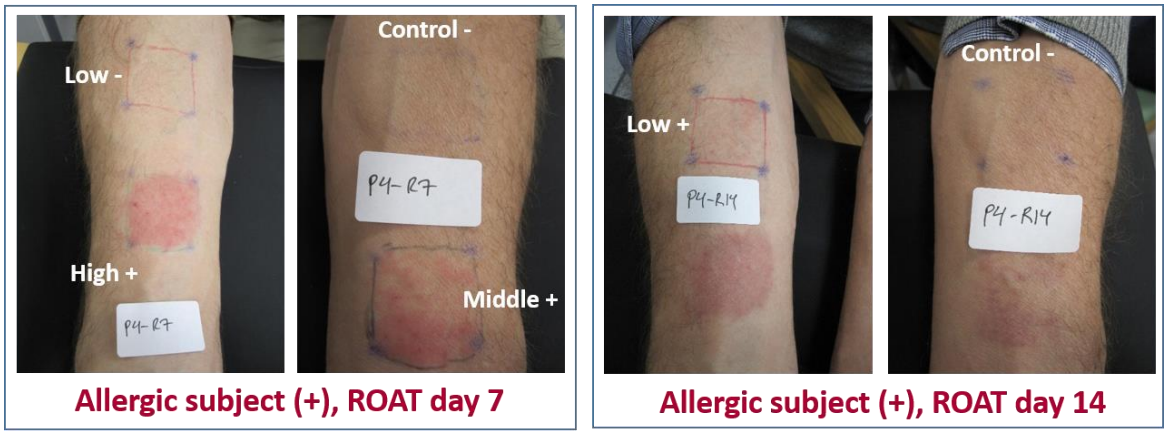


*Figure 3: Example of positive ROAT reactions in a subject with a weak positive (+) confirmatory patch test to Lim-OOHs 0.3% in pet. After seven days of exposure in the ROAT (left-hand side), the subject had positive reactions to the highest and middle dose of exposure to Lim-OOHs in the simulated fine fragrance. After 14 days of exposure in the ROAT (right-hand side), the subject also reacted to the lowest applied dose, while no reaction was seen to the vehicle control.*

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# Title: Allergic contact dermatitis to hydroperoxides of limonene and dose-response relationship – a repeated open application test (ROAT) study.

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**Running head:** ROAT limonene hydroperoxides.

**Abstract (max 200 words, currently 197 words)**

**Background:** Contact allergy to oxidized limonene, with hydroperoxides of limonene (Lim-OOHs) as main allergens, is common. However, high proportions of weak positive and doubtful patch test reactions have been reported.

**Objectives:** To determine clinical relevance, elicitation threshold, and dose-response relationship of Lim-OOHs in individuals with a positive or doubtful patch test to standard Lim-OOHs 0.3% in petrolatum (pet.).

**Methods:** A multicentre 3-week double-blinded vehicle-controlled repeated open application test (ROAT) study with a simulated fine fragrance containing Lim-OOHs at 1260 ppm, 420 ppm, and 140 ppm, equal to a dose/area per application of Lim-OOHs of 3.0 μg/cm$^2$, 0.99 μg/cm$^2$, and 0.33 μg/cm$^2$.

**Results:** Among 11 subjects allergic to Lim-OOHs, 11 (100%), 7 (64%), and 3 (27%) reacted to the applied doses. No reactions were seen in 17 healthy controls exposed to the highest dose. This difference in reactivity was statistically significant ($p$<0.0001). Among 13 subjects with a doubtful patch test to Lim-OOHs, two (15%) had a positive ROAT to the highest Lim-OOHs dose applied ($p$=0.36 compared to controls).

**Conclusions:** Contact allergy to Lim-OOHs is of clinical relevance in patients with a positive patch test. A doubtful patch test to Lim-OOHs 0.3% in pet. can be of clinical relevance.

**Keywords:** Allergic contact dermatitis, clinical relevance, contact allergy, fragrance substance, dose-response relationship, limonene hydroperoxides, oxidized limonene, ROAT.

## Introduction

Limonene, a cyclic monoterpene, is the major constituent of peel oil from citrus fruit and is extensively used as a fragrance chemical in scented household and cosmetic products for its fresh citrus odour (1,2). Experimental studies have established pure limonene as a prehapten (3) which can oxidize upon air exposure (autoxidation) to form sensitizing allergen-specific oxidation products. Based on the local lymph node assay in mice, the EC3 value for oxidized limonene is 10-fold lower than that of pure limonene, indicating a higher sensitizing potency. The main sensitizing haptens formed during autoxidation of limonene are hydroperoxides, including limonene-1-hydroperoxide and limonene-2-hydroperoxide, of which the former has been identified as the strongest sensitizer in oxidized limonene (4,5).

Oxidized limonene 3% in petrolatum (pet.), with a stable and standardized content of the main allergenic hydroperoxides of limonene (Lim-OOHs) of 0.3%, has been commercially available as the patch test preparation "Hydroperoxides of Limonene 0.3% in pet." from Chemotechnique Diagnostics (Vellinge, Sweden) since 2012 (6). High rates of contact allergy to Lim-OOHs 0.3% in pet. have been reported in consecutive dermatitis patients referred for patch testing, both in an international multicentre study with 5.2% positive overall (7), and lately in patch test clinics across Europe with 2.5% to 5.3% positive reactions (8–11). In most of these investigations, high rates of weak positive as well as high rates of doubtful and/or irritant patch test reactions to Lim-OOHs 0.3% in pet. have been reported, which has caused some concern about the nature and clinical relevance of positive reactions (12,13).

The relevance of a positive or doubtful patch test reaction can be assessed by the repeated open application test (ROAT) (14). The ROAT is a standardized exposure test mimicking daily use of a (cosmetic) product containing a contact allergen of interest (15). The aim of the ROAT is to investigate whether allergic contact dermatitis can be elicited following twice daily application for a period of two to four weeks. In an experimental setting, the ROAT can be used to elicit allergic contact dermatitis, under standardized conditions, to specified doses of a contact allergen to determine an elicitation threshold and dose-response relationship (16).

In the current study, we wanted to determine clinical relevance as well as the elicitation threshold and dose-response relationship, of Lim-OOHs in individuals with either a positive or doubtful patch test to standard Lim-OOHs 0.3% in pet. To do so, we performed a multicentre 3-week double-blinded vehicle-controlled ROAT study with a simulated hydroalcoholic leave-on cosmetic product containing Lim-OOHs in three different concentrations.

## Material and methods

### Test subjects

The study was conducted in accordance with the Declaration of Helsinki (17) and was approved by the regional ethical committees in Denmark and Sweden. The study was prospectively registered at www.clinicaltrials.gov (NCT03313232). We identified possible participants among patients who were patch tested with Lim-OOHs 0.3% in pet. (oxidized limonene 3% in pet., with a stable and standardized content of the main allergenic limonene hydroperoxides of 0.3%), as part of routine patch testing at the Department of Dermatology and Allergy, Gentofte University Hospital (DK) or the Department of Occupational Dermatology, Sahlgrenska University Hospital (SE) between 2012 and 2017. Patients with a previous positive patch test (at least one reading) and patients with a previous doubtful patch test (at least two readings), aged 18 years or older, were invited to participate. Exclusion criteria were active eczema on the upper back or forearms, pregnancy or breast feeding, recent application of topical immunosuppressant treatment to the upper back or forearms, systemic immunosuppressant treatment, and excessive UV exposure within three weeks of study start. Contact allergy to other allergens was not an exclusion criterion. Nineteen subjects (DK: 12, SE: 7) with a previous positive patch test and 12 subjects (DK: 10, SE: 2) with a previous doubtful patch test to Lim-OOHs 0.3% in pet. agreed to participate and fulfilled the inclusion criteria. Healthy volunteers with no history of eczema or known contact allergy to Lim-OOHs or other fragrance substances were included as a control group. Exclusion criteria were the same as for the previously patch tested subjects. Eighteen healthy controls (DK: 12, SE:6), with a similar age and sex distribution as the previously patch tested subjects, were included following advertisement on the website www.forsøgsperson.dk or were recruited among office workers at the Sahlgrenska University Hospital. All clinical investigations took part between October 2017 and April 2018.

At the day of inclusion, all participants had a clinical examination of their skin and filled out a questionnaire on history of eczema and consumer behaviour regarding use of fragranced products. A diagnosis of atopic dermatitis (AD) was made according to the UK Working Party's diagnostic criteria (18).

### Chemicals and test preparations

Limonene ("pure") and oxidized limonene with a documented content of Lim-OOHs of 12.6% (limonene-1-hydroperoxide and limonene-2-hydroperoxide) from the same batch were purchased from Chemotechnique Diagnostics (Vellinge, Sweden), and stored under argon or nitrogen at -20 °C. Concentrations and doses of oxidized limonene in the test solutions used in the study, and their content of Lim-OOHs, are shown in Table 1. For the ROAT, solutions of oxidized limonene at 1.0%, 0.33%, and 0.11% respectively in ethanol/water (80:20) were produced to simulate a fine fragrance as the model for a leave-on cosmetic product. The highest concentration was based on the known concentration of limonene determined by chemical analyses in leave-on

cosmetic products intended for non-occlusive use (19). Prior to initiation of the ROAT, all participants were patch tested with 20 mg of standard Lim-OOHs 0.3% in pet., obtained from Chemotechnique Diagnostics, as well as a pet. control. In addition, subjects with a previous positive or doubtful patch test to this test preparation were also patch tested with a dilution series (15 µl on filter discs) of Lim-OOHs in ethanol/water. With respect to oxidized limonene, the highest concentration in the dilution patch test series corresponded in dose per area (1.2 mg/cm$^2$) to the concentration of oxidized limonene in the standard patch test of Lim-OOHs 0.3% in pet. The concentration of Lim-OOHs in the oxidized limonene used for the dilution patch test and ROAT solutions (Lim-OOHs content of 12.6%) was higher than that normally found in the standard patch test material of oxidized limonene (Lim-OOHs content ≈ 10%). Hence, calculated concentrations and doses of Lim-OOHs in the test preparations are presented to correctly quantify allergen exposure.

Oxidized limonene diluted to 1% (w/w) in ethanol/water (80:20, v/v) was investigated for the stability of Lim-OOHs. The formation of Lim-OOHs was investigated in limonene ("pure") diluted to 1% in ethanol/water. Aliquots were taken out from each sample and stored in triplicate at either room temperature or at -20 °C under argon. After two and three weeks, the samples were analysed using reversed-phase high-performance liquid chromatography/electrospray ionisation–tandem mass spectrometry (HPLC/ESI-MS/MS). No degradation of Lim-OOHs was observed in the aliquots of oxidized limonene stored at -20 °C. A tendency of degradation of Lim-OOHs was observed in oxidized limonene stored at room temperature, however the decrease over time was not significant and within the relative standard deviation (RSD <22%) of the analyses. The ratio of limonene-2-hydroperoxide to limonene-1-hydroperoxide in the oxidized limonene was between 6:1 and 5:1 across storage conditions and sampling time. In limonene ("pure"), from which the oxidized limonene was produced, limonene-2-hydroperoxide was detected at a level of 0.28% (w/w), corresponding to 0.0028% in the 1% ethanol/water solution, at time of purchase. This level remained constant, independent of storage conditions and sampling time. Limonene-1-hydroperoxide was below the detection limit at all measurements and thus there was no evidence for hydroperoxide formation. For the clinical part of the study, both ROAT solutions and the patch test dilution series were prepared weekly to avoid any changes in Lim-OOHs concentrations.

**Patch testing procedure**

A flowchart for the study participants is shown in Figure 1. Prior to inclusion in the ROAT, patch testing was done using Finn Chambers® (8 mm; Smartpractice, Phoenix, AZ, USA) applied on the upper back for two days with Scanpor® tape (Norgesplaster, Vennesla, Norway). Placement and reading of the dilution patch test series were blinded to the investigators. Scoring of patch test reactions was done at day (D) 3 or 4 and D7 (16). In addition, patch test reactions were also scored using an extended reading scale: 0 = no reaction; 1 = few (≥5) papules with no erythema, no infiltration; 2 = faint erythema with no infiltration or papules; 3 = faint erythema with few papules and no homogeneous infiltration; 4 = erythema, homogeneous infiltration; 5 = erythema,

infiltration and a few papules; 6 = erythema, infiltration and papules; 7 = erythema, infiltration, papules and a few vesicles; 8 = intensive erythema, infiltration and vesicles. This was done to detect weaker allergic reactions with regard to defining the Minimal Eliciting Concentration (MEC) for the dilution patch test series, that is: The threshold concentration defined as the lowest dose giving a visible reaction (minimum score of 1 on the extended reading scale), if there is a continuous line of reactions from the highest dose and down (20,21).

Based on results of the confirmatory patch test with Lim-OOHs 0.3% in pet., previously patch tested subjects were categorized as either *allergic* (positive patch test reaction: +/++/+++) or *doubtful allergic* (doubtful patch test reaction: ?+). A total of 11 subjects had a positive reaction and 13 subjects had a doubtful reaction to the confirmatory patch test and advanced to the ROAT (see supplementary Table 1 for individual patch test reactions). Previously patch tested subjects who had a negative confirmatory patch test to Lim-OOHs in 0.3% pet. (n=7) did not advance to the ROAT. This included four participants with a previous weak positive patch test to Lim-OOHs 0.3% in pet., of whom three participants did have at least a doubtful patch test reaction to one of the two highest applied concentrations of Lim-OOHs in the dilution patch test series. However, this information was not available to the investigators until after the study had ended due to blinding. Among the *healthy controls*, one subject showed a positive patch test reaction to Lim-OOHs 0.3% in pet. and did not advance to the ROAT.

**Repeated open application test (ROAT) procedure**

In the ROAT, subjects were exposed twice daily for up to 21 days to one (*healthy controls*) or three (*allergic* and *doubtful allergic* subjects) concentrations of Lim-OOHs in the simulated fine fragrance, as well as a vehicle control. At the start of the ROAT and at weekly evaluations, participants were provided with 500 µl of each test solution in colour coded 1.5-ml Eppendorf Tubes®. Participants were thoroughly instructed on how to apply 25 µl to each 3x3 cm colour coded test area on the forearms using a micropipette (DK: LGG-Labware, Meckenheim, Germany. SE: VWR International, Spånga, Sweden). The applied volume of the simulated fine fragrance corresponded to 2.4 mg/cm$^2$, which is comparable to the expected daily exposure to hydroalcoholic products on unshaved skin (22). Test solutions were applied in the centre of the test areas, distributed evenly using the side of the pipette tip, and allowed to dry by evaporation. Participants could shower during the ROAT but were not allowed to use scented products or wash the test areas directly. Placement of test areas on the forearms was randomized for each subject, and the individual content of the colour coded test solutions was blinded to both participants and investigators (separate blinding codes for *allergic/doubtful allergic* subjects and *healthy controls*). Eppendorf Tubes® were weighed before and after being provided to the participants to estimate compliance.

Reactions on the ROAT test areas were scored clinically for development of dermatitis after 7, 14, and 21 days of exposure, or when the participant observed a reaction. Scoring of ROAT reactions involved assessment of involved area of application, presence and strength of erythema, level of infiltration and possible presence of

vesicles (16,23). Application of individual test solutions was continued until a positive ROAT reaction was observed, corresponding to a minimum ROAT score of 5 (≥25 % involved area, presence of erythema, and signs of infiltration, i.e. at least one papule), up to a maximum of 21 days of application. All readings were documented by photography.

**Statistical analyses**

Data management and statistical analyses were performed with SAS® Enterprise Guide®, version 7.1 (SAS Institute Inc., Cary, NC, USA) and two-sided p-values < 0.05 were considered statistically significant. Graphing was done using GraphPad Prism® version 7 (GraphPad software, La Jolla, California, USA). *Allergic* and *doubtful allergic* subjects were compared to *healthy controls* using the Mann-Whitney U test for continuous variables, and Fisher's exact test for categorical variables. Compliance in the ROAT was based on weighing of test solutions and expressed as the mean percentage of actual use compared to expected use. In exploratory analyses, the dose-response relationship in Lim-OOHs *allergic* subjects was estimated with threshold logistic regression analyses. The predicted eliciting doses ($ED_{xx}$), including 95% confidence intervals (95% CIs), that would elicit a patch test reaction or ROAT reaction respectively in 10%, 25%, 50%, 75%, and 90% of *allergic* subjects were calculated according to Fieller's method. This was done using probability unit (probit) analyses (24), and fitted four-parameter dose-response curves with constraints on minimum and maximum response frequencies were drawn.

## Results

Baseline characteristics for *allergic* subjects, *doubtful allergic* subjects, and *healthy controls* who advanced to the ROAT are shown in Table 2. The distribution of strength of reaction to the confirmatory patch test with Lim-OOHs 0.3% pet. in the 11 *allergic* subjects was (+) in three (27.3%), (++) in seven (63.6%), and (+++) in one (9.1%). Compared to the *healthy controls*, significantly more *allergic* subjects had a current eczema, all with only minor involvement, and significantly fewer *allergic* subjects were exposed to fragranced products in daily life. Compared to the *healthy controls*, *doubtful allergic* subjects were significantly more likely to have a history of atopic dermatitis.

The proportions of observed reactions to the applied patch test and ROAT solutions are summarized in conjunction with the selected exposures in Table 1. All *allergic* and *doubtful allergic* subjects reacting to the confirmatory patch test also reacted to the highest applied dose of Lim-OOHs in the patch test dilution series. Among *allergic* subjects, 3 (27%) reacted continuously down to the lowest dose applied (0.65 µg Lim-OOHs/$cm^2$), corresponding to a MEC in *allergic* subjects of 0.0024%, equal to 24 parts per million (ppm). Among the *doubtful allergic* subjects, one participant (7.7%) reacted to the second lowest applied patch test dose (1.9 µg Lim-OOHs/$cm^2$), corresponding to a MEC of 0.0073% (73 ppm). No reactions were seen to the vehicle control.

In the ROAT, the 17 *healthy controls* were exposed to the highest dose of 3.0 µg Lim-OOHs/cm$^2$ (1260 ppm) and none reacted. Eleven (100%) *allergic subjects* reacted to the highest dose, after a median of seven days of exposure, which was significantly different from the *healthy controls* ($p$<0.0001). This included one *allergic* subject who displayed a positive ROAT to the highest applied dose 12 days after exposure had ended (see additional information in supplementary Table 1). Seven (64%) *allergic* subjects reacted in the ROAT to the middle dose of 0.99 µg Lim-OOHs/cm$^2$ (420 ppm) after a median of 14 days of exposure. Three (27%) *allergic* subjects had a positive ROAT to the lowest dose of 0.33 µg Lim-OOHs/cm$^2$ (140 ppm) after 14 days (n=2) and 18 days (n=1) of exposure, respectively. Among *doubtful allergic* subjects, two (15%) had a positive ROAT to the highest Lim-OOHs dose applied, which was not significantly different compared to the *healthy controls* ($p$=0.36). Of the two *doubtful allergic* subjects did one also react to both the middle and lowest dose of Lim-OOHs after 14 days of exposure. Among all participants, no reactions were seen to the vehicle control in the ROAT. Positive ROAT reactions in *allergic* and *doubtful allergic* subjects are illustrated in Figures 2 and 3. Compliance in the ROAT, in terms of actual usage of individual test solutions compared to expected usage, was 116-125% in *allergic subjects*, 112-116% in *doubtful allergic* subjects, and 119-123% in the *healthy controls*. Compared to the *healthy controls*, no significant differences in compliance were observed for *allergic* or *doubtful allergic* subjects, respectively (see supplementary Table 2).

Fitted dose-response curves based on frequencies of observed reactions to Lim-OOHs in *allergic* subjects in the patch test and ROAT are shown in Figure 4. The effect of repeated exposure compared to single patch test exposure is quantified in Table 3, displaying calculated doses ($ED_{xx}$) that will elicit a response in the specified proportions of *allergic* subjects. Following repeated exposure in the ROAT, the calculated $ED_{10}$ for Lim-OOHs was 0.20 µg Lim-OOHs/cm$^2$ per application (95% CI: 0.016 - 0.37 µg Lim-OOHs/cm$^2$), corresponding to an estimated concentration of Lim-OOHs in the simulated fine fragrance of 85 ppm (95% CI: 6.8 – 157 ppm). Significantly lower elicitation doses, i.e. non-overlapping 95% CIs, were estimated for $ED_{50}$ and $ED_{75}$ in Lim-OOHs *allergic* subjects when exposed in the ROAT compared to the single patch test exposure.

## Discussion

In recent years, Lim-OOHs present in oxidized limonene has emerged as a very frequent cause of contact allergy in consecutively patch tested dermatitis patients (7–11). However, high rates of weak positive and doubtful reactions have caused some concern regarding the clinical relevance of a positive patch test reaction (12,13). We studied this question and found that all patients with a currently positive patch test to standard Lim-OOHs 0.3% in pet., and 15% (2 of 13) of those with a currently doubtful patch test reaction at D3/4 or D7, developed allergic contact dermatitis when exposed daily to realistic doses of oxidized limonene. This substantiates the clinical relevance of a positive patch test reaction to standard Lim-OOHs 0.3% in pet., and further indicates that even

some patients with only a doubtful patch test reaction have a weak, but clinically relevant, allergy. No reactions, neither allergic nor irritant, were observed in the *healthy control* group.

This ROAT study was conducted in a blinded and randomized fashion, using three concentrations of oxidized limonene with a content of Lim-OOHs of 1260 ppm, 420 ppm, and 140 ppm, corresponding to a dose per area per application of 3.0, 0.99, and 0.33 µg Lim-OOHs/cm$^2$ respectively. This was formulated in ethanol/water to simulate exposure to a fine fragrance, with 100%, 64%, and 27% of patients with a positive patch test to standard Lim-OOHs 0.3% in pet. developing allergic contact dermatitis at these exposures. The selected concentrations and doses were chosen to simulate real life daily exposure to limonene in a hydroalcoholic leave-on cosmetic product, however quantitative assessment of exposure to Lim-OOHs in commercial fragranced consumer products containing limonene is challenging (25). A recently published method for selective analysis of hydroperoxides in perfumes investigated the content of limonene-2-hydroperoxide in 10 fine fragrances kept and used under normal conditions by consumers for one to five years after purchase (26). In four of these fine fragrances, limonene-2-hydroperoxide was detected at concentrations up to 56 ppm, while the levels of the more sensitizing limonene-1-hydroperoxide were not determined. In the current study, we estimated the dose, following repeated exposure in the ROAT, that will elicit allergic contact dermatitis in 10% of sensitized individuals to be 0.20 µg Lim-OOHs/cm$^2$, corresponding to a concentration of Lim-OOHs in the simulated fine fragrance of 85 ppm. With the known ratio of limonene-2-hydroperoxide to limonene-1-hydroperoxide in the oxidized limonene used for this study, this corresponds to a concentration of limonene-2-hydroperoxide of ≈ 70 ppm and a concentration of limonene-1-hydroperoxide of ≈ 15 ppm, with the former being comparable to this documented exposure to limonene-2-hydroperoxide in leave-on cosmetic products.

These levels of exposure to Lim-OOHs are in contrast to data published by the fragrance industry, showing that limonene-2-hydroperoxide could be indirectly detected only up to 5 µg/g (ppm) in 14 of 39 aged fine fragrances recalled from consumers (27). Based on stability analyses performed in the same study, the authors argued that the formation of Lim-OOHs could be impaired or slowed down when formulating limonene into a hydroalcoholic solution such as a fine fragrance. Even so, sensitizing Lim-OOHs can be present already from time of purchase of the limonene added to a cosmetic product. Experimental oxidation studies with the essential oil sweet orange oil (main compound being limonene) has shown that limonene-2-hydroperoxide was present at a concentration of 0.05% (500 ppm) at time of purchase. Following one year of storage at 4 °C in darkness, the concentration of limonene-2-hydroperoxide had increased significantly to 0.29% (2900 ppm) (28,29). In a clinical setting, other cosmetic products than fine fragrances, such as deodorants, are more common causes of allergic contact dermatitis in patients with fragrance contact allergy (30). Furthermore, although several possible sources of exposure to oxidized limonene exist, cosmetic products labelled to contain limonene are by far the most common type of exposure causing allergic contact dermatitis in patients with a positive patch test to Lim-OOHs 0.3% in pet (31).

Future research into formation and stability of Lim-OOHs over time, in cosmetic products with different matrices containing limonene from different sources, would be of interest to further elucidate this complex matter.

The challenges encountered in quantifying the content of Lim-OOHs in both the added limonene and the finished consumer product are reflected in the current legislation and industry guidelines. In the European Commission's Regulation no. 1223/2009 on cosmetics, the limit reported regarding limonene is "a peroxide value less than 20 mmoles/L" which applies to the added substance and not to the finished cosmetic product (32). The standard for use concentrations of limonene in scented consumer products published by the International Fragrance Association (IFRA) states that: "Such products should have a peroxide value of less than 20 millimoles peroxides per liter, determined according to the FMA method" (33). This method seems to identify all types of peroxides and not just Lim-OOHs, and further the accuracy and adequacy of this method is unknown (34). Hence it is unknown to which extent both the legislation and the IFRA standard limit exposure to Lim-OOHs in cosmetic products containing limonene.

In addition to repeated exposure in the ROAT in the current study, a serial dilution patch test was performed with concentrations of Lim-OOHs from 0.59% to 0.0024% (24 ppm). Among 11 patients with a positive patch test to standard Lim-OOHs 0.3% in pet., 27% reacted continuously down to the lowest applied concentration, and hence displayed a MEC of 24 ppm. This finding supports the evidence that sensitized individuals can react to even lower concentrations of exposure to Lim-OOHs than investigated in the ROAT. However, it is important to remember that the applied dose per area when patch testing is higher than in the ROAT for a specific concentration of Lim-OOHs. Overall, the dose-response relationship in patients with contact allergy to Lim-OOHs 0.3% in pet., following both single patch test exposure as well as repeated exposure in the ROAT, resembles that of other well-established fragrance contact allergens such as cinnamal (35), isoeugenol (36,37), and hydroxyisohexyl-3-cyclohexene carboxaldehyde (HICC/Lyral®) (23,38). The results of the current study are also in accordance with a previous ROAT study on another oxidized fragrance terpene, namely oxidized linalool. Following repeated exposure of allergic subjects, with both a simulated fine fragrance and a simulated cream, the lowest concentration of linalool hydroperoxides that elicited a positive ROAT reaction was 560 ppm (39). As previously established for these fragrance contact allergens, we demonstrated that repeated exposure is important for the elicitation of allergic contact dermatitis when exposing sensitized individuals to low doses of Lim-OOHs. It would have been desirable, had we exposed *allergic* subjects to even lower concentrations of Lim-OOHs, both in the ROAT and the dilution patch test, to provide an exposure estimate for a no-effect level with regards to elicitation of allergic contact dermatitis. As this is the first ROAT study to investigate an elicitation threshold for Lim-OOHs, we chose the selected dilutions based on pragmatic experience from previous investigations.

Seven participants with either a previous weak positive patch test (n=4, from the Swedish centre) or doubtful patch test (n=3, two from the Danish centre and one from the Swedish centre) to Lim-OOHs 0.3% in pet. had a

negative confirmatory patch test and were excluded from advancement to the ROAT. It is well known that patch test reactivity can vary over time and reactivity can be regained later in time (20,40,41). Importantly, three of the four participants with a previous weak positive patch test and a current negative confirmatory patch test to Lim-OOHs 0.3% in pet., did in fact have at least a doubtful patch test reaction to one of the two highest applied concentrations of Lim-OOHs in the dilution patch test series. However, as placement and concentrations of these were blinded to the investigators, eligibility for advancement to the ROAT could not be based on these.

To conclude, patients with a positive patch test to standard Lim-OOHs 0.3% in pet. develop allergic contact dermatitis when exposed to realistic doses of Lim-OOHs in a simulated leave-on cosmetic product. Furthermore, a doubtful reaction to this patch test preparation can be of clinical relevance in a subgroup of patients. In patients sensitized to Lim-OOHs, the dose-response relationship, following both single patch test exposure as well as repeated exposure, resemble that of other well-established fragrance contact allergens. Elicitation of allergic contact dermatitis in individuals sensitized to Lim-OOHs was observed to all applied concentrations in the ROAT, with a lowest observed effect-level of exposure to Lim-OOHs of 140 ppm.

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## Legends

Table 1: Overview of used concentrations and doses of oxidized limonene, including content of limonene hydroperoxides, and proportions of observed reactions in allergic and doubtful allergic subjects.

Table 2: Baseline characteristics for participants advancing to the ROAT.

Table 3: Calculated eliciting doses ($ED_{XX}$) and corresponding 95% confidence intervals (95% CI) for specified proportions of limonene hydroperoxide allergic subjects reacting in the patch test and ROAT.

Figure 1: Study flowchart for participants with a previous positive or doubtful patch test to limonene hydroperoxides 0.3% pet., as well as healthy controls. *One participant developed a clearly positive reaction on the ROAT test area 12 days after exposure had ended in the study (see supplementary table 1 for additional details).

Figure 2: ROAT reactions in subject with a positive (+) confirmatory patch test to limonene hydroperoxides 0.3% pet. At day 7 (a), the subject had a positive ROAT to the dose of 3.0 µg (high) and 0.99 µg (middle) limonene hydroperoxides/$cm^2$. At day 14 (b), the subject had a positive ROAT to the dose of 0.33 µg (low) limonene hydroperoxides/$cm^2$. No reaction was seen to the vehicle control (control).

Figure 3: ROAT reactions in subject with a doubtful (?+) confirmatory patch test to limonene hydroperoxides 0.3% pet. At day 6 (a), the subject had a positive ROAT to the dose of 3.0 µg (high) limonene hydroperoxides/$cm^2$. At day 14 (b), the subject had a positive ROAT to the dose of 0.99 µg (middle) and 0.33 µg (low) limonene hydroperoxides/$cm^2$. No reaction was seen to the vehicle control (control).

Figure 4: Observed and predicted dose–response curves in limonene hydroperoxide allergic subjects following exposure in the patch test dilution series and repeated open application test (ROAT).

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**Table 1: Overview of used concentrations and doses of oxidized limonene, including content of limonene hydroperoxides, and proportions of observed reactions in *allergic* and *doubtful allergic* subjects**

| | Concentration | | Dose | | Observed reactions, n(%) | |
|---|---|---|---|---|---|---|
| Test solution (vehicle) | Concentration of oxidized limonene, % | Content of limonene hydroperoxides, % (ppm) | Dose per application of oxidized limonene, µg/cm$^2$ | Content of limonene hydroperoxides per application, µg/cm$^2$ | Allergic subjects (n=11) | Doubtful allergic subjects (n=13) |
| Confirmatory patch test (petrolatum)* | 3 | 0.3 (3000) | 1200 | 120 | | |
| Patch test dilution series (80% ethanol) | 4.7 | 0.59 (5922) | 1206 | 152 | 11 (100) | 13 (100) |
| | 1.6 | 0.20 (1974) | 400 | 50 | 10 (91) | 8 (62) |
| | 0.52 | 0.066 (658) | 133 | 17 | 10 (91) | 5 (38) |
| | 0.17 | 0.022 (219) | 44 | 5.5 | 6 (55) | 1 (7.7) |
| | 0.058 | 0.0073 (73) | 15 | 1.9 | 4 (36) | 1 (7.7) |
| | 0.019 | 0.0024 (24) | 5.1 | 0.65 | 3 (27) | 0 |
| | Vehicle | - | - | - | 0 | 0 |
| ROAT solutions (80% ethanol) | 1.0 | 0.13 (1260) | 24 | 3.0 | 11** (100) | 2 (15) |
| | 0.33 | 0.042 (420) | 7.9 | 0.99 | 7 (64) | 1 (7.7) |
| | 0.11 | 0.014 (140) | 2.6 | 0.33 | 3 (27) | 1 (7.7) |
| | Vehicle | - | - | - | 0 | 0 |

*The confirmatory patch test material was not from the same batch as the oxidized limonene used in the ROAT and patch test dilution series, but from the commercially bought batches used in the participating departments at the time of the study. **Including one participant who displayed a positive ROAT 12 days after exposure had ended (see supplementary table 1 for additional details).

**Table 2: Baseline characteristics for participants advancing to the ROAT**

| | Allergic subjects (n=11) | | Doubtful allergic subjects (n=13) | | Healthy controls (n=17) |
|---|---|---|---|---|---|
| | | Compared to healthy controls, p-value | | Compared to healthy controls, p-value | |
| Age in years, mean(SD) | 52 (15) | 0.41 | 40 (17) | 0.27 | 47 (17) |
| Age range, years | 30-73 | | 20-78 | | 23-73 |
| Female, n(%) | 9 (82) | 1.0 | 8 (62) | 0.44 | 13 (76) |
| History of atopic dermatitis, n(%) | 0 (0) | - | 6 (46) | **0.003** | 0 (0) |
| Eczema currently, n(%): | 6 (55) | **0.007** | 5 (38) | 0.06 | 1 (5.9) |
| -Facial eczema, n | 1 | | 2 | | 0 |
| -Hand eczema, n | 2 | | 3 | | 1 |
| -Truncal eczema, n | 1 | | 1 | | 0 |
| -Leg eczema, n | 2 | | 1 | | 0 |
| Exposed to fragranced products in daily life, n(%) | 2 (18) | **0.002** | 9 (69) | 0.67 | 14 (82) |

**Table 3: Calculated eliciting doses ($ED_{XX}$) and corresponding 95% confidence intervals (95% CI) for specified proportions of limonene hydroperoxide allergic subjects reacting in the patch test and ROAT**

| | Patch test | | ROAT | |
|---|---|---|---|---|
| | Dose (µg limonene hydroperoxides/cm$^2$) | 95% CI | Dose (µg limonene hydroperoxides/cm$^2$) | 95% CI |
| $ED_{10}$ | 0.27 | (0.017-0.79) | 0.20 | (0.016-0.37) |
| $ED_{25}$ | 0.89 | (0.15-1.9) | 0.35 | (0.076-0.57) |
| $ED_{50}$ | 2.9 | (1.1-5.9) | 0.61 | (0.30-1.0) |
| $ED_{75}$ | 9.4 | (4.7-31) | 1.1 | (0.67-3.3) |
| $ED_{90}$ | 31 | (13-254) | 1.9 | (1.1-15) |

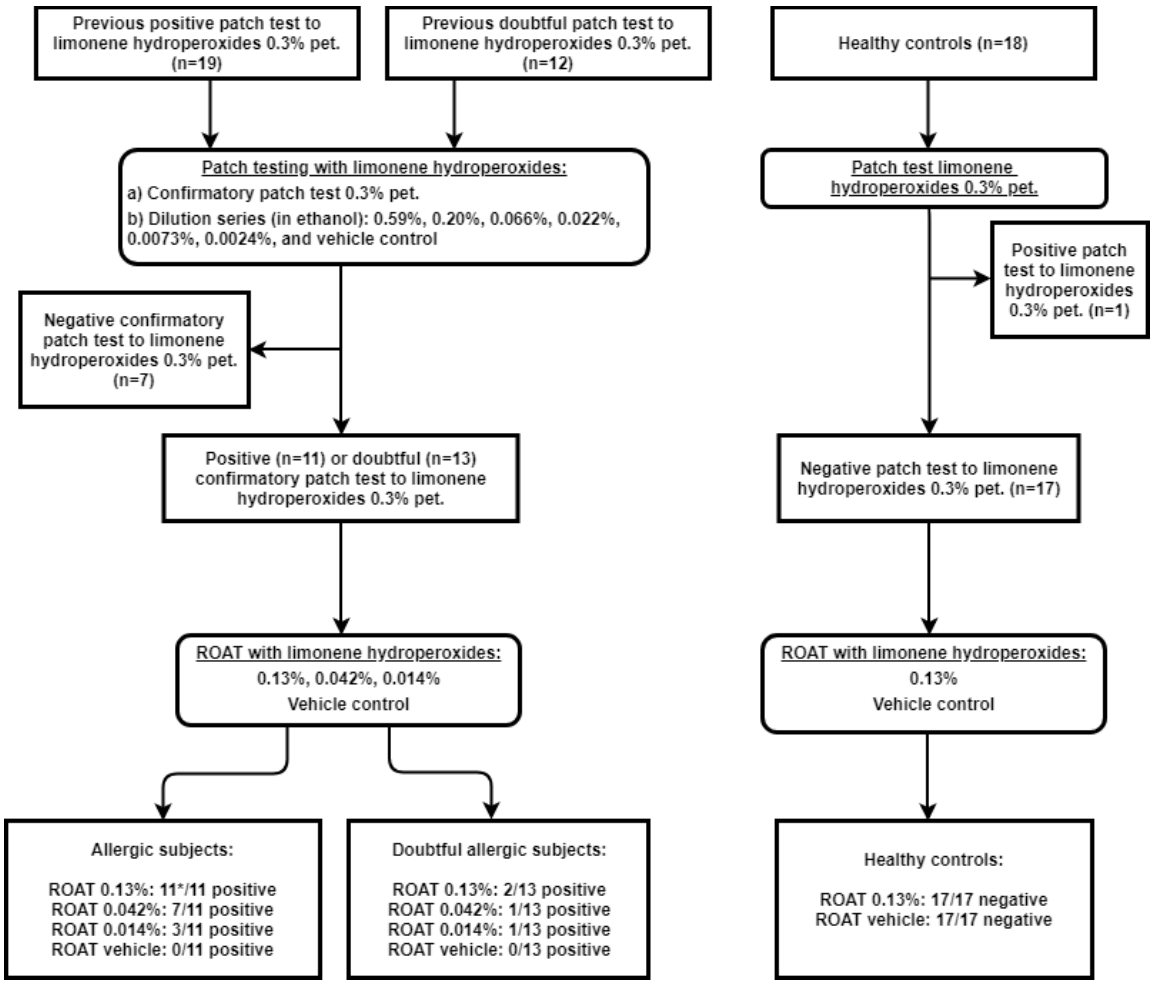


*Figure 1: Study flowchart for participants with a previous positive or doubtful patch test to limonene hydroperoxides 0.3% pet., as well as healthy controls. *One participant developed a clearly positive reaction on the ROAT test area 12 days after exposure had ended in the study (see supplementary table 1 for additional details).*

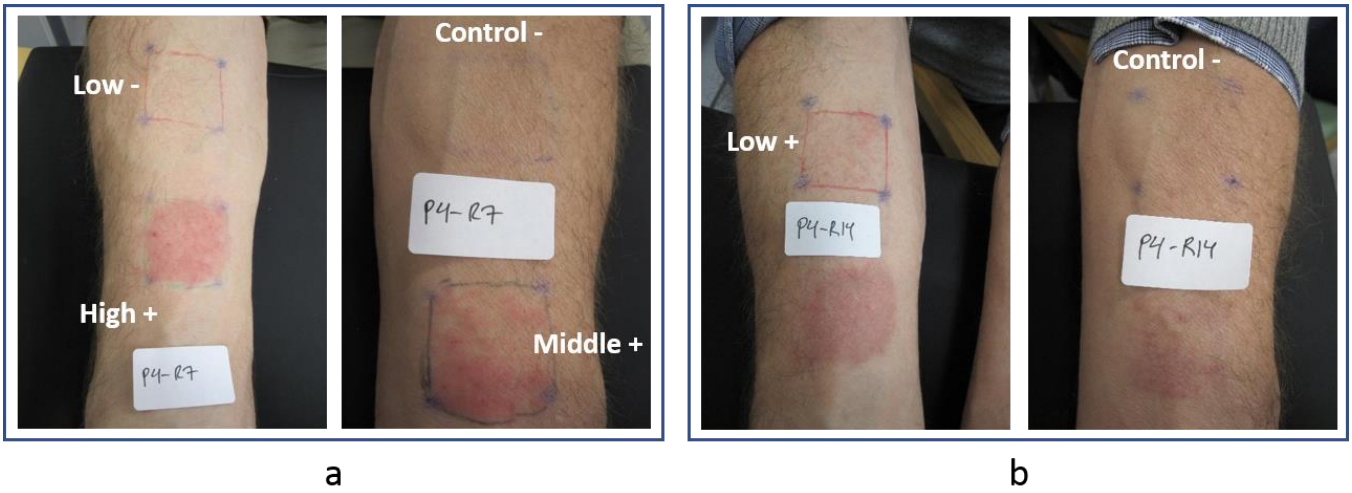


*Figure 2: ROAT reactions in subject with a positive (+) confirmatory patch test to limonene hydroperoxides 0.3% pet. At day 7 (a), the subject had a positive ROAT to the dose of 3.0 µg (high) and 0.99 µg (middle) limonene hydroperoxides/cm$^2$. At day 14 (b), the subject had a positive ROAT to the dose of 0.33 µg (low) limonene hydroperoxides/cm$^2$. No reaction was seen to the vehicle control (control).*

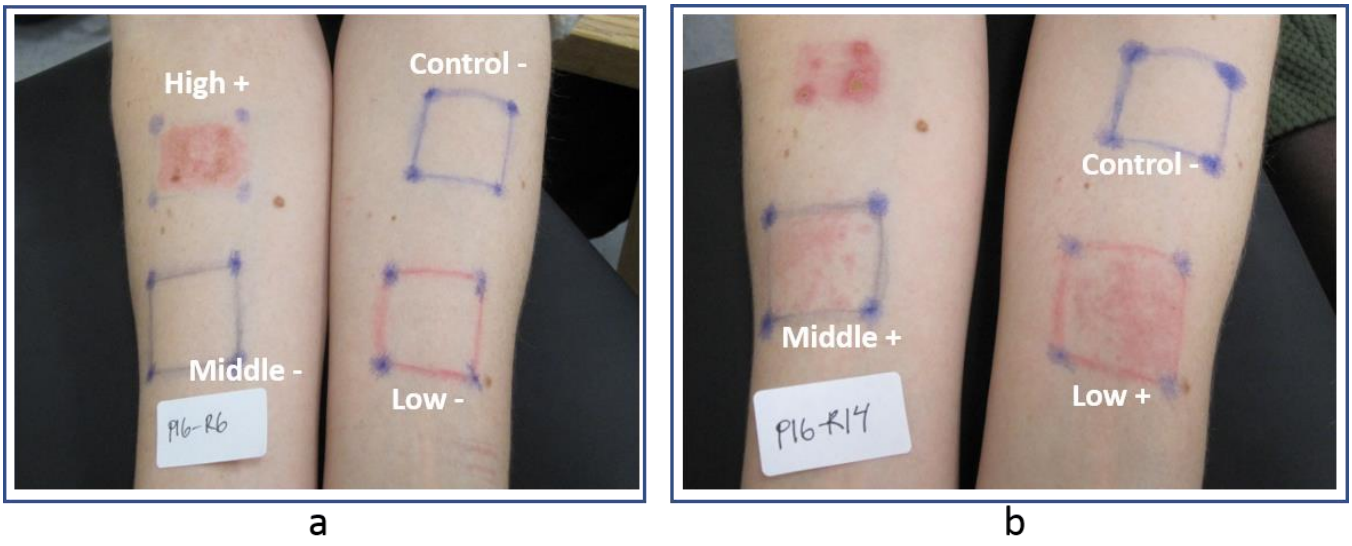


*Figure 3: ROAT reactions in subject with a doubtful (?+) confirmatory patch test to limonene hydroperoxides 0.3% pet. At day 6 (a), the subject had a positive ROAT to the dose of 3.0 µg (high) limonene hydroperoxides/cm$^2$. At day 14 (b), the subject had a positive ROAT to the dose of 0.99 µg (middle) and 0.33 µg (low) limonene hydroperoxides/cm$^2$. No reaction was seen to the vehicle control (control).*

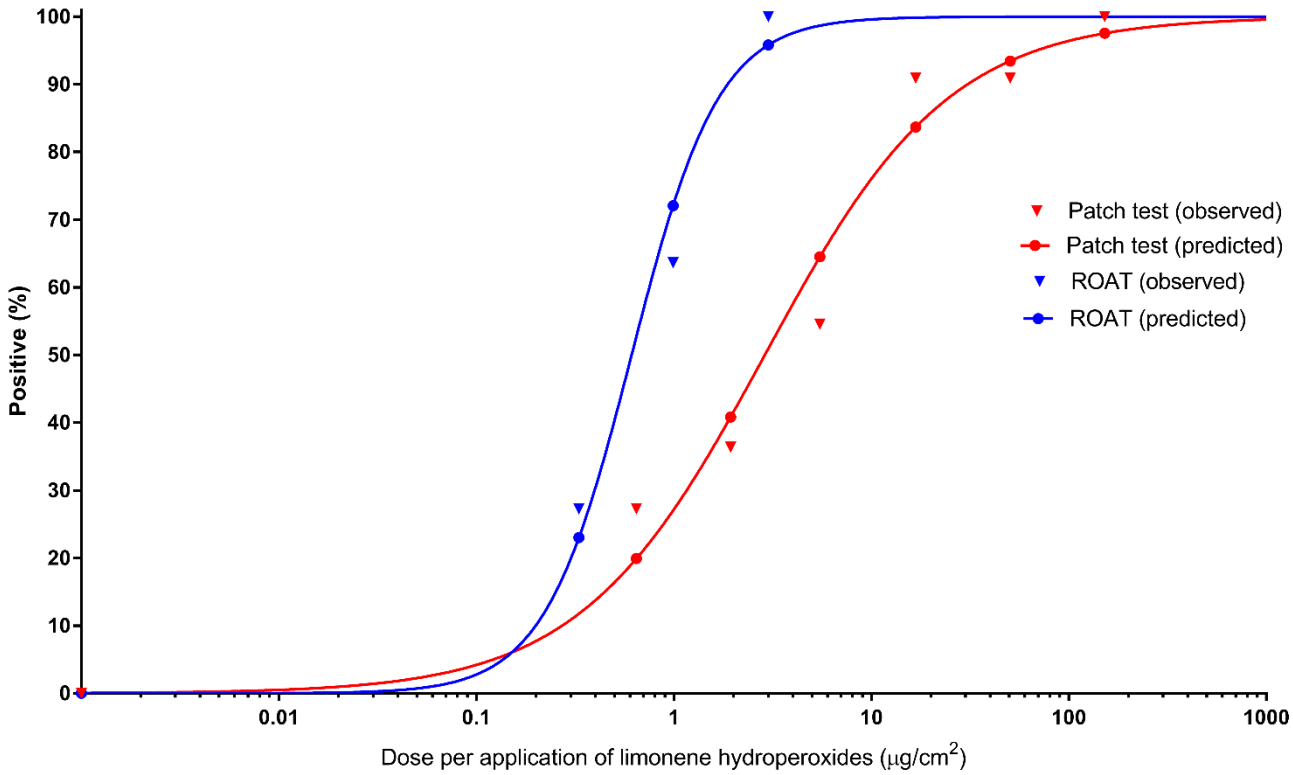


*Figure 4: Observed and predicted dose–response curves in limonene hydroperoxide allergic subjects following exposure in the patch test dilution series and repeated open application test (ROAT).*

## Supplementary material

**Supplementary table 1: Individual results (excluding healthy controls) to previous and confirmatory patch testing with limonene hydroperoxides 0.3% pet., as well as individual reactions in the ROAT.**

| Participant | Age & sex | Atopic dermatitis (UK Working Party criteria) | Previous patch test limonene hydroperoxides 0.3% pet. (test year) | Confirmatory patch test limonene hydroperoxides 0.3% pet. | Positive ROAT, concentration of limonene hydroperoxides (day) |
|---|---|---|---|---|---|
| DK-P1 | 55 F | No | ++ (2016) | ++ | 0.13% (21) |
| DK-P2 | 73 F | No | ++ (2016) | ++ | 0.13% (7), 0.042% (14) |
| DK-P3 | 47 M | No | ++ (2016) | ++ | 0.13% (7), 0.042% (7) |
| DK-P4 | 54 M | No | + (2012) | + | 0.13% (7), 0.042% (7), 0.014% (14) |
| DK-P5 | 40 M | No | + (2014) | ?+ | No reactions |
| DK-P6 | 23 F | Yes | + (2015) | ?+ | No reactions |
| DK-P7 | 30 F | No | + (2013) | + | 0.13% (7), 0.042% (14), 0.014% (18) |
| DK-P8 | 35 F | No | +++ (2014) | ++ | 0.13% (7) |
| DK-P9 | 20 F | No | ?+ (2017) | ?+ | No reactions |
| DK-P10 | 68 F | No | ++ (2015) | +++ | 0.13% (11), 0.042% (21) |
| DK-P11 | 45 F | No | ?+ (2015) | ?+ | No reactions |
| DK-P12 | 59 F | No | ++ (2015) | ++ | 0.13% (7), 0.042% (21) |
| DK-P13 | 61 M | No | ?+ (2015) | ?+ | No reactions |
| DK-P14 | 37 M | Yes | ?+ (2015) | ?+ | No reactions |
| DK-P15 | 34 F | No | ?+ (2016) | Negative | - |
| DK-P16 | 40 F | No | + (2014) | ?+ | 0.13% (6), 0.042% (14), 0.014% (14) |
| DK-P17 | 70 F | No | + (2014) | ++ | 0.13% (7), 0.042% (7), 0.014% (14) |
| DK-P18 | 57 F | Yes | ?+ (2014) | Negative | - |
| DK-P19 | 44 M | Yes | ?+ (2014) | ?+ | No reactions |
| DK-P20 | 28 F | Yes | ?+ (2014) | ?+ | No reactions |
| DK-P21 | 57 F | Yes | ?+ (2012) | ?+ | No reactions |
| DK-P22 | 24 F | Yes | ?+ (2013) | ?+ | 0.13% (21) |
| SE-P1 | 46 F | No | + (2017) | + | 0.13% (21+12*) |
| SE-P2 | 35 F | Yes | + (2017) | Negative | - |
| SE-P4 | 74 F | No | + (2016) | Negative | - |
| SE-P5 | 28 F | No | + (2017) | ?+ | No reactions |
| SE-P6 | 78 M | No | ?+ (2016) | ?+ | No reactions |
| SE-P8 | 46 F | Yes | + (2015) | Negative | - |
| SE-P10 | 28 F | No | ?+ (2017) | Negative | - |
| SE-P11 | 50 F | No | + (2016) | Negative | - |
| SE-P12 | 38 F | No | +++ (2015) | ++ | 0.13% (7) |

*The participant developed a clearly positive reaction on the ROAT test area 12 days after exposure had ended in the study. She had not used any scented products on the test area between finishing the ROAT and developing a reaction.

**Supplementary table 2: Compliance in the ROAT based on weighing of test solutions before and after administration by participants**

| | Actual use compared to expected use, mean percentage (SD)* | | | | |
|---|---|---|---|---|---|
| **Test solution** | **Allergic subjects** | | **Doubtful allergic subjects** | | **Healthy controls** |
| | | **Compared to healthy controls, p-value** | | **Compared to healthy controls, p-value** | |
| Oxidized limonene 1.0 % | 116% (9.9) | 0.38 | 112% (21) | 0.23 | 119% (9.3) |
| Oxidized limonene 0.33 % | 125% (11) | - | 116% (21) | - | - |
| Oxidized limonene 0.11 % | 119% (12) | - | 112% (20) | - | - |
| Vehicle | 116% (6.0) | 0.09 | 112% (22) | 0.11 | 123% (12) |

* Two *allergic subjects* and one *healthy control* did not return one or more test solutions during the study period.

# 6. Considerations on material and methods

The following section elaborates on aspects and considerations of the material and methods which are not covered in full in manuscripts I-III.

## 6.1 Study part 1: Observational registry studies (manuscripts I and II)

### *6.1.1 Material and study population*

For study part 1 of this thesis (manuscript I and II), data were extracted from the Danish national database for contact allergy only for consecutive dermatitis patients patch tested at the Department of Dermatology and Allergy, Copenhagen University Hospital Herlev and Gentofte. With regard to manuscript I, in addition to the diagnostic considerations discussed below, this was done as the database holds electronic records of patch test results for all patients patch tested at Herlev and Gentofte Hospital since 1979[118], which is longer than for any other contributors to the database. With regard to manuscript II, a unique feature at the Department of Dermatology and Allergy, Herlev and Gentofte University Hospital is that consecutive adult dermatitis patients have been continuously patch tested with the 26 fragrance contact allergens with mandatory labelling within the EU since July 2009[119], in addition to being patch tested with the European baseline series and a local extended baseline series.

### *6.1.2 Diagnostic considerations*

Globally acknowledged criteria for scoring of a positive patch test reaction have been implemented since the 1970s[63,64]. If not performed by experienced personnel, inter-individual variation in the performance of the patch test procedure and interpretation of patch test reactions can occur[120,121]. This includes variation in the applied patch test dose, which is vital for elicitation of a positive patch test reaction in a sensitized individual[122]. In addition, several factors related to the chemico-physical properties of individual contact allergens can influence the patch test result. Contact allergens, such as fragrance substances, with a high vapour pressure, can be unstable even over short periods of time, and hence require specific storage and handling conditions in order not to degrade and give false negative patch test reactions[123–125]. Additionally, environmental factors such as immunosuppressive treatment of the patient[126], and possibly seasonal variation[127] and ultraviolet (UV) exposure [128] can also influence the reproducibility of a positive patch test reaction. For the whole study period covered in manuscript I and II, patch test preparations and patch test readings at the Department of Dermatology and Allergy, Herlev and Gentofte University Hospital, have continuously been performed by few trained specialist nurses, to minimize the influence of the above-mentioned factors. This is of special importance regarding trend analyses spanning several years (and decades) as reported in manuscript I.

### *6.1.3 Study design and statistical considerations*

The results presented in study part 1 are mainly descriptive, however as manuscript I and II present data from observational registry studies, inherent epidemiological limitations apply to these. Specifically, established statistical associations between possible risk factors, such as clinical characteristics, and outcomes, i.e. a positive patch test, can only be interpreted as associations without the possibility of addressing causality.

In manuscript I, the prevalence of contact allergy to FMI is presented in an aggregated form for 5-year and 10-year periods, respectively. This was done to improve clarity and avoid over-interpreting random variation in the prevalence of contact allergy to FMI, which can be quite substantial, if comparing results on a year-to-year basis[36,129,130]. To investigate whether the conclusions of manuscript I on temporal trends in contact allergy to FMI were robust to statistical manipulation, a post-hoc analysis was performed. As shown in Figure 4, addressing the prevalence of contact allergy to FMI on a year-to-year basis from 2006 to 2015 did not change the conclusions presented in manuscript I. Applying the Cochrane-Armitage trend test on the year-to-year sex-specific prevalence estimates, a significant increase in the prevalence of contact allergy to FMI was observed for both female and male dermatitis patients for the 10-year period 2006 to 2015. On the contrary, no significant decrease in FMI sensitization could be found within the last 5-year period, for neither female nor male dermatitis patients.

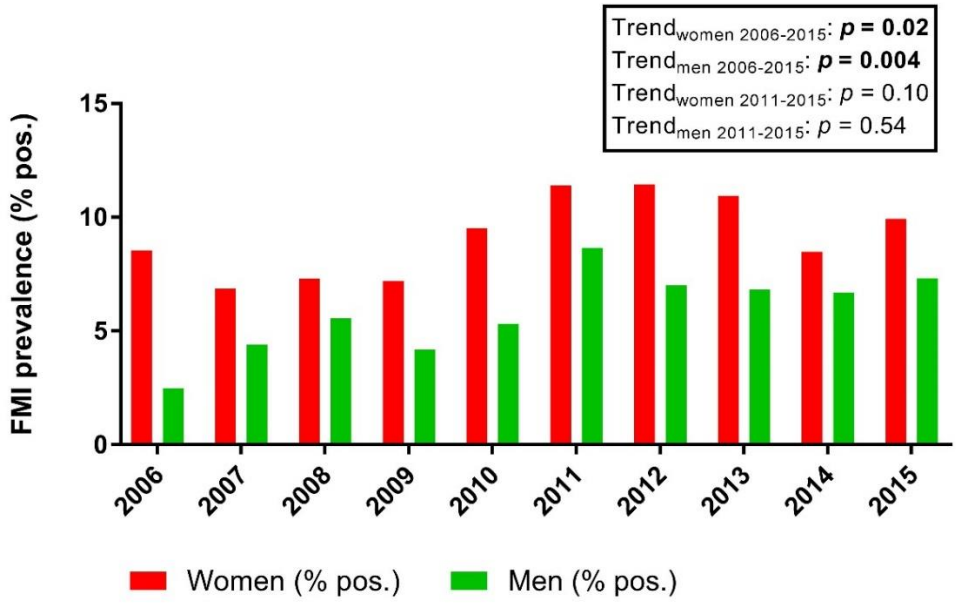


*Figure 4: Sex-specific yearly prevalence rates of FMI sensitization for the material presented in manuscript 1 for the period 2006 to 2015. Post-hoc trend analyses were done with the Cochrane-Armitage trend test.*

In manuscript II, results on contact allergy to the screening markers of fragrance allergy balsam of Peru 25% pet. and colophonium 20% pet., both present in the current European baseline series, were not included in the analyses. This was done to provide a more "clean" estimate on the prevalence of contact allergy to fragrance substances. In a recent British investigation on consecutive patch testing with the 26 EU-labelled fragrance contact allergens, including the oxidized versions of linalool and limonene, it has been estimated that up to 59% of patients with contact allergy to one of the 26 fragrances would be missed if only patch testing with FMI 8%, FMII 14%, HICC 5% and balsam of Peru 25% as part of the European baseline series[84]. Retrospectively, it would have been interesting to provide an estimate of this proportion for our patch test population for comparison, although the British study performed patch testing with the single constituents of FMI with double the concentration compared to that present in the mix.

Finally, it should be emphasized that Thyssen et al. has previously published data on contact allergy to FMI for the period 1985 to 2007 for dermatitis patients patch tested at the Department of Dermatology and Allergy, Copenhagen University Hospital Herlev and Gentofte[129], however with slightly different inclusion criteria than those applied in manuscript I.

## 6.2 Study part 2: Clinical experimental study (manuscript III)

### *6.2.1 Study population*

Participants (allergic and doubtful allergic subjects) for the study were recruited among dermatitis patients who had been previously patch tested with Lim-OOHs 0.3% in pet. at the two participating hospital clinics. It is possible that the invited patients who agreed to participate were more heavily affected by their allergic disease than individuals who did not wish to participate. Indeed, it proved more challenging to include patients with a previous doubtful patch test to Lim-OOHs 0.3% in pet. Regarding the included participants with a previous positive patch to Lim-OOHs 0.3% in pet., it should be noted that the positivity ratio[131], that is the proportion of weak positive (+) reactions among all positive (+, ++, +++) reactions, was 63% (12 of 19). This is identical (63%) to the positivity ratio observed among consecutive dermatitis patients with a positive patch test to Lim-OOHs 0.3% in pet. presented in manuscript II (64 of 102).

### *6.2.2 The test material and exposure related factors*

The content of Lim-OOHs (limonene-1-hydroperoxide and limonene-2-hydroperoxide in a ratio between 6:1 and 5:1) in the weekly prepared test solutions for the dilution patch test series and ROAT was calculated, based on knowledge of the established content of Lim-OOHs in the oxidized limonene purchased from Chemotechnique Diagnostics. It was not feasible to have all weekly prepared test solutions analyzed for their individual content of Lim-OOHs. Also, the stability analyses performed prior to study start confirmed that any degradation of Lim-OOHs in the ethanol/water vehicle at room temperature (which was the storage condition used by participants) was within the relative standard deviation of the analyses.

We aimed at simulating realistic exposure to oxidized limonene through the daily use of a leave-on cosmetic product. The highest concentration of oxidized limonene used in the ROAT (1.0%) was based on the established concentration (0.77%) of limonene in leave-on cosmetic products on the Danish market intended for non-occlusive use (found in a hand lotion)[132]. The content of a single fragrance substance in a fine fragrance, such as simulated in the current study, is typically higher than the one found in creams and lotions[133]. With the oxidation protocol utilized by Chemotechnique Diagnostics, this resulted in a content of Lim-OOHs in the three ROAT solutions of 1260 ppm, 420 ppm, and 140 ppm. Another approach could have been to base exposure on the established content of Lim-OOHs in cosmetic products determined by chemical analyses. However, at the time of study start, only limited literature on this was available, in which indirect detection of Lim-OOHs through a reduction-assay had estimated levels only up to 5 ppm of limonene-2-hydroperoxide in aged perfumes[134]. As discussed in manuscript III, a newer analytical method has detected levels, only looking at Limonene-2-hydroperoxide, up to 56 ppm in aged perfumes[87], which is only ~2 times lower than the lowest applied concentration of limonene-2-hydroperoxide in the ROAT.

It would have been desirable, had all applications in the ROAT been performed under supervision of the study personnel, however for practical reasons this was not possible. As reported in the compliance data in manuscript III, there was a tendency for participants to apply more test solution than expected using the micropipette, however with no differences comparing allergic and doubtful allergic subjects respectively to the healthy controls. In addition to receiving thorough verbal and written instructions, as well as hands-on demonstrations by the principal investigators, a YouTube® demonstration video was also prepared and used by some participants: (https://www.youtube.com/watch?v=-zBbTa9nEfs&t=6s)

### *6.2.3 Stratification of allergic and doubtful allergic subjects*

For presentation of data from the dilution patch test series and ROAT with Lim-OOHs, subjects were stratified as either allergic or doubtful allergic based on results of their confirmatory patch test to Lim-OOHs 0.3% in pet. As shown in the supplementary material for manuscript III, this included one Swedish and three Danish participants with a previous (+) reaction who had a (?+) reaction to the confirmatory patch test with Lim-OOHs 0.3% in pet. Among these were one of the two doubtful allergic subjects who displayed a positive ROAT reaction (to all applied ROAT doses). This subject's confirmatory patch test to Lim-OOHs 0.3% in pet. and dilution patch test series with Lim-OOHs is shown in Figure 5 (left-hand side), in comparison to a subject with a previous (+) and confirmatory (+) reaction to Lim-OOHs 0.3% in pet. (right-hand side). It could be speculated that the confirmatory patch test to Lim-OOHs 0.3% in pet. in the subject on the left-hand side in Figure 5 was false "not-positive". However, based on the reaction pattern to the dilution patch test series to Lim-OOHs, it is evident that the patch test response is markedly different compared to the subject on the right-hand side (see Figure 5 text for additional details). As no photo documentation of the previous patch test reactions to Lim-OOHs 0.3% in pet. was available for participants, subjects were stratified into allergic and doubtful allergic solely based on their reaction to the confirmatory patch test with Lim-OOHs 0.3% in pet., that was performed as part of the study.

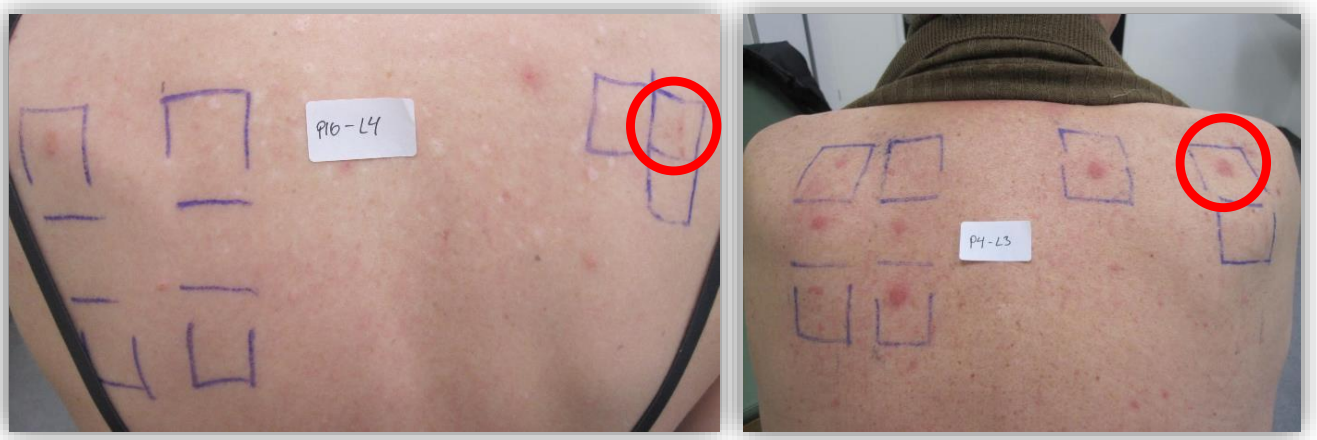


*Figure 5: Confirmatory patch test to Lim-OOHs 0.3% in pet. (red circle with a pet. control immediately below) and dilution patch test series to Lim-OOHs (placed in randomized order) for two subjects both with a previous (+) reaction to Lim-OOHs 0.3% in pet. Based on the confirmatory patch test, the test subject on the left-hand side was categorized as doubtful allergic, and the subject on the right-hand side as allergic. The reaction pattern to the dilution patch test series with Lim-OOHs is markedly different between the two individuals, with the subject on the left-hand side only reacting to the highest concentration (top left corner), and the subject on right-hand side reacting with decreasing intensity to all applied concentrations of Lim-OOHs (except the ethanol/water vehicle control which is at the top of the second row from the left).*

# 7. Discussion

Below, the results obtained in manuscripts I-III are discussed in a broader context of the research field.

## 7.1 Trends in contact allergy to FMI (manuscript I):

A trend is defined as "*a general direction in which something is developing or changing"*[135]. The aim of manuscript I was primarily to investigate whether introduction of the industry-promoted QRA in 2008 had resulted in a general decrease in contact allergy to FMI. The QRA should allegedly protect consumers from being sensitized to fragrance contact allergens in consumer products, including those present in FMI. However, we found no indication of any decrease in sensitization to FMI. In female patients, a significant trend was observed with regards to an increase in the prevalence of contact allergy to FMI across three decades. Most importantly, a significant increase in the prevalence of FMI sensitization was observed for both male and female dermatitis patients within the last 10 years. Even among younger dermatitis patients (≤40 years), we found both absolute and relative increases in the prevalence of sensitization from 2011-2015 compared to the previous 5-year period, although these results did not reach statistical significance.

The Danish national database for contact allergy does not contain specific information on the time duration from patients' first symptoms of dermatitis (and presumably induction of sensitization) to the patient being patch tested for their symptoms. Hence, it could be speculated that there is a delay in seeing the effect of preventive initiatives on the prevalence of disease. However, looking at the preservative methylisothiazolinone (MI), which caused an epidemic of contact allergy and allergic contact dermatitis from its use in cosmetic products over the last decade, two things are to be mentioned: Firstly, patients with allergic contact dermatitis from contact allergens found in cosmetic products are usually patch tested within 1-2 years following onset of their dermatitis[136]. Secondly, effective regulation of the allowed use concentrations of (cosmetic) contact allergens can result in a demonstrable decline in the prevalence of disease within only a few years[137].

Although the findings of manuscript I are from a single university clinic, similar increases in FMI sensitization have also been observed within recent years in several other European patch test populations. In 4430 consecutive Swedish dermatitis patients patch tested between 2009 and 2015, a significant increase in FMI sensitization was observed from 5.6% in 2009-2012 to 7.3% in 2013-2015[138]. The Information Network of Departments of Dermatology (IVDK) has published data on more than 130,000 dermatitis patients patch tested with FMI between 1999 and 2012 in Germany, Switzerland and Austria[36]. For the whole study period, a total of 8.7% had a positive patch test to FMI. Stratification by sex and into several age groups revealed significant increases in FMI sensitization from 2007 to 2012 across all examined stratifications, including in patients younger than 37 years of age. The European Surveillance System on Contact Allergies (ESSCA)[139] is an international network that since 2001 has collected routine patch test data from collaborating patch test clinics across Europe, including from the Department of

Dermatology and Allergy, Copenhagen University-Hospital Herlev and Gentofte. Acknowledging that the clinics contributing to ESSCA has varied to some extent over time, the age and sex standardized prevalence of FMI contact allergy within this large network increased from 6.9% (95% CI: 6.7-7.1%) in 2009-2012 to 7.3% (95% CI: 7.0-7.6%) in 2013-2014[140,141]. Taken together, these findings indicate continued exposure to well-established fragrance contact allergens that results in increasing prevalence rates of contact allergy to FMI among consecutive patch tested dermatitis patients.

Compared to the above-mentioned studies reporting on the prevalence of contact allergy to FMI over time, the material presented in manuscript I includes several unique features. Most importantly, clinical relevance was established in 78% of patients with a positive patch test to FMI for the period 2006-2015, with no temporal changes between 2006-2010 and 2011-2015. Specifically, the proportion of FMI contact allergy with a current clinical relevance remained high and unchanged over time, which contrasts with what would be expected, had the QRA been effective at preventing sensitization to the fragrance contact allergens present in FMI. As observed for the (cosmetic) preservative methyldibromo glutaronitrile, effective prevention of sensitization, in the form of a complete ban in cosmetic products, results in a significant decrease within only a few years in the proportion of individuals with a current relevance of their positive patch test[142].
In manuscript I, it was also possible to adjust the observed increase in FMI sensitization within recent years for concomitant sensitization to the emulsifier SSO 20% in pet., which can potentially cause false-positive patch test reactions to FMI 8% in pet. Excluding all patients sensitized to SSO, since consecutive patch testing with the emulsifier was initiated in 2010, did not change the overall conclusions of an observed increase in contact allergy to FMI within recent years. Data from the IVDK network have otherwise indicated that not controlling for SSO sensitization can affect the outcome of patch testing with FMI[80,143], however, these investigation were done in a subgroup of FMI positive patients. We have recently shown that for the patch test population described in manuscript I, only 0.2% of consecutively patch tested patients from 2010-14 had a positive patch test to SSO 20% in pet., corresponding to only 1.4% of FMI positive patients having a concomitant positive patch test to SSO[144]. This finding highlights the importance of consecutive patch testing to provide an unbiased estimate of a specific sensitization burden.

When the original QRA model was introduced in 2008, it was praised by the fragrance industry as a *"major improvement"* in terms of establishing safe use concentrations of sensitizing fragrance substances in scented consumer products[145]. However, already from the time the model was introduced, it was heavily criticized for being a theoretical unvalidated model with lack of in-depth method descriptions[106]. From the period 2008 to 2015 as covered in manuscript I, the use concentrations in scented consumer products of the sensitizing fragrance substances present in FMI, have been based on the QRA for IFRA associated members (90% of the global market for fragrance substances). We and others have not been able to demonstrate any preventive effect on the risk of sensitization to FMI in consecutive dermatitis patients

referred for patch testing. On the contrary, the prevalence of sensitization to FMI, the most important marker of fragrance contact allergy, seems to be increasing with the exposure levels allowed by the QRA. The inadequacy of the original QRA also seems to have been, at least partly, accepted by supporters of the model, quoting Kimber, Gerberick, and Basketter for: '*Naturally, just as with computer systems, skin sensitization QRA can suffer from the "rubbish in, rubbish out" phenomenon'*[146].
As mentioned in the introduction of this thesis, IFRA and the fragrance industry have recently introduced QRA2 which includes several changes to the safety factors applied to calculate acceptable exposure levels to sensitizing fragrance substances. Nonetheless, the SCCS has still concluded that it is not possible to establish a concentration based on the QRA2, at which induction of sensitization to a fragrance contact allergen is unlikely to occur[107].

## 7.2 Improved screening with the 26 EU-labelled fragrance contact allergens (manuscript II)

The results of manuscript II confirmed that a high proportion of sensitized individuals will be missed if diagnosing fragrance contact allergy by the mere use of the screening markers FMI and FMII. Although other studies have previously reported on patch testing consecutive dermatitis patients with the 26 EU-labelled fragrance contact allergens[75,119,147–149], manuscript II was the first published report to include results on the oxidized versions of linalool and limonene, that is Lin-OOHs and Lim-OOHs. Along with *E. furfuracea* (tree moss) and HICC, these were the single fragrance substances with the highest rates of contact allergy observed in the study. Concomitant positive patch test reactions to either FMI or FMII were only observed in approximately 30% of patients sensitized to Lin-OOHs and Lim-OOHs, respectively.

Since manuscript II was published, a ban as of August 2017 has been imposed by the European Commission on the use of HICC as well as the two potent sensitizers atranol and chloroatranol, which are both present in *E. furfuracea* (tree moss) and *E. prunastri* (oak moss), in cosmetic products[150]. However, due to a strong industry, these three potent sensitizers can be formulated into cosmetic products until August 2019, and furthermore products containing these are allowed to be sold on the European market until August 2021. These bans build on a large body of scientific evidence gathered by international patch test clinics in addition to well conducted experimental elicitation studies. Regarding HICC, these combined investigations resulted in the SCCS concluding in their 2012 opinion on fragrance contact allergens in cosmetics that "*the number of cases of HICC allergy documented over the last decade is exceptionally high and that continued exposure to HICC by the consumer is not considered safe*"[25]. As observed in manuscript II, the prevalence of contact allergy to HICC seems to have declined over the last years, which could indicate a decrease in consumer exposure. Exposure to HICC has especially been associated with deodorant use[151], and we have recently shown that the proportion of deodorants on the Danish market labelled to contain HICC has decreased significantly within recent years, compared to previous investigations[13].

The high rates of contact allergy to *E. furfuracea* (and *E. prunastri*) are not reflected in frequent exposure to these based on labelling in cosmetic products in the EU[13,32]. This could indicate that exposure to the natural extracts occur through other sources than cosmetics. Another plausible explanation is that these natural extracts, containing atranol and chloroatranol to a variable degree, are used in such low concentrations that they are not to be labelled by their individual INCI name in cosmetic products. In a ROAT performed in patients with contact allergy to *E. prunastri* (oak moss) and chloroatranol, elicitation of allergic contact dermatitis was seen following repeated exposure to as low concentrations as 5 ppm of chloroatranol[152].

The findings of manuscript II seem to favor that breakdown testing with the constituents of FMI (except cinnamal) are done at double the concentration compared to that found in the mix, in accordance with how breakdown testing with FMII constituents is done. Cinnamal patch tested at 2% in pet. has previously shown high rates of irritancy[153]. As observed also among British dermatitis patients[75,84], increasing the test concentration of the FMI constituents results in an increased proportion of consecutive FMI-positive patients with a concomitant positive patch test to at least one mix constituent. This is of benefit to the patient in terms of avoiding exposure to a single fragrance contact allergen instead of all eight constituents of FMI. Furthermore, increasing the test concentration of the individual FMI constituents seems to detect more cases of fragrance contact allergy, that is the proportion of "mix negative but constituent positive" patients would increase, as observed for FMII in manuscript II. Of note, we found that 30% of patients with contact allergy to farnesol 5% in pet. had a concomitant negative patch test to FMII 14% in pet, which is a markedly higher proportion compared to the other FMII constituents. This could indicate that the concentration of farnesol in FMII (2.5%) maybe on the verge of being too low. None of the previous studies on consecutive patch testing with the 26 EU-labelled fragrance contact allergens[75,84,119,147,149] have described concomitant reactivity to the mixes and their constituents as detailed as in manuscript II. Whether this finding on farnesol contact allergy can be reproduced in other patch test populations would be of interest, especially as the prevalence of contact allergy to farnesol was only second to HICC for the individual FMII constituents.

In manuscript II, it was confirmed in consecutive dermatitis patients that the strength of patch test reactivity to FMI and FMII is the strongest predictor for a (concomitant) positive patch test to a mix constituent. This has previously been shown in selected FMI/FMII positive patients[80]. Although increasing the patch test concentration of the FMI constituents could detect additional patients with a specific fragrance contact allergy as discussed above, there is still a high proportion of especially patients with only a (+) reaction to FMI and FMII who has a negative breakdown test. The majority of sensitizing fragrance substances are only categorized as moderate sensitizers when tested individually in the LLNA[11]. However, exposure to a combination of two fragrance contact allergens can produce an additive or synergistic effect on elicitation in patients sensitized to both, in comparison to the expected response when testing with these

substances individually[57]. Mice studies have further shown that fragrance contact allergens in a mix, such as present in a cosmetic product, increases the risk of sensitization as well as the elicitation response, in comparison to being exposed to individual fragrance contact allergens[154]. These effects of simultaneous exposure to multiple fragrance contact allergens, occurring in most scented cosmetic products and simulated in FMI and FMII, could explain some of the residual negative breakdown tests to the fragrance mixtures, especially in patients with only a weak contact allergy.

Patch testing with the 26 EU-labelled fragrance contact allergens identifies a substantial number of additional cases of fragrance contact allergy, compared to testing only with the fragrance screening markers present in the European baseline series. However, as identified in the 2012 SCCS opinion on fragrance contact allergens in cosmetics, 82 fragrance substances are categorized as established contact allergens in humans[25]. Further, the SCCS opinion identified an additional 18 fragrance chemicals that were classified as established contact allergens based on LLNA data, however sufficient evidence from human clinical studies were lacking for these. Several of these 18 fragrances are among the top 100 fragrance chemicals worldwide, in terms of volume used, as declared by IFRA[25]. Among these high volume fragrance chemicals, IFRA has as well developed standards for the use levels for three of these compounds (see Table 2) in cosmetic products[155–157]. This indicates that consumers are widely exposed to these fragrances in scented consumer products such as cosmetics. Whether these three fragrance chemicals, proven to have a sensitizing potency in the LLNA, cause fragrance contact allergy in humans due to unrecognized but present exposure is unknown. Contact allergy to these three fragrance substances is currently being investigated at the Department of Dermatology and Allergy, Copenhagen University Hospital Herlev and Gentofte. The optimal patch test concentrations for these are being investigated by patch testing consecutive adult dermatitis patients, following informed consent, with increasing concentrations according to a published protocol[158]. The study will finish in 2018.

| **Individual fragrance chemical (INCI name)** | **CAS no.** | **Human clinical evidence** | **LLNA values[25]** | | **IFRA Standard** |
|---|---|---|---|---|---|
| | | | % | M* | |
| Cyclamen aldehyde | 103-95-7 | None | 22 | 1.64 | Yes[155] |
| Hexyl salicylate | 6259-76-3 | Limited[159] | 0.18 | 0.008 | Yes[156] |
| alpha-Methyl-1,3-benzodioxole-5-propionaldehyde (MMDHCA) / Helional® | 1205-17-0 | None | 16.4 | 0.85 | Yes[157] |

*Table 2: Fragrance substances established as allergens in the LLNA, which have an IFRA standard and are among the "Top 100" fragrances worldwide with regards to volume used by the fragrance industry. *EC3 based on molecular concentration*

### 7.3 Allergic contact dermatitis to hydroperoxides of limonene (manuscript III)

The results of manuscript III demonstrated that patients with contact allergy to Lim-OOHs 0.3% in pet. develop allergic contact dermatitis when exposed to realistic daily doses of oxidized limonene, containing Lim-OOHs, in a simulated hydroalcoholic leave-on cosmetic product. It was further established that the overall dose-response relationship in patients with contact allergy to Lim-OOHs 0.3% in pet. resembles that of other well-characterized sensitizing fragrance substances. Development of allergic contact dermatitis was also observed following repeated exposure to Lim-OOHs in the ROAT, in 15% of patients with a doubtful patch test reaction to Lim-OOHs 0.3% in pet., indicating the presence of a weak, clinically relevant contact allergy. This is in accordance with findings from previous patch test dose studies of other oxidized fragrance terpenes, where 25% and 60% of patients with doubtful patch test reactions to oxidized linalool and oxidized geraniol at 4.0% pet. respectively showed simultaneous positive reactions to the same oxidized terpene at 6.0% pet.[160,161]. Hence, if clinical relevance is suspected in a patient with a doubtful patch test to Lim-OOHs 0.3% in pet., a use test with the relevant (cosmetic) product should be carried out[162].

The oxidized limonene purchased from Chemotechnique Diagnostics and used in the ROAT and dilution patch test series had a documented content of Lim-OOHs of 12.6%. This consisted of limonene-2-hydroperoxide and limonene-1-hydroperoxide in a ratio between 6:1 and 5:1. Although limonene-1-hydroperoxide (EC3-value of 0.33%) has been shown to be significantly more sensitizing than limonene-2-hydroperoxide (EC3-value of 0.83%)[163], both contact allergens are classified as strong sensitizers according to the LLNA[45]. Although these are the main allergen-specific haptens present in oxidized limonene, several other both primary and secondary oxidation products, with a variable degree of sensitizing potency, can be formed[164]. In a Swedish study, patch testing 763 consecutive patients separately with oxidized limonene 3% in pet., limonene-1-hydroperoxide, and limonene-2-hydroperoxide (each at 0.5% in pet.) identified most reactions to limonene-1-hydroperoxide, followed by limonene-2-hydroperoxide and oxidized limonene. Most patients reacted to more than one of the three patch test preparations, however within each group of positive reactions were a minor fraction that only reacted to that one specific marker[165]. Accordingly, we do not know whether allergic subjects in manuscript III were sensitized to limonene-1-hydroperoxide and/or limonene-2-hydroperoxide or other haptens in the oxidized limonene.

The analytical difficulties in quantifying exposure to Lim-OOHs are reflected in the current legislation within the EU, as well as industry standards regarding the use of limonene in cosmetic products and its content of sensitizing oxidation products. As discussed in manuscript III, it is not specifically specified to what extend exposure to Lim-OOHs is limited in cosmetic products containing limonene used by the consumer[102,166]. We found that 27% of subjects with a positive patch test to Lim-OOHs 0.3% in pet. developed allergic contact dermatitis to the lowest applied concentration of Lim-OOHs of 140 ppm in the

ROAT. Based on the proportions of observed ROAT reactions, we also calculated the dose that would elicit allergic contact dermatitis in 10% of sensitized individuals, the $ED_{10}$, equal to 0.20 µg Lim-OOHs/$cm^2$. This corresponds to a concentration of Lim-OOHs in the simulated fine fragrance of ~85 ppm. Given the limitations and uncertainties discussed above, the calculated $ED_{10}$ in sensitized individuals would correspond to a concentration of limonene-1-hydroperoxide of ~15 ppm and a concentration of limonene-2-hydroperoxide of ~70 ppm in the simulated leave-on cosmetic product investigated in manuscript III. The $ED_{10}$ value has previously been suggested in safety assessment for preventive purposes for other contact allergens[25,167]. The calculated $ED_{10}$ value for limonene-2-hydroperoxide is very close to the detected concentration (56 ppm) found among a few (n=10) aged perfumes recalled from consumers[87]. As none of the exposure studies performed to date have investigated the specific levels of the more sensitizing limonene-1-hydroperoxide in aged fine fragrances recalled from consumers[87,134], there is a major research need to further elucidate exposure to this strong sensitizer.

## 8. Conclusions

In this thesis, it was shown that contact allergy to FMI, the most important marker of fragrance contact allergy, has increased in prevalence among both female and male dermatitis patients within recent years. No preventive effect of the industry-promoted and unvalidated QRA risk assessment model could be demonstrated on the prevalence of FMI sensitization. Contact allergy to FMI continues to be frequent, with the majority of positive patch test reactions being of clinical relevance. These observations indicate that consumers continue to be exposed to well-established fragrance contact allergens at concentrations that cause sensitization to FMI. Cosmetic products constituted 95% of relevant exposures in patients with a current clinical relevance of their contact allergy to FMI.

Fragrance contact allergy is diagnosed in one out of every six dermatitis patients when patch testing consecutively with the 26 fragrance contact allergens with mandatory labelling in cosmetic products in the EU, in addition to the fragrance screening markers FMI and FMII. Contact allergy to the oxidized fragrance terpenes Lin-OOHs and Lim-OOHs is common, albeit high rates of weak positive and doubtful patch test reactions to these patch test preparations were seen. Only around 30% of patients sensitized to Lin-OOHs and Lim-OOHs would be diagnosed with fragrance contact allergy if only patch testing with FMI and FMII. In addition to Lin-OOHs and Lim-OOHs, the highest rates of contact allergy were observed to HICC and *E. furfuracea* (tree moss) among the 26 EU-labelled fragrance contact allergens. When patch testing with the single constituents of FMI, results from this thesis support the use of double the concentration (2%, except for cinnamal) compared to that found in the mix, as already recommended for FMII. This will increase both the proportion of FMI sensitized patients with a positive breakdown test and increase the proportion of patients diagnosed with fragrance contact allergy that would otherwise have a negative patch test to FMI.

In the experimental ROAT study, it was demonstrated that all patients with a positive patch test, as well as a subgroup of patients (15%) with a doubtful patch test, to standard Lim-OOHs 0.3% in pet. develop allergic contact dermatitis when exposed daily to realistic doses of oxidized limonene. These findings support the clinical relevance of a positive patch test to this patch test preparation, and even some patients with only a doubtful patch test have a clinically relevant contact allergy. A total of 27% of patients with a positive patch test to Lim-OOHs 0.3% in pet. reacted to the lowest applied concentration of 140 ppm Lim-OOHs in the ROAT. This exposure consisted of the two main sensitizers limonene-2-hydroperoxide and limonene-1-hydroperoxide in a ratio between 6:1 and 5:1 in the simulated fine fragrance. Overall, the dose-response relationship in individuals sensitized to Lim-OOHs resembles that of previously well-characterized fragrance contact allergens, following both single patch test exposure as well as repeated exposure in a ROAT.

## 9. Future perspectives

The current self-regulated risk management imposed by the fragrance industry has failed in terms of establishing safe use concentrations of well-established fragrance contact allergens in scented consumer products. Primary prevention of contact allergy to fragrance substances is best achieved by eliminating exposure, and with the vast majority of exposure to sensitizing fragrance substances being through cosmetic products, this would and should be feasible. The recent ban within the EU on the use of HICC, atranol, and chloroatranol in cosmetic products, with the two latter being the main sensitizers in the natural extracts *E. furfuracea* (tree moss) and *E. prunastri* (oak moss), is to be considered a milestone. This ban outlines the extensive scientific documentation needed for an established fragrance contact allergen to become effectively regulated. This includes continuous documentation of high rates of contact allergy in several patch test populations over time, quantitative exposure assessment through chemical analyses of relevant (cosmetic) exposures, and clinical experimental investigations of elicitation threshold and dose-response relationship. However, the overall burden of contact allergy to fragrance substances remains high, with HICC, atranol, and chloroatranol only constituting a minor fraction of the established fragrance contact allergens being extensively used in scented consumer products. For other established fragrance contact allergens present in FMI such as cinnamal and isoeugenol, which still frequently cause sensitization in dermatitis patients, all the scientific data listed above have been provided by the independent scientific community. Hence an additional ban on these fragrance contact allergens would be a “low hanging fruit” in terms of reducing the overall burden of contact allergy to fragrance substances even further.

The diagnosis of fragrance contact allergy is markedly improved by screening with the 26 fragrance contact allergens with mandatory labelling in cosmetics within the EU, in addition to the screening markers present in the current European baseline series. Also, in terms of secondary prevention of allergic contact dermatitis in sensitized individuals, a specific diagnosis is of vital importance. In this sense, it is paramount that labelling of all sensitizing fragrance substances used in consumer products, irrespective of use concentrations, is made mandatory. This would be of importance to both the clinician in terms of improving the diagnosis of fragrance contact allergy, as well as the sensitized individual seeking to avoid future exposure.

With regards to oxidized limonene and Lim-OOHs, further work into quantifying exposure to the individual sensitizing limonene hydroperoxides is needed, especially for the more sensitizing limonene-1-hydroperoxide. Conceptually, oxidized limonene, with its content of a wide and varying array of sensitizing oxidation products, could be viewed upon as a natural extract. e.g. like *E. furfuracea* (tree moss) or *E. prunastri* (oak moss). It is the individual limonene-1-hydroperoxide and limonene-2-hydroperoxide, paralleling atranol and chloroatranol that are to be restricted or banned from being present in scented consumer products. An additional ROAT study in patients with known specific contact allergy to limonene-1-hydroperoxide or limonene-2-hydroperoxide exposed to relevant concentrations of the respective hydroperoxide would be of benefit to support the results of the ROAT study performed as part of this thesis.

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