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Multiple aspects of contact allergy

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Multiple Aspects of Contact Allergy

Immunology, patch test methodology and
epidemiology

Daan Dittmar

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**university of
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Multiple Aspects of Contact Allergy

Immunology, patch test methodology and
epidemiology

Phd thesis

to obtain the degree of PhD at the
University of Groningen
on the authority of the
Rector Magnificus prof. C. Wijmenga
and in accordance with
the decision by the College of Deans.

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CHAPTER

1

General introduction

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This thesis consists of research performed as part of the 'Expert Center for Eczematous and Occupational Dermatoses' at the department of Dermatology, University Medical Center Groningen, in the Netherlands. The focus of this thesis is on contact allergy and covers a broad range of topics, including immunology, diagnostics, and epidemiology. The following introduction to this thesis functions as a general introduction to the field of contact allergy and related research, and introduces specific terms/concepts vital for a correct understanding and interpretation of the chapters which follow.

Dermatitis is inflammation of the skin, which presents with erythema, papules, vesicles (sometimes bullae in contact dermatitis), oozing, and excoriations in the acute phase, and with lichenification, squamae, and fissures in the chronic phase. Contact dermatitis is caused by contact of the skin with (mostly) harmful substances in our surroundings. Different etiological subtypes of contact dermatitis have been identified; irritant contact dermatitis (ICD), allergic contact dermatitis (ACD), and protein contact dermatitis.⁽¹⁾ The latter is a rare entity and will not be discussed any further, although it must be considered, for example, in occupations which involves food handling. The main difference between ICD and ACD is that ICD is caused by substances identified as 'irritants', and the resulting dermatitis is due to the toxic effects of said irritants, while ACD is caused by 'contact allergens', and is a T cell driven skin inflammation. Substances can be both an irritant and a contact allergen, and the latter most likely requires an irritant effect on the skin in order to activate the innate immune system (see below).⁽²⁻⁴⁾ Because of this it is probably not uncommon in patients diagnosed with ACD for their dermatitis to be partially caused by exposure to irritants.⁽⁵⁾ Irritants can range from the extremely harmful (e.g. a strong corrosive chemical), in which a short moment of contact results in ICD, to the relatively benign (e.g. water), to which frequent and prolonged exposure can ultimately result in ICD as well. Properties that make a chemical an 'irritant' include cytotoxicity through pH disturbances, acting as a surfactant, and presence of alkylating properties, among others.^(6, 7) The same is true for contact allergens, although allergenic potential (or rather, sensitising potential) is not defined as how much damage the contact allergen can do to the skin, but rather how sensitising it is, i.e. strong sensitisers can induce sensitisation at a lower dose (and shorter contact) than weak sensitisers. However, it is important to realise that the irritant effect of contact allergens is needed for sensitisation, and that therefore the sensitising potential is also dependant on the irritant properties.^(6, 7) Management of both ICD and ACD are the avoidance of the causative exposure. Depending on the

severity and location of the contact dermatitis, topical corticosteroids of different potencies can be indicated.

ICD and ACD are among the major causes of occupational skin disease and therefore cause a high socio-economic burden, and can also greatly reduce quality of life in patients.(8) If the causative substance cannot be avoided at their current work, sometimes there is no choice but to change occupation.

GENERAL INTRODUCTION TO CONTACT ALLERGY AND ALLERGIC CONTACT DERMATITIS

In the simplest of terms, contact allergy is the immunological state in which re-exposure to the causative contact allergen above a threshold dose results in dermatitis 24 to 72 hours after re-exposure. Within the classification of hypersensitivity by Gell and Coombs, contact allergy is classified as a type IV delayed type hypersensitivity; a T cell mediated immune response.(9) An important distinction exists between contact allergy, an immunological state, and allergic contact dermatitis (ACD), the clinical consequence of a contact allergy in case of repeated exposure. Allergic contact dermatitis is always caused by a contact allergy, while individuals with a contact allergy might go their entire lives without ever experiencing skin complaints if they are never exposed again to the responsible contact allergen above the required dose for elicitation. Acquiring a contact allergy is called the sensitisation phase (also referred to as the induction of contact allergy), while ACD represents the elicitation phase. Contact hypersensitivity represents the mouse model of ACD.

Haptens, pro-haptens, and pre-haptens

In the field of contact allergy, contact allergens are also referred to as haptens, to avoid confusion with aeroallergens (causing immediate type allergic reactions, type I according to the Gell and Coombs classification). In order to function as a hapten, a molecule needs to have a specific molecular structure. Firstly, haptens have low molecular weight, meaning they are generally smaller than 500 daltons (Da, equivalent to g/mol), in order to allow for effective penetration of the stratum corneum.(10) To contextualize; a hydrogen atom is about 1 Da, and the hair dye allergen *para*-phenylenediamine (PPD, CAS no. 106-50-3) is about 108 Da.(10) Secondly, haptens have to be protein reactive. The reason for this is twofold; to activate the innate immune system, and to either be recognized as a hapten-protein complex by DC receptors or react with surface proteins on dendritic cells (DCs), after which it can be

presented to naïve T cells.(11, 12) This process of binding to proteins in the human skin is called haptenization (or haptentation) and is vital, as the haptens themselves are too small to be recognized by the immune system. This concept of haptenization as a fundamental requirement of a molecule to act as a sensitizer was first proposed back in 1936 by Landsteiner and Jacobs, and has been supported by many investigations since. (11, 13) There are over 4000 chemicals and metal ions that can function as haptens; however, the majority of contact allergies are caused by a small fraction of all these possible haptens.(14)

It must be stated however, that sometimes the word 'haptent' is used to indicate the fragment bound to the protein after haptenization, which can be very different than the original chemical, as it can undergo transformation in the process of haptenization. (15) In the current thesis the term 'haptent' refers to a chemical with the potential to induce contact allergy, i.e. before haptenization. In this introduction, the term 'contact allergen' will be used to denote haptens.

There is a group of molecules which do not initially possess a sensitizing potential, but can become a contact allergen either through transformation by enzymatic processes in the skin or by transformation in the outside world by for example air oxidation or ultra-violet irradiation. These were initially collectively named 'prohaptens', but later the term 'prehaptens' was proposed for the latter category (not-enzymatic transformation).(16) However, this categorization has been argued against as there is not enough knowledge yet to completely distinguish between 'prohaptens' and 'prehaptens', and some chemicals can be both.(15)

Pathogenesis

The immunological aspect of contact allergy and ACD has been investigated extensively over the previous decades, and advancements in technology has coincided with incremental steps taken in its understanding. The more we learn about the pathogenesis of ACD (or any disease for that matter), the more complex it becomes, and the task of understanding all facets can become daunting. Therefore, we should start with a bird-eye view of the whole process, step by step:

Sensitisation:

- 1) Skin exposure to a hapten with consequent penetration through the stratum corneum (henceforth referred to as the skin barrier).
- 2) Binding of hapten to epidermal proteins (haptenization).
- 3) Activation of the innate immune system creating an inflammatory environment (xenoinflammation).

- 4) Binding of hapten-protein complex to DCs (or direct haptization inside the MHC groove on DCs).
- 5) Migration of dendritic cells carrying the hapten-protein complex via afferent lymphatics to regional lymph nodes.
- 6) Recognition of the hapten-protein complex presented by the DC by naïve T cells.
- 7) Proliferation of hapten-specific memory, effector, and even regulatory T-cells in the lymph node and subsequent systemic dissemination by release of the proliferated T-cell progeny into the blood circulation.

Elicitation:

- 8) Re-exposure to the causative hapten, after which the hapten is again presented by DCs to memory/effector T cells present in the skin, causing them to secrete pro-inflammatory cytokines and chemokines, causing local dermatitis.

Step 3, in which the innate immune system is activated, is thought to be caused by the irritant properties of a hapten, and the term *xenoinflammation* has been proposed.⁽⁴⁾ The exact process has not yet been completely elucidated, although key players such as reactive oxygen species (ROS), Toll-like receptor 4 (TLR₄), and the NOD-like receptor protein 3 (NLRP₃) inflammasome have been identified.^(2, 17, 18) The subsequent inflammatory environment that is created activates DCs and other epidermal cells.⁽⁴⁾ Besides activation of the innate immune system, irritants which have a solvent/surfactant effects can disrupt the skin barrier, allowing for more efficient penetration by haptens.⁽¹⁹⁾ Xenoinflammation also helps recruitment of effector T cells to the skin in the elicitation phase, and prior/concomitant exposure to other haptens/irritants can decrease the threshold for sensitisation and elicitation and/or increase the strength of elicitation response.^(4, 20-22) More detail on how the innate immune system is activated is provided in chapter 2 of this thesis, with a focus on the cytokine tumor necrosis factor (TNF), and the role it plays in the pathogenesis of contact allergy and ACD.

Cross-reactivity and co-sensitisation

An important concept in contact allergy is cross-reactivity, in which an individual who is sensitised to hapten A develops an elicitation response when exposed to hapten B, to which there was no prior exposure. The explanation is that hapten B has the same chemical group and spatial geometry as hapten A and can therefore be recognized by the same hapten A-specific T cells.⁽²³⁾ Two haptens which are different from each other can still cross-react if one of the two is metabolized in the skin (or transformed non-enzymatically outside the skin due to for example oxidation) so that the resulting

molecule is similar to other haptens.(24) The pro- and prehaptens concepts play an important role in understanding cross-reactions between initially chemically unrelated molecules. The reverse can also be true; two haptens which, although are of similar structure, do not cross-react as they are metabolized in the skin by two different pathways, an example being eugenol (CAS no. 97-53-0) and isoeugenol (CAS no. 97-54-1).(25)

Individuals allergic to PPD can cross-react to another hair dye allergen 2,5-toluenediamine (TDA, CAS no. 95-70-5, also known as *para*-toluenediamine, PTD), but also to benzocaine (CAS no. 94-09-7), a local anaesthetic which is ethyl ester of *para*-aminobenzoic acid, and to N-isopropyl-N'-phenyl-*para*-phenylenediamine (IPPD, CAS no. 101-72-4), a stabilizer in rubber, among others.(26, 27) Although the term cross-reactions is used in a clinical setting to denote elicitation reactions to two or more chemically similar haptens, it is almost impossible to ascertain whether these constitute true cross-reactions or if the patient has been co-sensitised, i.e. exposed to both haptens, especially if the two haptens are often found together in products (e.g. PPD and *p*-aminophenol, both ingredients present concomitantly in hair dyes) or separately but in similar products (PPD and TDA, both hair dyes). Only a limited number of cross-reacting haptens have been identified as true cross-reactions. A famous example are corticosteroids, in which four groups have been identified based on structure-activity relationship analyses (group A to D). It was hypothesized that cross-reactivity occur within corticosteroid groups, which was confirmed for groups A, B, and D by analysing patch test data.(28, 29)

Risk factors for sensitisation

Exposure

The largest determinant of whether a person becomes sensitised is exposure, as opposed to susceptibility. The area and concentration of the hapten in the substance to which the skin is exposed can be expressed as 'dose per unit area', and corresponds to the intensity of the sensitisation stimulus.(30) Exposure to specific contact allergens can be higher in specific occupations or in specific conditions, for example leg stasis dermatitis, in which there is high exposure to topical medicaments containing corticosteroids, preservatives, and fragrances as potential contact allergens.(31) Risk factors for having a contact allergy are being of the female sex and having old age; these can also both be explained more by exposure than by differences in innate susceptibility, although sex influences some aspects of the immune response. For example, the influence of the menstrual cycle on the elicitation phase has been

explored - often of nickel contact allergy - and results suggest an inhibitory effect during the ovulatory phase (high oestrogen) and an increased immune response during the progestinic phase (high progesterone).(32, 33) Increased exposure in females is due to more occupations with high exposure (health care workers, hairdressers) and habits and clothing (more fragrances, jewellery, etc.).(34) Although with age comes senescence of the immune system, i.e. it is theoretically more difficult to acquire a new contact allergy, this is countered by cumulative lifetime exposure.(34)

Additional risks other than susceptibility that influence sensitisation are exogenous factors which influence the penetration of the hapten through the skin barrier, for example the physiochemical properties of the chemical in question (e.g. the pH and the lipophilicity), the properties of the vehicle, and whether there is occlusion or not.(35, 36) Topical preparations that stay on the skin also transform over time, with evaporation of for example water leading to more concentrated preparations the longer it stays on the skin.(37) Depending on the hapten, higher or lower levels of exposure are required to reach the threshold for sensitisation or elicitation; a lower level of exposure is required for 'strong' sensitisers compared to 'weak' sensitisers.

Skin barrier

A second important determinant for sensitisation is the skin barrier; when compromised, it is easier for haptens to penetrate into the epidermis. There are inter- and intra-individual variations in the skin barrier. A method by which this was investigated was by measurements of the trans epidermal water loss (TEWL), an *in vivo* measurement in which passive water diffusion through the stratum corneum is measured.(38) Its most common use is to assess skin responses to irritants. TEWL has been found to be inversely correlated with the size of the flattened corneocytes and the number of cell layers of the stratum corneum in the respective skin area.(39) As most molecules penetrate in an intercellular fashion (through the lipid layer between the corneocytes), a higher TEWL implies higher permeability, i.e. TEWL is inversely correlated with barrier function. Some suggest that this might be true for hydrophilic chemicals, but that it is not known yet whether it is also true for lipophilic chemicals.(7) There are no large sex- or race-dependent differences in TEWL.(40) A variation in TEWL between individuals has been observed as well, with a coefficient of variation ranging from 31% to 47% for the palm and from 35% to 57% for the forearm.(41) A possible explanation for these inter-individual differences might be mutations in the filaggrin gene (*FLG*), mainly found in populations of North-European descent and Asian populations.(42) Filaggrin (filament-aggregating protein) plays an important role in

the formation of corneocytes and in the generation of intracellular metabolites, such as natural moisturizing factor, contributing to hydration and lowering the pH of the stratum corneum.(42) As a result, loss-of-function mutations in the filaggrin gene are associated with reduced hydration, increased TEWL, and an increased skin pH, which in turn leads to reduced resistance against pathogenic microorganisms and activation of pH-sensitive serine proteases.(43) The activated serine proteases can cause, among others, compromised intercellular connections and can activate the pro-inflammatory cytokines interleukin-1 α (IL-1 α) and IL-1 β . Filaggrin has been argued to be at the centre of atopic dermatitis pathogenesis.(43) One study found that the R501X and 2282del4 mutations in *FLG* were strongly associated with contact allergy in individuals with (atopic and/or hand) dermatitis, but not in individuals without dermatitis.(44) The most likely explanation for this is that active inflammation can cause down regulation of filaggrin. Prior or concomitant exposure to irritants during exposure to contact allergens can also negatively influence the skin barrier, as can psychological stress. (22, 45)

Genetic predisposition

Besides the above mentioned *FLG* mutations, other genes have been investigated and specific mutations/genotypes have been found to contribute to a higher susceptibility for developing contact allergy. A more complete overview is provided in (46). There is evidence that mutations in the genes encoding for the pro-inflammatory cytokines IL-10, IL-16, and TNF pose a risk factor for sensitisation.(47-49) Many of these studies are however hampered by small sample sizes and other methodological flaws. Studies investigating *TNF* variants are discussed critically in chapter 2 of this thesis.

Another line of defence, besides the skin barrier, are drug-metabolizing/detoxifying enzymes present in the epidermis that metabolize and/or detoxify exogenous molecules.(50) Two well-known examples are glutathione-S-transferase (GST) and N-acetyltransferase 1 (NAT1), phase 2 enzymes of the cytochrome P450 superfamily. Specific genotypes for these enzymes may make an individual more or less susceptible to develop contact allergy to specific haptens, as has been suggested for GST and contact allergy to chromium and thiomersal.(51) N-acetyltransferases are enzymes with activity towards aromatic amines, and NAT1 had been shown to be able to detoxify unoxidized PPD into mono-acetyl-*para*-phenylenediamine (MAPPD) and di-acetyl-*para*-phenylenediamine (DAPPD), which do not possess any sensitising potential.(52, 53) Although in vitro studies have shown that NAT2 is also able to acetylate PPD, little to none NAT2 activity has been observed in the skin.(54) Variants in *NAT1* can result

in rapid or slow acetylation phenotypes, the hypothesis being that slow acetylation phenotype results in a higher susceptibility to develop PPD contact allergy. Different studies investigating the role of *NAT* genotypes in contact allergy to PPD or *para*-amino-substituted aryl compounds have produced contradictory results, however. (53, 55, 56)

DIAGNOSING CONTACT ALLERGY: PATCH TESTING

Originally conceived by Joseph Jadassohn (and Jean-Henri Fabre) at the end of the 19th century and later expanded by Bruno Bloch (among others) in the early 20th century, patch testing is the golden standard diagnostic test to diagnose contact allergy.(57) Contact allergens dissolved or dispersed in a vehicle (often petrolatum or water) are applied to the skin under occlusion for a standard amount of time. In its essence patch testing is an attempt at recreating the elicitation phase of contact allergy in a controlled setting.

The main indication for patch testing is suspected ACD.(58) It can, however, also be indicated in patients with atopic dermatitis, especially if it is refractory to treatment and an underlying ACD is suspected.(59) Besides attempting to detect any contact allergies, patch testing is also used to investigate the safety of alternative products (topical medicaments, cosmetics, gloves, medical adhesives such as glucose monitors in diabetes patients or TENS (see chapter 8a)) in patients with suspected/proven contact allergies. This use of patch testing allows for adequate advice to be given to the patient on which products to avoid and which products are safe to use.

Patch test technique

The patch test is most often performed on the upper back of a patient for practical reasons, as it provides a large and relatively flat surface area. Sometimes a patch test can also be performed on the upper arms or thighs, for example if a large variety of contact allergens are suspected and the back is not large enough to test all preferred contact allergens, or if there is active dermatitis or other skin diseases on the back which make it impossible to perform the patch test (although postponing the patch test might be preferred in this situation; differences in skin barrier between anatomical sites make patch testing elsewhere less reliable (see above)).(58) Additional reasons for postponing a test are if topical corticosteroids are applied to the back - which implies active dermatitis on the back, another reason to postpone patch testing - or

if the patient is on immunosuppressive medication, as this can inhibit an elicitation reaction, with therefore a high risk of false negatives.(60)

Patch test materials and series

The contact allergens dissolved in a vehicle are applied to the back in patch test chambers; different test chambers exist and differ mostly in size (area ranging from 0.5cm² to 0.68cm²) and material (polyethylene foam in IQ Ultra™ chambers, aluminium in Finn Chambers®, polypropylene in Van der Bend chambers). Due to different sizes different amounts of contact allergen containing vehicle have to be applied so the 'dose per unit area' is equal.(61) These are loaded by the investigator with a standardized amount of contact allergen containing vehicle. For aqueous solutions it is recommended to load the chambers using a micro-pipette in order to minimize variation in dose between chambers; for petrolatum a syringe is often used, which is slightly more difficult and more dependent on the experience of the investigator.(62) An attempt to standardize patch testing, especially the dose applied, resulted in the true test, a preloaded, ready-to-apply, patch test in which the allergens are dissolved in a hydrophilic polymer vehicle that turns into a gel following contact with perspiration of the skin.(63)

In order to screen dermatitis patients for the most common and frequent contact allergens baseline series were developed, with variants for different continents and/or geographic regions. The most commonly applied in Europe is the European baseline series (EBS), consisting of contact allergens responsible for the most frequent contact allergies based on epidemiological data.(64) Inclusion in the EBS is warranted if a contact allergen shows more than 1% positive reactions in consecutively patch tested patients. A proposal for an update of the EBS was recently published, with inclusion of new allergens and exclusion of old allergens.(65) Besides the EBS country specific series have been developed as well (eg the Italian Group of Research in Environmental and Contact Dermatitis (GIRDCA) Series).(66) Most countries in Europe however opt to use the EBS supplemented with contact allergens relevant to exposures in that country. Besides these baseline series, additional series exist for patch testing in patients with specific (occupational) exposure to contact allergens not present in the EBS (or other baseline series). Examples of additional series containing a specific group of contact allergens include a cosmetic series, a fragrances series, and a corticosteroids series. Examples of additional series aimed at specific occupational exposures include a bakery series and a hairdressing series. Besides patch testing contact allergens at standardized concentrations, the clinician can also consider to patch test the patient's

own material to which he/she has developed a skin reaction. It must be noted that this requires a lot of experience, and testing 'as is' can lead to active sensitisation or a strong irritant reaction (false positive), so often the substance has to be diluted, with the risk of obtaining a false negative result.(58) Testing the separate ingredients of a product can be very useful in diagnosing ACD, assuming this information can be obtained through an ingredient list or a material safety data sheet (MSDS).

Patch test method and interpretation

The optimal patch test technique as recommended by the European Society of Contact Dermatitis (ESCD) is to have an occlusion time of 48 hours with visual readings performed at day 2, day 3 or 4, and day 7.(58) Some patch test centres prefer to use an occlusion time of 24 hours for practical reasons. Studies have shown that for some contact allergens, for example nickel, more positive reactions are seen with 48 hours occlusion as compared to 24 hours occlusion.(67) A longer occlusion time might increase the chance of active sensitisation however, but this is rarely reported, and concentrations of contact allergens in the vehicle are chosen thusly to minimize this complication (but allowing for the maximum amount of elicitation reactions). Readings are often performed on two occasions instead of the optimal three, again because of logistical and practical considerations. Not performing a day 7 reading risks missing late positive reactions for some allergens, leading to a lower diagnostic sensitivity.(68) As interpreting a patch test reaction is difficult and requires experience, in particular being able to differentiate between borderline positive reactions (termed doubtful) and irritant reactions, criteria were formulated by the ICDRG to facilitate interpretation and limit intra-observer variation (table 1).(58) Especially this latter issue is of large importance to increase validity of multicentre patch test studies (e.g. chapter 7).(69) The reading criteria are based on morphology of the skin reaction and it is important for an assessor to avoid interpreting a skin reaction by what is expected based on the patients exposure patterns.

Besides assessing the morphology of a patch test reaction and distinguishing between true allergic reactions and non-allergic reactions, the clinical relevance of a positive patch test reaction has to be assessed. As stated earlier, a contact allergy does not necessarily imply that the dermatitis is caused by it; there has to be current exposure to the contact allergen, and the dermatitis has to be explainable with regard to localisation and course of the dermatitis relative to the exposure.(70) A contact allergy that is not of current clinical relevance might be of previous clinical relevance, i.e. there

has only been exposure in the past but not anymore, or it might not be relevant at all; the result of limited exposure only leading to sensitisation or a cross-reaction.

Table 1: reading criteria of the ICDRG (58)

Symbol	Morphology	Assessment
-	No reaction	Negative reaction
?+	Faint erythema only	Doubtful reaction
+	Erythema, infiltration, possibly papules	Weak positive reaction
++	Erythema, infiltration, papules, vesicles	Strong positive reaction
+++	Intense erythema, infiltrate, coalescing vesicles	Extreme positive reaction
IR	Various morphologies, e.g. soap effect, bulla, necrosis	Irritant reaction

Additional diagnostic tests in detecting contact allergy

Patch testing is the golden standard in diagnosing a contact allergy, however, a few additional tests exist, to be viewed as complimenting patch testing rather than replacing it. The first is the repeated open application test, or ROAT, in which a test substance is applied twice daily to a small surface area of between 3x3 cm to 5x5 cm on the volar aspect of the forearm for about seven days, or longer if no dermatitis is seen yet but is expected to develop.(71) It is especially useful when patch-testing with a product or its ingredients is negative in spite of the suspicion that exposure to this product is the cause of the patient’s dermatitis. A scale for evaluating ROAT skin reaction was developed by Johansen *et al*, in which the area, erythema, infiltration, and number of vesicles are scored.(72) Besides the ROAT there are the semi-open test and open test, in which a small amount of product is applied to the skin and subsequently covered with permeable tape or left open, respectively.(58) The semi-open use test is suggested for products with a suspected irritant effect, and the open use test is useful to test whether it is safe to patch test a patient’s own material.

EPIDEMIOLOGY OF CONTACT ALLERGY

Contact allergy is a very common condition (or rather, immunological state), and in theory everybody is able to become sensitised to one or more contact allergens, if exposure is high enough.(73) Most epidemiological studies in the field of contact allergy provide us with prevalences of contact allergy overall and to specific contact

allergens; the number of people with a contact allergy divided by the total population tested. Other important epidemiological outcomes are incidence and cumulative incidence; the number of new cases over a specified period of time in a population, and the proportion of new cases of a fixed, non-diseased, population per specified unit of time. It is important for both outcome measures that the source population is well defined in regard to size, time period, method of patch testing (consecutive testing or aimed testing), whether all individuals are tested with the same allergens in the same concentration, etc. The majority of epidemiological studies in the field of contact allergy focus on prevalence rather than incidence. For a cumulative incidence to be investigated a specific population has to be patch tested twice over a period of time, in order to reveal how many new cases of contact allergy have been developed over that time. One such example is the Copenhagen study, in which 12% of a randomly selected population developed one or more contact allergies over a period of 8 years.⁽⁷⁴⁾ Epidemiological studies with a focus on prevalence are mostly based on data from patch test clinics, and serve as an important surveillance method of observing trends in contact allergy to specific contact allergens, whether it be contact allergens with established high sensitisation rates, or rising sensitisation rates to new contact allergens, for example the preservative methylchloroisothiazolinone/methylisothiazolinone (MI/MCI).⁽⁷⁵⁾ Another purpose in epidemiological studies is to determine whether there are specific risk factors, or specific groups with a high risk, for developing contact allergy. For example, as mentioned above, women and individuals of older age have a higher prevalence of contact allergies.⁽³⁴⁾ Most of the epidemiological data available on contact allergy are derived from research performed in patient populations, that is, patients suffering from dermatitis in which ACD is suspected. It is important to consider that these results cannot be extended to all dermatitis patients, as it more likely represents a subset of patients with more severe dermatitis, i.e. those referred to a dermatologist. More rare, and more difficult to perform, are prevalence studies focussing on contact allergy in the general population. Reported prevalences are often age- and sex-standardized in order to allow for better comparability between studies.⁽⁷⁶⁾ Specific characteristics of the population which can influence the reported prevalences are covered by the MOAHLFA-index, in which the proportions of the following patient demographics are provided: male sex, occupational cause of dermatitis, (a history of) atopic dermatitis, hand, leg, and face, respectively, as primary sites of dermatitis, and age above 40 years old.⁽⁷⁷⁾ For example, in a population with a high proportion of leg dermatitis, a higher prevalence of contact allergy to ingredients of topical medicaments can be expected, or, in a

population with a high proportion of occupational dermatitis, higher prevalences of contact allergy to occupational contact allergens (e.g. epoxy resins) can be expected. (31, 78) By reporting these characteristics the data can be correctly interpreted and correct comparisons between different studies is facilitated.

The prevalence of contact allergy is based on patch test results, and as with all diagnostic tests, patch testing carries the risk of false positives but more importantly the risk of false negatives.(79) Possible pitfalls of patch testing are important to keep in mind when interpreting the prevalence of contact allergy and ACD (see above). The majority of epidemiological studies concerning contact allergy are performed in Europe and North America. These results cannot be used to infer the prevalence of contact allergy overall and to specific contact allergens to other continents, as exposure to contact allergens differ between countries. Some examples of factors which influence the differences in exposure to contact allergens between countries are: products available on the consumer market, legislation which regulates presence and concentration of contact allergens in products, occupations with specific exposures (e.g. Batik industry in Indonesia), climate, culture (treatment of skin disease with natural products), etcetera.(80, 81)

As stated before, only a small fraction of all possible contact allergens are responsible for the majority of contact allergy. The allergen groups responsible for the majority of contact allergies are metals, fragrances, and preservatives.(82) The contact allergen with the highest reported sensitisation rate is nickel.(82, 83)

The most recent study investigating the prevalence of contact allergy in the general population in Europe (five countries participated: Sweden, the Netherlands, Germany, Italy and Portugal) found an overall contact allergy prevalence of about 27% (n=841/3119; 95% confidence interval 25.5–28.5) to one or more allergens from EBS. (83) A recent meta-analysis on contact allergy prevalence in the general population found that about 20% have a contact allergy.(84) Although this number appears alarmingly high, the majority are caused by metals (mostly nickel), preservatives, and fragrances, and a large majority are most likely not of any real significance, i.e. have not resulted in actual morbidity (ACD). It should also be considered that the rate of false positive reactions is higher in a population with a lower disease prevalence.(85) A strength of this study is that the population which was patch tested has a similar age and sex distribution as the total European Standard Population. As expected, differences in contact allergy to specific contact allergens were seen between the countries. A clear example is the lower sensitisation rate of nickel in the general population in Sweden compared to for example Portugal. This is in all likelihood the

result of legislation implemented in 1990/1991 in Denmark, Germany and Sweden, prohibiting the sale of products containing high levels of nickel.(86)

Epidemiological studies in the field of contact allergy are of the utmost importance, and the data that is collected serves as surveillance of contact allergy epidemics, the most illustrative example being the methylchloroisothiazolinone/methylisothiazolinone (MI/MCI) epidemic in the last decades.(75) New products are constantly introduced into the consumer market, and it is vital to remain vigilant as each new product carries the risk of causing contact allergy. Case reports are therefore also important in the contact allergy field, specifically for reporting new contact allergens or new exposures.

OUTLINE OF THIS THESIS

The field of contact allergy and ACD is constantly evolving and contains many facets, reflected in the outline of this thesis. The facets which were investigated were the immunology and genetics of contact allergy, the diagnostic patch test, and the epidemiology of contact allergy.

The first section concerns immunology and genetics of contact allergy. In **chapter 2**, attempts at finding a role of variants in *TNF*, specifically *TNF* – 308G>A in increased susceptibility to develop contact allergy either in general or to specific contact allergens, are reviewed. The role of TNF in the pathophysiology of contact allergy is explored to provide context, and suggestions for future studies are provided.

PPD is a common contact allergen, causing ACD in hair dyeing and henna tattoos, and by investigating the pathways in which it either is detoxified or causes sensitisation, prevention and/or safe alternatives can be explored. One such attempt at primary prevention of PPD contact allergy is the development of 2-methoxymethyl-*para*-phenylenediamine (ME-PPD). Cross-elicitation patterns to ME-PPD in PPD allergic patients is investigated in **chapter 3**, by performing open use testing, and the dose-response is investigated by patch testing with different concentrations.

The second section focusses on different aspects of patch testing; reliability and technique. **Chapter 4** investigates whether a positive patch test reaction is persistent upon repeated testing, exploring not only whether a contact allergy is a permanent state or a transient one, but also how patch test results can change over time depending on exogenous and endogenous factors. The influence of vehicle and patch test system on a patch test outcome is by comparing MCI/MI tested in aqua versus MCI/MI as tested in the TRUE test in **chapter 5**.

The third section is about the epidemiology of contact allergy, with **chapter 6** investigating prevalences of polysensitisation (≥ 3 positive patch test reactions to contact allergens in the EBS) throughout Europe based on data from the European Surveillance System on Contact Allergy (ESSCA). Furthermore, specific risk factors for becoming polysensitised are analysed. **Chapter 7** reports prevalences of contact allergy to hydroperoxides of limonene and/or linalool in consecutively patch tested patients in our centre, and characterizes this group compared to individuals allergic to other fragrances and non-allergic individuals.

Chapter 8 reports two case studies; the first presents a patient who developed ACD to his transcutaneous electrical nervous stimulation (TENS), the second presents two patients with allergic contact dermatitis to ethylenediamine, both working at the same ethylene amines producing factory.

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CHAPTER

2

Immunology and genetics of tumour necrosis factor in allergic contact dermatitis

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SUMMARY

During the sensitization phase of allergic contact dermatitis, the proinflammatory cytokine tumour necrosis factor (TNF) plays an important role by promoting epidermal Langerhans cell migration to draining lymph nodes. It also plays a role during the elicitation phase. The TNF gene (*TNF*) is located within the major histocompatibility complex region. Many single-nucleotide variants exist in the promoter region of *TNF*, and these may either increase or decrease RNA transcription and therefore lead to higher or lower levels of TNF. The most extensively studied single-nucleotide variant of *TNF* is a base pair substitution in the promoter region at location –308 relative to the transcription start site (rs1800629, *TNF* –308G>A), which is believed to increase transcription and lead to higher TNF levels. The role of TNF in allergic contact dermatitis and the functionality of *TNF* –308G>A are reviewed in this article. The association between genetic variants and disease can be studied in a case–control design. Only a few case–control studies investigating the association between *TNF* –308G>A and allergic contact dermatitis have been published, with contradictory results. These are reviewed critically, and suggestions for future case–control studies on this topic are made.

Allergic contact dermatitis (ACD) is a delayed-type hypersensitivity reaction caused by exposure of the skin to allergens (1). It starts with a sensitization phase, in which a person gains the potential to develop a cutaneous allergic reaction to a certain allergen, i.e., acquires contact allergy. Repeated exposure to this allergen (or a cross-reacting allergen) in a sensitized person causes inflammation of the skin. This is called the elicitation phase. The level of exposure, determined by dose and time of exposure, is the most important exogenous factor determining whether an individual becomes sensitized. However, a certain amount of interindividual variability exists in the susceptibility to develop ACD independently from exposure (2), some examples being sex, ethnicity, and age (3, 4). There may, however, also be a genetic basis for this variability in susceptibility, as has been suggested by family and twin studies (5–8). Knowledge of the genetic factors that lead to an increased susceptibility to develop ACD could help in identifying individuals at risk. Genetic factors for the development of ACD that have been investigated so far have previously been reviewed by Schnuch et al. and Friedmann et al. (9, 10)

A large part of genetic research is focused on ‘candidate genes’, that is, genes that encode specific proteins relevant to the disease pathophysiology (11). One such

important protein is tumour necrosis factor (TNF) (sometimes still referred to as tumour necrosis factor alpha, or TNF- α) (12). TNF is a proinflammatory cytokine with important roles in many inflammatory diseases, for example in psoriasis and rheumatoid arthritis (12). The role of TNF in ACD has been extensively investigated. The most important function seems to be to promote migration of the antigen-presenting Langerhans cells (LCs) to draining lymphnodes during the sensitization phase (13–15). TNF also has an important role during the elicitation phase of ACD (16). Genetic variants in the promoter region of the TNF gene (*TNF*) can lead to either an increase or a decrease in transcription of *TNF*, and can therefore influence TNF production (17). They can therefore theoretically lead to a higher or lower susceptibility to develop contact allergy and subsequent ACD. The role of these genetic variants of *TNF* in ACD has so far been studied in a few different clinical studies (18–24).

This review aims to elucidate the role of TNF in ACD, look at the different variants in the promoter region of *TNF* and their biological relevance, take a critical look at the clinical studies performed on *TNF* variants in ACD, and give suggestions for future research on variants in *TNF*.

THE ROLE OF TNF IN CONTACT HYPERSENSITIVITY

TNF is an important player in the development of contact hypersensitivity by inducing LC migration

One of the essential steps in the sensitization phase of ACD is considered to be the migration of hapten-carrying LCs from the epidermis to draining lymph nodes. LCs belong to the dendritic cell (DC) family, and are the main antigen-presenting cell residents in the epidermis. When they arrive in the paracortical area of the lymph node, LCs present the hapten to naive T lymphocytes. The T lymphocytes then differentiate into antigen-specific type 1 T helper (Th) cells/type 1 cytotoxic T (Tc) cells and Th17/Tc17 cells, and proliferate. More recent evidence suggests that dermal DCs are vital for hapten presentation to T cells and their differentiation into effector T cells, with LCs having a more tolerogenic role (discussed in more detail below) (25, 26).

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LC migration is initiated by altered expression of its surface adhesion molecules. This maturation process is induced by cytokines, among them TNF, released in the epidermis after penetration of a hapten into the epidermis. Experimental studies have shown the important role of TNF: intradermal injection of TNF induced DC accumulation in regional lymph nodes, and injection with anti-TNF inhibited LC migration (13–15). The following sections will examine the role of TNF in ACD more deeply, and discuss how TNF production in the epidermis is caused by exposure to haptens, how TNF induces LC migration, and the role of TNF in the elicitation phase.

TNF production and secretion occurs after activation of the innate immune system by the hapten

TNF secretion in the epidermis occurs early in the sensitization phase. Its secretion is a result of the activation of the innate immune system by haptens (27). Two concomitant pathways have been identified; one involving activation of the inflammasome, and

another involving activation of toll-like receptors (TLRs). These two pathways are illustrated in Fig. 1.

After a hapten penetrates into the epidermis, it induces keratinocytes to produce reactive oxygen species (ROS). One study showed that dose-dependent application of the chemical allergens 2,4,6-trinitrochlorobenzene (TNCB), 4-ethoxymethylene-2-phenyloxazol-5-one (oxazolone, or OXA) and mercaptobenzothiazole induced ROS production both in vitro and in vivo (28). ROS cause cellular damage, resulting in the release of extracellular ATP, which, in turn, activates the NOD-like receptor protein 3 (NLPR3) inflammasome. Inflammasomes are multiprotein complexes that are able to detect pathogens, irritants, and endogenous danger signals (29, 30). ATP can activate the NLPR3 inflammasome by binding to the transmembrane purinergic receptor P2X7, located on DCs (31). One study showed that nickel can directly activate the NLPR3 inflammasome independently of ATP, although ROS were still required (32). Activation of the NLPR3 inflammasome activates caspase-1, an enzyme that is responsible for the maturation of the cytokines pro-interleukin (IL)-1 β and pro-IL-18 (27, 31, 33). Keratinocytes produce both IL-1 β and IL-18, and LCs produce only IL-1 β (34, 35). Together, they activate keratinocytes to produce TNF and granulocyte-macrophage colony-stimulating factor (16, 36).

The second pathway involves the extracellular TLRs, which belong to the 'pattern recognition receptor' family. In the epidermis, they can be found on keratinocytes, DCs, fibroblasts, and mast cells. They are able to bind to pathogen-associated molecular patterns, for example lipopolysaccharide (LPS), a bacterial endotoxin, and provide a first line of defence against invading pathogenic microorganisms. Binding of ligands to TLR4 induces the production and secretion of Th1 and Th17 cytokines, among them TNF, IL-1 β , and IL-18 (37). In vivo experiments have shown the importance of TLRs in contact hypersensitivity (CHS) the mouse model of human ACD. One study showed that mice lacking both TLR2 and TLR4 did not develop CHS after repeated application of TNCB (38). The role of LPS as a ligand was investigated in germ-free mice (lacking a microbial skin flora) (38). These mice were still able to develop CHS. This led to the notion that the binding of endogenous ligands to TLR2 and TLR4 plays a vital role in the development of CHS. Both TLR2 and TLR4 are capable of binding to endogenous ligands, known as damage-associated molecular patterns (DAMPs). In CHS, both oxidative degradation (through ROS) and enzymatic degradation of hyaluronic acid (HA; also known as hyaluronan) contribute to the production of HA fragments (28, 39). HA fragments are proinflammatory molecules, and can act as ligands for TLR2 and TLR4 (40), causing maturation of DCs (41, 42). TNF secreted by keratinocytes

is required for HA fragment-induced maturation of DCs (41). The importance of HA fragments and TLR4 in DC migration, and in CHS overall, was further shown in mice overexpressing human hyaluronidase 1 (encoded by *HYAL1*), which showed increased HA degradation (43). Other examples of DAMPs that are TLR4 ligands in CHS are fibronectin extra type III domain A, heparan sulfate, fibrinogen, and heat shock proteins (44). Besides activation of TLR4 by binding with DAMPs, it has been shown that both nickel and cobalt can bind directly to human, but not to mouse, TLR4 (45, 46).

Figure 1.

Activation of the innate immune system after a hapten penetrates the skin. After haptens penetrate the epidermis, they, besides binding to Langerhans cells, activate the innate immune system through a series of steps. First, keratinocytes are induced to produce reactive oxygen species (ROS), which cause cellular damage that leads to the formation of damage-associated molecular patterns (DAMPs) and the release of ATP. ATP activates the NOD-like receptor protein 3 inflammasome by binding to, for example, the transmembrane purinergic receptor P2X₇ on dendritic cells. The activated inflammasome causes caspase-1 activity. Caspase-1 is responsible for the maturation of pro-interleukin (IL)-1 β and pro-IL-18, and subsequently the cytokines IL-1 β and IL-18 are released. At the same time, DAMPs, among them hyaluronic acid (HA) fragments, bind to toll-like receptors (TLR) 2 and 4, on either keratinocytes (as pictured here) or dendritic cells. Nickel and cobalt can bind directly to TLR4. Activation of TLR2 and TLR4 leads to secretion of IL-1 β , IL-18, and tumour necrosis factor (TNF). IL-1 β and IL-18 stimulate keratinocytes to produce more TNF.

Besides keratinocytes (the main producers of TNF in the epidermis), more recent evidence suggests that epidermal LCs may also be responsible for increased TNF secretion in CHS (47, 48). Experimental studies showed that CD34⁺ DCs (progenitors of both DCs and LCs) produced TNF when stimulated with certain chemicals (49–51). For example, nickel, oxazolone and hexyl cinnamal increased TNF production in CD34⁺ DCs (49, 50). Conversely, eugenol and 2,4,6-trinitrobenzene sulfonic acid decreased TNF production (50). Besides being hapten-dependent, TNF production by CD34⁺ DCs also showed individual variation (50). There is also evidence that mast cell-derived TNF plays a role in CD8⁺ DC maturation and migration in CHS (52).

Binding of TNF to TNF-R2 induces maturation of LCs and subsequent migration to draining lymph nodes

There are two TNF receptors: TNF-R1 (or p55TNFR) and TNF-R2 (or p75TNFR). LCs in the skin express TNF-R2 but not TNF-R1 (53). In accordance with this, one group showed that LC migration was normal in TNF-R1 knockout mice when they were challenged with fluorescein isothiocyanate (FITC), but abnormal in TNF-R2 knockout mice when they were injected with TNF (54, 55). When TNF-R2 knockout mice were challenged with oxazolone, they showed a decreased CHS response (54). Another study showed that TNF-R1 also plays an important role in CHS, as TNF-R1-deficient mice did not develop CHS in response to TNCB (56). Further investigation showed that TNF-R1 did not influence DC migration, but increased antigen uptake by DCs (56).

TNF influences LC migration in various ways through binding to TNF-R2. It induces a change in the expression of surface molecules of LCs in the maturation process. Among these changes are: decreased E-cadherin surface expression, and increased expression of CD44, major histocompatibility complex (MHC) II, intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and chemokine receptor type 7 (CCR7) (57–60). LCs are positioned in the suprabasal layer of the epidermis, and are bound to the extracellular matrix and keratinocytes through E-cadherin. A decrease in E-cadherin surface expression therefore leads to separation of the LCs (60). CD44 plays an important role in the interaction between LCs and T lymphocytes (61, 62). CCR7-deficient mice show impaired LC migration upon skin painting with FITC (63). One study showed that, although CCR7 expression on maturing LCs is strongly dependent on TNF when they are stimulated with DNCB, it is mostly independent of TNF when they are stimulated with NiSO₄ (64). One study showed that DC stimulation with TNF caused large changes in the actin-based cytoskeleton and highly increased motility (65). In order to reach the lymphatic vessels in the dermis, LCs have to move

through the basal membrane. TNF induces LCs to secrete matrix metalloproteinase 9 (also known as gelatinase), which helps to degrade the basal membrane (66).

The role of TNF in the elicitation phase

The elicitation phase of ACD consists of the migration of circulating primed memory/effector T lymphocytes into the (epi)dermis upon re-exposure to an allergen. The T cells are presented with the hapten by local DCs, and are subsequently reactivated and release proinflammatory cytokines, among them TNF. In order to show the relevance of TNF in the elicitation phase, one group treated sensitized mice with systemic anti-TNF antibodies immediately before challenge with TNCB; the CHS response was reduced significantly (16). Similarly, the CHS response decreased significantly in TNF^{-/-} mice (although it was not absent), and an enhancing role of TNF was postulated (67).

Besides having a cytotoxic effect, TNF has other ways of influencing the elicitation phase. TNF causes increased expression of the adhesion molecules ICAM-1 and VCAM-1, and endothelial leukocyte adhesion molecule (also known as E-selectin), on endothelial cells, increased expression of ICAM-1 and VCAM-1 on DCs, and increased expression of ICAM-1 on keratinocytes (27, 68). These adhesion molecules help the recruitment of circulating effector/memory T lymphocytes into the epidermis. The expression of vascular endothelial growth factor, whose action contributes to oedema by way of increased vascular permeability, was shown to be reduced in TNF^{-/-} mice as compared with wild-type mice (67). TNF also induces IL-33 production in keratinocytes. Blocking IL-33 in mice diminished the CHS response, showing its role in the elicitation phase (69).

Recent discoveries that challenge this model of CHS

Although ample evidence exists to support the role of TNF in inducing LC maturation and migration, more recent evidence suggests that certain allergens, for example 2,4-dinitrochlorobenzene and 2,4-dinitrofluorobenzene (DNFB), can induce LC migration independently of TNF (70). Other investigated allergens, such as oxazolone and *p*-phenylenediamine (PPD), did require TNF, however (70).

The essential role of LCs in CHS was also questioned after studies performed in LC-deficient mice. These studies showed both increased and decreased CHS responses in the absence of LCs (71–73). Despite this absence, the mice were still able to be sensitized, suggesting that dermal DCs are able to mediate a CHS response. The differences in CHS responses seen in these studies performed on LC-deficient mice might be explained by the use of different allergens. Decreased CHS responses were

seen in response to TNCB and oxazolone, whereas increased CHS responses were seen in response to DNFB, FITC, and oxazolone (71–73).

One study suggested a tolerogenic role for LCs through their secretion of IL-10 after interaction with CD4⁺ T cells (72). IL-10 influences LC migration negatively, inhibits effector/memory T cells, and induces the development of regulatory T (Treg) cells (74, 75). One group aimed to clarify this perhaps paradoxical role of LCs, in that they appear both to contribute to, and to counteract, CHS responses. It showed that, at steady state, human skin LCs induce the proliferation of skin-resident Treg cells, thereby mediating tolerance to endogenous antigens (76). However, when LCs were subjected *in vitro* to *Candida albicans*, an increase in the number of antigen-specific memory/effector T cells was seen (76). Another recent study showed how LC migration in response to contact with a weak allergen (2,4-dinitrothiocyanobenzene) led to tolerization *in vivo*. LCs were able to present the hapten to both CD8⁺ and CD4⁺ Foxp3⁺ Tregs, leading to deletion or anergy of the former and activation of the latter (25). All in all, the role of LCs in CHS remains controversial (26).

THE SINGLE-NUCLEOTIDE VARIANTS (SNVS) IN THE PROMOTER REGION OF *TNF*

The various SNVs in the promoter region of *TNF*

TNF is located on chromosome 6p21.3, in the MHC III region, situated between the MHC I and MHC II regions, which encode the human leukocyte antigen (HLA) surface molecules (17). Many SNVs¹ of varying importance have been identified in the promoter region of *TNF* (17). SNVs in the promoter region can lead to either an increase or a decrease in *TNF* mRNA production and TNF release by different cell types (17, 78). Interestingly, one study investigated the genetic influence on individual variation in cytokine production, and found this to be ~60% for TNF (79).

The SNV in the *TNF* promoter region at location –308 relative to the transcription start site (officially called rs1800629, but also referred to as –308G>A, 308.2, or *TNF2*), in which a guanine (G) is substituted by an adenine (A), was the first SNV to be discovered (80). Many others have since been found, including SNVs at the locations –1031, –863, –857, –575, –376, –238, –163, and –49 relative to the transcription start site (80–82).

1 We have attempted to adhere to the most currently used genetic terminology and nomenclature in this review. Therefore, the term 'single nucleotide variant' or 'SNV' is used in this article, instead of single nucleotide polymorphism or 'SNP'. An up-to-date source for genetic nomenclature can be accessed at: <http://varnomen.hgvs.org/> (last accessed on 30-09-2016) (77).

The SNVs at locations –308 and –238 (rs361525) are the most thoroughly investigated, concerning both function (or pathogenic effects) and their relevance in different diseases. Case–control studies of TNF promoter variants in ACD have focused almost solely on the SNV at the –308 location, which we will refer to as *TNF* –308G>A (18–23).

Is *TNF* –308G>A functional?

Whether or not *TNF* –308G>A has a functional effect has been investigated extensively. Investigations have focused on differences in transcriptional activity and/or TNF production. These studies have been previously reviewed by different groups, and these have, surprisingly, come to conflicting conclusions (78, 83, 84). We will discuss the different types of studies performed on this topic and the reasons why they might have come to contradictory conclusions.

Promoter activity can be measured in vitro by using reporter gene constructs. In this process, the promoter region of interest is attached to a reporter gene, so that it controls the expression of the reporter gene (85). Examples of reporter genes are those encoding chloramphenicol acetyltransferase and luciferase (86, 87). Luciferase is a firefly enzyme that generates light. The expression of the luciferase gene can therefore be quantified by measuring emitted light with a luminometer. By measurement of this light, the influence of SNVs on activity of the promoter region can be investigated. Transcription can be artificially amplified by challenging the reporter gene construct with a stimulant, for example phorbol myristate acetate, LPS, or TNF itself. Another in vitro method is to measure *TNF* mRNA with an enzyme-linked immunosorbent assay. There are approximately as many in vitro gene reporter construct studies that report significantly increased transcription activity of *TNF* –308G>A as there are that do not show any significant increase or decrease (81, 88–98). A possible explanation for these discrepancies is that different methodologies are used. Some examples of factors that can influence the results of reporter gene assays investigating *TNF* –308G>A are the amount of DNA used, the amount of transfection control vector used, and the method of transfection (98). One group developed a new reporter system that circumvents these influences, and the results suggested that *TNF* –308G>A is functional (98). Another reason for diverging results might be that different cell lines are used to investigate the functionality of *TNF* –308G>A. The first study investigating the functionality of *TNF* –308G>A found a sixfold to sevenfold increase in transcription activity in Raji cells (B lymphocytes) that carry the SNV as compared with the wild type (88). Since then, studies investigating the effect of *TNF* –308G>A in Raji cells, Jurkat cells (T lymphocytes) and U937 cells (pre-monocytes) have found both increased

transcription activity and no change in transcription activity. This suggests that the functionality of this variant is perhaps more dependent on the context than on cell type. The choice of stimulant used to amplify transcription can also influence the results.

A second method of investigating the functionality of the *TNF* promoter region variants is to compare differences in TNF production between the different alleles/genotypes *ex vivo* or *in vivo* (79, 99–111). These studies have focused on both healthy populations and populations with specific illnesses, for example diabetes (99), multiple sclerosis (101), and sepsis (104, 105, 109). These studies also produced varying results, again most likely because of the varying methods that were used. Which type of study is able to draw more meaningful conclusions about whether *TNF* –308G>A is functional or not; *in vitro* or *in vivo* studies? One group found that *in vitro* quantification of mRNA was more accurate and sensitive than *in vivo* protein measurement (112). However, if a variant is shown to be functional *in vitro*, this does not necessarily mean that it will be functional *in vivo* (113), as expression of a gene *in vivo* might also be dependent on the tissue type, environmental context, and the genetic background of an individual.

Inheritance of variants in TNF

Different variants in the *TNF* promoter region are not inherited independently from each other, or independently of the rest of the MHC complex. Certain alleles within the MHC complex are inherited together as a haplotype. One example of such a haplotype is HLA-A1/HLA-B8/HLA-DR3 (114). An important concept in genetics is linkage disequilibrium. This is the association between a variant and a nearby marker at a population level (115). Alleles that are in close proximity to one another are more likely to be inherited together through linkage disequilibrium. As *TNF* is situated within the MHC III region, alleles in the promoter region are also associated with certain alleles in the MHC I and MHC II regions. The aforementioned haplotype HLA-A1/HLA-B8/HLA-DR3 has been found to be associated with *TNF* –308G>A (114). Certain HLA alleles, for example HLA-DR1, HLA-DR3, and HLA-DR4, have been found to be associated with increased TNF secretion by monocytes. Others, however, for example HLA-DR2 and HLA-DR5, have been found to be associated with lower TNF secretion (99, 116–118). These findings might be explained by the presence or absence of linkage disequilibrium of HLA alleles with *TNF* –308G>A. Another option is that *TNF* –308G>A is a marker for increased TNF production through linkage disequilibrium with an as yet unidentified functional SNV. It is, however, important to keep in mind that linkage disequilibrium

is not always necessarily complete; certain alleles might be inherited together often, but not always (119).

The SNVs that have been identified so far in the promoter region of *TNF* have also been found to be associated with each other, to varying degrees. There are, however, differences between different ethnicities in how frequent the SNVs are and how strong the linkage between them is. One study found that *TNF* –238G>A is often found together with *TNF* –376G>A in people from Kenya, but not in people from Gambia (120). The same association has been found in Caucasians from New England, where the linkage was complete (121). A Dutch study looked at different combinations of *TNF* –238 and *TNF* –308 (together with two alleles in the lymphotoxin α gene) in the Dutch population, and found five different possible haplotypes. In this population, the combination of *TNF* –238G>A and *TNF* –308G>A did not occur (122). Because of these differences between different ethnic groups, it is important to focus on a well-defined homogeneous population when performing genetic association studies, in order to avoid confounding (123).

Investigating the relevance of *TNF* –308A in disease

An appropriate study design for investigating the relevance of an SNV in a specific disease is the case–control design. Some of the requirements for this study design are that the variant under investigation has to have a functional effect, and that the protein produced by its gene plays an important role in the pathophysiology of the disease. In other words, *TNF* –308A carriers have to have a significantly higher level of TNF at the site of the disease than *TNF* –308G carriers. The SNV also has to be functional in the cell type responsible for TNF secretion in the disease under investigation. Differences can be assessed at either the allele level (comparing –308G carriers with –308A carriers), or at the genotype level, for example by comparing *TNF* –308A/A homozygotes with *TNF* –308G/A and *TNF* –308G/G carriers.

Case–control Studies of *TNF* –308G>A in ACD

There are, to the best of our knowledge, seven studies that have investigated *TNF* –308G>A in ACD (18-24). In the following section, we will take a critical look at these studies to learn from them and gain insights in how to improve future research on this topic. General limitations of these studies are then discussed. Table 1 gives an overview of the methodology of these seven studies, and Tables 2 and 3 show genotype and allele frequencies, respectively.

Table 1. Overview of methodology of studies investigating the *TNF*-308G>A polymorphism in allergic contact dermatitis

Study	Year	Study design	Ethnic group	Patch testing	Contact allergen	description controls	Genotyping method
Westphal <i>et al.</i> ¹²⁶	2003	Case-control	(German) "Caucasian patients"	"standard series"	Para-substituted aryl compounds	Recruited from blood donor registry	PCR-RFLP
Wang <i>et al.</i> ¹²⁷	2007	Cross-sectional	"Taiwanese construction workers"	European Standard Tray ¹ and construction-related substances ²	Potassium dichromate	Cement workers	PCR-RFLP
Blömeke <i>et al.</i> ⁴⁷	2009	Case-control	"Caucasian individuals from Germany and the Netherlands"	No information provided	<i>p</i> -phenylenediamine	"unrelated Caucasians with no history of sensitization to PPD or ACD"	PCR-hybridization probes
Ertam <i>et al.</i> ¹²⁸	2009	Case-control	"Turkish patients"	TRUE test ³	Allergens from the TRUE test	No information provided	ASPCR
Khatr <i>et al.</i> ¹²⁹	2014	Case-control	Indian population*	Indian Standard Series ⁴	Parthenium hysterophorus	"Healthy individuals" recruited from different departments at the All India Institute of Medical sciences"	ARMS-PCR

Table 1. Continued

Study	Year	Study design	Ethnic group	Patch testing	Contact allergen	description controls	Genotyping method
Westphal et al.(2) ¹³⁰	2016	Case-control	(German) "white patients"	"standard series" "cosmetic biocide series"	Allergens from standard series or he cosmetic biocide series	"Healthy unrelated Caucasian blood bank donors"	Real-time PCR
Colagiovanni et al. ¹³¹	2016	Case-control	Not mentioned	"European Standard Series"	Nickel	"Healthy controls" "selected from the Division of Clinical Nutrition and Nutrigenomics"	PCR

ARMS-PCR , amplification refractory mutation system-polymerase chain reaction; ASPCR, allelic-specific polymerase chain reaction; ACD, allergic contact dermatitis; PPD, Para-phenylenediamine; PCR-RFLP, polymerase chain reaction assay with specific fluorescence-labeled hybridization probes; TRUE test, Thin-Layer Rapid Use Epicutaneous test.

¹ Chemotechnique Diagnostic AB, Vellinge City, Sweden

² Trolab, Montreal City, Canada

³ Mekos Laboratoriet, Hillerod, Denmark

⁴ Performed by using Van der Bend square chambers, includes standardized aqueous extract of *Parthenium hysterophorus*

* Information not provided by article, but reasonable assumption

Table 2. Overview of the *TNF* -308 genotype frequencies in the case-control studies

Study	Number of individuals, n		ACD group, n (%)			Control group, n (%)		
	Case	Control	GG	GA	AA	GG	GA	AA
<i>Westphal et al.</i> ¹²⁶	86	310	57 (66.3)	26 (30.2)	3 (3.5)	226 (72.9)	84 (27.1)	0 (0.0)
<i>Wang et al.</i> ^{127 a}	17	130	11 (64.7)	6 (35.3)	0 (0.0)	113 (86.9)	17 (13.1)	0 (0.0)
<i>Blömeke et al.</i> ⁴⁷	181	161	110 (61)	62 (34)	9 (5)	124 (77)	34 (21)	3 (2)
<i>Ertam et al.</i> ¹²⁸	50	100	34 (68)	14 (28)	2 (4)	31 (31)	54 (54)	15 (15)
<i>Khatiri et al.</i> ¹²⁹	60	60	43 (71.7)	13 (21.7)	4 (6.6)	44 (73.3)	16 (26.7)	0 (0.0)
<i>Westphal et al.</i> ^{(2) 130 b}	239	345	161 (67.4)	69 (28.8)	9 (3.8)	232 (67.3)	104 (30.1)	9 (2.6)
<i>Colagiovanni et al.</i> ¹³¹	41	40	35 (85.4)	5 (12.2)	1 (2.4)	30 (75%)	(25%)	0 (0.0)

^aDue to the cross-sectional study design, genotype frequencies were not provided in the original article; the numbers shown in this table are extracted from the data provided in the original article.

^bCases are polysensitized individuals.

Table 3. Overview of the allele frequencies in the case-control studies

Study	Cases		Control	
	A%	G%	A%	G%
Westphal <i>et al.</i> (1) ¹²⁶ *	18.6	81.4	13.55 ^a	86.45 ^a
Wang <i>et al.</i> ¹²⁷ *	17.65	82.35	7.82	92.17
Blömeke <i>et al.</i> ⁴⁷	22.1	77.9	12.42	87.58
Ertam <i>et al.</i> ¹²⁸	18	82	42	58
Khatrri <i>et al.</i> ¹²⁹	17.5	82.5	13.33	86.67
Westphal <i>et al.</i> (2) ¹³⁰	18.2	81.8	17.7	82.3
Colagiovanni <i>et al.</i> ¹³¹ *	8.5	91.5	12.5	87.5

*Allele frequencies not mentioned in original article, therefore, allele frequencies were calculated using the genotype frequencies with the Hardy-Weinberg principle: $p^2 + 2pq + q^2 = 1$ (and $p + q = 1$), where $p = G$ and $q = A$, and $p^2 = GG$, $2pq = GA$, and $q^2 = AA$.

^a Genotype frequencies within this control group deviated from the Hardy-Weinberg equilibrium and are therefore not representative of the whole population.

Westphal *et al.* ¹ (18)

This was the first case–control study to focus on genetic variants in ACD. Besides investigating genotype frequencies of *TNF*–308G>A and *TNF*–238G>A, the study also investigated genotype frequencies of variants in the genes encoding IL-1 β , IL-1 receptor antagonist, and IL-6. Cases were subdivided into monosensitized and polysensitized individuals. Monosensitization was defined as having a positive patch test (PT) reaction to a p-substituted aryl compound (e.g. PPD), and polysensitization as having at least one additional positive PT reaction to a chemically unrelated allergen. Patch testing was performed with a ‘standard series’. Controls were subdivided into individuals with and without a history of eczema. No patch testing was performed in controls. The difference in *TNF*–308G>A genotype distribution between polysensitized cases and controls without eczema was significant ($p = 0.038$), but no significant differences were found in any of the other investigated variants, including *TNF*–238G>A.

The main strength of this study is that it investigated well-defined phenotypes. Polysensitized patients were compared with healthy controls without eczema, and these phenotypes represent subjects with high and low susceptibility, respectively (124). One limitation is that, in the control group, the *TNF*–308G>A genotype frequencies were not within the Hardy–Weinberg equilibrium, implying that the frequencies of the alleles are not in concordance with those in the general population, and are therefore not fully representative.

Wang et al. (19)

In contrast to the other studies discussed here, this cross-sectional study investigated risk factors for the development of ACD in response to chromate in Taiwanese cement workers. Variants of the *TNF*–308 allele and in the glutathione-S-transferase (GST)-M1 and GST-T1 genes were analysed as risk factors. All subjects underwent patch testing with the European baseline series and construction-related substances. Carrying the *TNF*–308G/A genotype was found to be a significant risk factor for having a positive PT reaction to potassium dichromate [odds ratio (OR) 3.9, CI: 1.14–13.2]. None of the subjects carried the *TNF*–308A/A genotype.

Although this was not a case–control study, and therefore lacked a well-defined control group for comparison, a major strength of this study is that exposure to cement, and therefore to chromate, was equal in all subjects. Because of this, any difference between subjects with a positive PT reaction to chromate and those without can be explained by factors other than exposure, for example by variants in specific alleles. Besides those with allergy to chromate, there were also subjects with positive PT reactions to, for example, ammoniated mercury and nickel sulfate. It is unclear why *TNF* –308G>A was only assessed as a risk factor in chromate-allergic subjects, or what proportion of the chromate allergic-group also had positive PT reactions to other allergens.

Blömeke et al. (20)

This case–control study investigating the association between *TNF* –308G>A and ACD compared patients with a history of ACD and a positive PT reaction to PPD with age-matched and sex-matched controls. It is unclear whether a patient's history of ACD had to be related to PPD exposure. The *TNF* –308A allele was significantly more frequent in cases than in controls (Fisher's two-sided exact test, $p = 0.0016$; logistic regression, $p = 0.0017$), in agreement with the results found by Westphal et al. (18). Analysis of subgroups showed that the association was even stronger in females aged >45 years.

In contrast to the study by Westphal et al., this study did not subcategorize monosensitized and polysensitized subjects, owing to the small number of polysensitized subjects. In comparison, the majority of the population studied by Westphal et al. were polysensitized. Controls had no history of ACD or sensitization to PPD, although it is unclear whether this implies that controls underwent patch testing or not.

Ertam et al. (21)

This Turkish case–control study looked at the association between *TNF* –308G>A and IL-1RA variants and ACD. Cases were defined as patients with ACD with a positive PT reaction to one or more allergens in TRUE Test®. More than half of the patients had a positive PT reaction to nickel sulphate. Controls consisted of individuals without a history of eczema and atopy. Interestingly, the frequency of the *TNF* –308G/G genotype was significantly higher in cases than in controls, which is in contradiction to the results found in the previous studies. The proportion of *TNF*–308A/A homozygotes in the control group (15%) is remarkable.

These results might be explained by the different genotype frequencies found in different ethnicities, although the reverse association of the A allele with ACD is still in contradiction to the current theory that the *TNF* –308G>A variant is functional and increases transcription of *TNF* mRNA. If *TNF* –308G>A is only a marker in linkage with the actual disease-causing gene, then the results can be explained by the study population being of a different ethnicity than in previous studies.

Khatri et al. (22)

A case–control study from India investigated the association between *TNF*–308G>A and airborne ACD to the plant *Parthenium hysterophorus*, in which the hapten parthenin, a sesquiterpene lactone, is the relevant allergen (125). The *P. hysterophorus* plant is common in India and Australia. Cases were defined as patients with ACD caused by *P. hysterophorus*, proven by a positive PT reaction. Cases were only included if they did not have any respiratory allergy and did not receive immunosuppressive drugs. Controls were healthy individuals, not further described. Perhaps surprisingly, no significant difference in genotype frequency between cases and controls was found. This might again be attributable to the difference in ethnicity studied as compared with previous studies in which a significant association with the *TNF*–308A allele was found. Westphal et al.(2) (23)

The second case–control study by the group of Westphal et al. focused on multiple genetic variants in ACD, investigating a total of 15 variants on 13 genes. For the *TNF* gene, only the SNV at location –308 was investigated. Again, cases were subdivided into monosensitized and polysensitized individuals, but these were defined differently; monosensitization was defined as having one or two positive PT reactions, and polysensitization as having positive PT reactions to three or more unrelated allergens. Patch testing was performed with a baseline series and a cosmetic preservatives series. Within the polysensitized group, a ‘high-risk’ subgroup was

selected, which included patients with at least one strong or extremely positive (++ or +++) PT reaction. There was no significant difference in *TNF*–308 genotype distribution between either polysensitized or the ‘high-risk’ polysensitized cases and controls. The only significantly more frequent variant was the homozygous A/A variant of CXCL11, which encodes a chemokine ligand, although this difference was no longer significant after correction for multiple statistical testing.

Colagiovanni et al. (24)

The most recent study investigated the association between *TNF*–308G>A and ACD caused by nickel in 41 cases compared to 40 controls. Patch testing was performed with the European baseline series, and both cases and controls were limited to females aged between 18 and 40 years. Patients with severe chronic disease (including autoimmune diseases) were excluded. There was no significant difference in genotype distribution, except in a small subgroup of polysensitized patients ($n = 4$, with 3 carrying a variant). Polysensitization was, however, not defined, and the 1 patient carrying the rare *TNF*–308A/A genotype had positive PT reactions to nickel, cobalt, and chrome. These positive reactions are more likely to be attributable to either co-exposure or cross-elicitation than to increased susceptibility.

Discussion of the genetic association studies in ACD

Although the studies described above had varying strengths in their design, there were some (common) limitations. One major limitation was that in none of the case–control studies patch testing was performed in the control group, although this might have been done in the study by Blömeke et al. Because of this, subjects with one or more undiagnosed contact allergies might have been included in the control group. Additionally, subjects in the control group might have been highly susceptible to develop sensitization but did not because of low exposure to allergens. None of the case–control studies attempted to assess the level of exposure in its population, because of the difficulty of this task. Exposure assessment would have increased the validity of the studies. Ideally, the level of exposure should be equal in both cases and controls. In general, description and selection of controls was lacking, with only a few inclusion or exclusion criteria being described. Meta-analyses show that the frequency of *TNF*–308G>A is increased in many autoimmune diseases (126, 127). Therefore, subjects with concomitant autoimmune diseases should be excluded, as this could be a confounding factor, and, in most studies, it was unclear whether these patients were excluded or not.

Of the seven studies performed on *TNF* variants in ACD so far, three studies found an association between ACD and *TNF*-308G>A, three found no association, and one found an inverse association. The studies of Westphal et al.(1) and Blömeke et al. are the most similar in ethnicity studied and the allergen to which the cases were allergic to, and both found a positive association (18, 20). However, the second study by Westphal et al., which contained the largest cohort, found no association. Because of these varying results, no definitive conclusion can yet be drawn on whether *TNF*-308G>A is associated with increased susceptibility to develop ACD. Non-replication is a common phenomenon in genetic association studies, and can often be explained by reasons such as small sample sizes, genotyping errors, and population stratification, among others (123, 128).

FUTURE RESEARCH ON *TNF* VARIANTS IN ACD

Basic requirements for a good case–control study

Because of the large number of case–control studies with varying methodological weaknesses, the editors of Nature Genetics wrote an editorial about the basic requirements for genetic association studies (129). These include: large sample sizes, small p-values, high risk/ORs, associations that are biologically plausible, investigating alleles that have a relevant physiological function, initial studies accompanied by an independent replication study, and the association being reproducible in both population and family studies. Sample size is one of the most important determinants of quality in case–control studies investigating associations between genotype and disease. Hattersley and McCarthy calculated required sample sizes according to the frequency of the susceptibility allele in controls, and the desired power or OR (128). The frequency of the *TNF*-308A allele in the HapMap CEU population (Utah residents with northern and western European ancestry from the CEPH collection) is ~17.56%, which is approximately the same percentage as was found in the control group in the study by Westphal et al. (2, 23, 130). Hattersley and McCarthy calculated that, for an allele frequency of ~10% in controls, 3066 subjects are needed (or 1533 in each group) to achieve a power of 90% to obtain a p-value of 0.05 with an allelic OR of 1.3. For an allele frequency of 20%, the required sample size would be 1772 (or 886 subjects per group). To obtain smaller p-values, the sample size required increases even further. The genetic association studies with a focus on ACD performed so far all had relatively small sample sizes.

Phenotype definition

Definition of the phenotype under investigation is another important factor for the quality of a case–control study. Here, we give a suggestion of which phenotype to investigate. There are several different types of selective case–control studies, which differ in the selection of cases and controls (131). One type of selective case–control study is that of extreme discordance, and is especially suited to investigate an association between a specific allele or genotype and disease. In this type, ‘severely affected’ cases are compared with ‘extremely normal’ controls. One suggestion for defining ‘severely affected’ cases among patients with ACD would be polysensitization. The subject of polysensitization as a model of increased susceptibility has been reviewed by Schnuch et al. (124). Polysensitization is defined as being sensitized to more than one allergen. We propose that cases should be polysensitized, with at least three or more positive PT reaction to unrelated allergens in, for example, the European baseline series (132). For controls, we would propose healthy subjects with no history of eczema and negative PT results. Depending on which genes are being investigated, certain concomitant autoimmune diseases should be considered to constitute an exclusion criterion in both groups. Whether these phenotypes are feasible when one is trying to obtain a large sample size might be an issue, and there is no definitive answer to what is better: a small sample size of phenotypically homogeneous cases, or a large sample size of less well-characterized cases (128). A solution to increase statistical power could be to perform a meta-analysis of multiple studies that have been performed; however, these would need to be sufficiently homogeneous, for example similar phenotypes measured with the same criteria. Current studies differ too much with respect to the investigated ethnicity, the allergens to which the cases are allergic to, and possibly the levels of exposure, for a meta-analysis to be sensibly performed.

Which variant(s) to focus on

It is important that the variant under investigation be both functional and biologically relevant for the studied disease. The main cells believed to be responsible for TNF production and secretion in ACD are keratinocytes, together with epidermal LCs and mast cells (47–52). Studies investigating whether *TNF* –308G>A is functional have looked at pre-monocytes, T lymphocytes, and B lymphocytes, and have obtained conflicting results. It would appear to be important to investigate whether or not this variant is truly functional in keratinocytes and LCs, or in DCs in general.

All studies investigating genetic variants of TNF in ACD focused solely on *TNF*–308G>A, except for the study by Westphal et al.(1), which also looked at *TNF*–238G>A (18). This is most likely because *TNF*–308G>A is the most extensively investigated variant in the promoter region of TNF. It has the most theoretical support for being functional, as it is located within a binding site for a transcription factor (133). Another option would be to focus on extended haplotypes, including multiple variants in the TNF promoter region, as seen in the study by Bouma et al. (122). However, an important drawback of this approach is that it causes an increase in degrees of freedom with an increasing number of haplotypes, leading to loss of power (115).

CONCLUSION

TNF plays an important role in the sensitization phase of ACD by inducing LC maturation and subsequent migration to draining lymph nodes. It also plays a role in the elicitation phase by exerting a cytotoxic effect and by promoting migration of circulating primed lymphocytes to the skin. There are many SNVs in the promoter region of TNF, of which some are believed to be functional. The most extensively investigated SNV is *TNF*–308G>A. There is no general consensus yet on whether it is functional, a marker for increased TNF production through genetic linkage disequilibrium, or neither. Despite this fact, it has been the focus of many case–control studies in many different diseases and conditions. Seven studies have investigated the association between *TNF*–308G>A and ACD, with varying results. For future studies on this subject, a consensus should be reached on which phenotype to investigate, in order to enable the performance of future meta-analyses, so meaningful conclusions can be drawn. We suggest that polysensitized subjects are compared with healthy controls with preferably comparable exposure, although the feasibility of such a design in terms of sample size remains an issue.

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CHAPTER

3

Cross-elicitation responses to 2-methoxymethyl-*p*- phenylenediamine in *p*- phenylenediamine-allergic individuals: Results from open use testing and diagnostic patch testing

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ABSTRACT

Background

Allergic contact dermatitis caused by *p*-phenylenediamine (PPD) is a health concern for hair dye users. Because of its lower sensitization potency, the PPD derivative 2-methoxymethyl-*p*-phenylenediamine (ME-PPD) has been developed as an alternative hair dye for primary prevention. However, cross-elicitation responses can occur in PPD-allergic subjects.

Objectives

To compare cross-elicitation responses to ME-PPD in open use and diagnostic patch testing of PPD-allergic subjects with hair dye-related allergic contact dermatitis.

Methods

Reactions to ME-PPD were investigated in 25 PPD-allergic subjects by performing (1) 45-minute open use testing with a hair dye containing 2.0% of either ME-PPD or PPD, and (2) patch testing with increasing ME-PPD concentrations (0.1%–2.0% pet.).

Results

Of the 25 PPD-allergic subjects, 21 (84%) reacted to open use testing with a hair dye containing 2.0% PPD, and testing with 2.0% ME-PPD led to cross-elicitation in 12 (48%). When patch tested with increasing ME-PPD concentrations, 13 (52%) cross-reacted at 0.1% (lowest dose) and 21 (84%) at 2.0% (highest dose), indicating decreased reactivity as compared with published PPD dose-response data.

Conclusion

In line with the decreased cross-reactivity of ME-PPD in hair dye open use testing, PPD-allergic subjects show an attenuated cross-elicitation dose response to ME-PPD in patch testing.

INTRODUCTION

p-Phenylenediamine (PPD, 1,4-diaminobenzene, CAS no. 106-50-3) is a hair dye molecule with good hair-colouring performance, but is also an important contact allergen associated with hair dye-related allergic contact dermatitis. Historically, attempts have been made to develop hair dye molecules that keep the balance between good hair-dyeing performance and sufficiently low skin sensitization potency to avoid induction under use conditions. The resulting PPD derivatives were toluene-2,5-diamine (TDA, 1,4-diamino-2-methylbenzene, CAS no. 95-70-5; synonym *p*-toluenediamine) and hydroxyethyl-*p*-phenylenediamine sulfate (HE-PPD, CAS no. 93841-25-9): TDA has good performance, but may lead to the induction of sensitization under use conditions, owing to its strong to extreme sensitization potency, as determined with the murine local lymph node assay (LLNA) and the guinea-pig maximization test. HE-PPD has limited performance and strong sensitization potency (as determined with the LLNA).(1-3)

More recently, 2-methoxymethyl-*p*-phenylenediamine (ME-PPD, CAS no. 337906-36-2) has been developed by introducing a methoxymethyl side-chain into PPD, resulting in a hair-dyeing performance equivalent to that of PPD and TDA. Analysis of the skin sensitization potency of ME-PPD in *in vitro* studies indicated a lower skin sensitization potency than that of PPD and TDA. *In vivo*, the LLNA showed a moderate skin sensitization potency of ME-PPD. Therefore, induction of skin sensitization has been considered to be unlikely when ME-PPD is used to replace PPD or TDA in hair dyes.(3)

Accordingly, ME-PPD has been developed for the prevention of skin sensitization, and not for individuals who have already been sensitized to other hair dye precursors, such as PPD and TDA. However, it is known that many individuals who are allergic to PPD or TDA continue to dye their hair, and they may use hair dye products that contain ME-PPD instead of PPD or TDA. (4) Therefore, the current study investigated cross-elicitation responses to an ME-PPD-containing hair dye under open use test conditions in PPD-allergic individuals with a history of hair dye-related allergic contact dermatitis. Furthermore, their cross-elicitation dose response to ME-PPD was determined under diagnostic patch test conditions, and compared with PPD elicitation dose-response data previously published by Søsted et al.(5)

METHODS

Twenty-five adult individuals with a previously documented positive patch test reaction to PPD 90 µg/cm² (TRUE Test; SmartPractice Europe, Reinbek, Germany) or PPD 1% pet. (Chemotechnique Diagnostics, Vellinge, Sweden) and a history of allergic contact dermatitis caused by hair dye were included. The exclusion criteria were as follows: skin anomalies or active dermatitis on the volar aspects of the forearms or on the back, and the use of immunosuppressive medication (including, but not limited to, oral corticosteroids, cyclosporine, azathioprine, and methotrexate) during the 4 weeks prior to inclusion. All tests were performed at the Department of Dermatology, University Medical Center Groningen, The Netherlands, and assessed according to ESCD guidelines on day (D) 2, D3, and D7.⁽⁶⁾ Of the 25 subjects included in this present study, 8 had participated in a previous ME-PPD open use test study.⁽⁷⁾ In the previous study, 6 of these 8 reacted positively to ME-PPD under hair dye conditions (open use test), and the other 2 did not. The study was approved by the local ethics committee of the University Medical Center Groningen.

Open use testing

Open use tests were performed on the volar aspects of both forearms. The patch test preparations, the vehicle (Koleston Perfect formula without fragrance) containing the hair dye precursors (PPD 4.0% or ME-PPD 4.0%, free base) and couplers (1.9% 2-methyl-5-hydroxyethylaminophenol; 3.6% 2-methylresorcinol) and the hydrogen peroxide-based developer solution (6.0% wt/wt Welloxon) were provided by Procter & Gamble Service (now represented by Coty, Darmstadt, Germany). The couplers were selected on the basis of their negligible sensitization potency as determined with the LLNA, each with an EC₃ greater than 50. (8, 9) The hair dye test product was always freshly prepared by mixing the tint (containing PPD or ME-PPD, and the couplers) with the hydrogen peroxide solution by use of a small wooden stick (1:1, 90 µL each), resulting in solutions containing 2.0% PPD and 2.0% ME-PPD, respectively. A dye-free test product, also mixed with hydrogen peroxide as described above, was used as a negative control. A 100-µL aliquot of the final PPD-containing or ME-PPD-containing product was applied directly to the volar forearm with a micropipette in a 3.8-cm² area marked by a round adhesive tape with a diameter of 22 mm. PPD was tested on the volar aspect of 1 arm, and ME-PPD and the negative control were tested on the other, so that an extreme positive reaction to PPD would have no influence on a possible elicitation response to ME-PPD. The contours of the round adhesive tape

were marked with a Chemo Skin Marker (Chemotechnique Diagnostics), in order to enable recognition of the test site at follow-up. The test areas were rinsed off with water after 45 minutes of application to simulate hair-dyeing conditions.

Diagnostic patch testing

Patch testing was performed on the back with ME-PPD 0.1%, 0.25%, 0.5%, 1.0% and 2.0% pet. in Van der Bend chambers (Van der Bend, Brielle, The Netherlands), fixed with Fixomull Stretch (BSN Medical, Hamburg, Germany). In the first 10 subjects, ME-PPD was only patch tested in concentrations of 0.5%, 1.0% and 2.0% pet., after which the protocol was adapted to also include patch testing with ME-PPD 0.1% and 0.25% pet. The first 10 subjects were recalled for testing with these 2 concentrations, and all except for 2 subjects were additionally tested.

Evaluation and statistics

Responses to the open use test were recorded at 60 minutes after application (15 minutes after rinsing of the area), and, together with the patch test readings, on D2, D3, and D7, and graded according to ESCD guidelines.⁽⁶⁾ For data analysis, the maximum open use test/patch test reactions were aggregated as the outcome.

Results are presented as the proportion of PPD-allergic patients who responded with a cross-elicitation response to ME-PPD as indicated by a positive patch test reaction for each dose. Binary logistic regression was performed, and a dose-response curve was plotted in order to investigate the threshold dose for cross-elicitation for different proportions of the study population. The probability of a positive response $P(x)$, where x represents the natural logarithm of the dose, for a given dose is as follows:

$$P(x) = \frac{\exp(b_0 + b_1 \cdot x)}{1 + \exp(b_0 + b_1 \cdot x)}$$

The effective dose (ED_y), that is, the hypothetical dose at which a proportion y of the 25 PPD-positive patients have a positive cross-elicitation response to ME-PPD, was calculated for 50%, 75% and 90% of the patients. For example, ED_{50} is the expected dose at which 50% of the 25 PPD-positive patients will have a positive response to ME-PPD. The confidence interval (CI) for each ED_y is given. The dose-response curve is presented for all 25 PPD-positive patients, and compared with the dose-response curve for PPD as reported by Sørensen et al. ⁽⁵⁾ Dose-response curves were generated by

the use of graphpad prism 7.03, with non-linear regression with curve fitting (GraphPad Software, La Jolla, California).

RESULTS

Cross-elicitation response to ME-PPD-containing hair dye in open use testing

The strengths of the original patch test reactions to PPD were weak (+) positive (n = 5), strong (++) positive (n = 12), and extreme (+++) positive (n = 8). The characteristics of the 25 subjects are shown in Table 1. A detailed overview of the individual open use and diagnostic patch test results obtained from the 25 PPD-allergic subjects is shown in Table 2. Twelve of the 25 subjects (48%, 95%CI: 28%-68%) showed cross-elicitation reactions to the open use test with hair dye containing 2.0% ME-PPD (Table 2), whereas 13 of 25 (52%) showed no cross-elicitation response. In the 12 of 25 subjects reacting to ME-PPD, the strengths of the reactions to the ME-PPD open use test were generally weaker than those to the 2.0% PPD open use test, with 6 of 12 having weak positive reactions, 5 of 12 having strong positive reactions and 1 of 12 having an extreme positive reaction to ME-PPD, as compared with 2 of 21 having weak positive reactions, 10 of 21 having strong positive reactions and 9 of 21 having extreme positive reactions to PPD. Correspondingly, 11 of 25 had reduced strengths of reaction to the ME-PPD open use test as compared with the PPD open use test, not counting 2 subjects who showed doubtful reactions to PPD and did not react to ME-PPD.

Table 1. overview of subject included in the present study on cross-elicitation to ME-PPD in PPD positive individuals

	Total n=25
Original PPD patch test reaction	
+	5 (20.0 %)
++	12 (48.0%)
+++	8 (32.0%)
Tested with all 5 ME-PPD patch test conc.	23
Only tested with ME-PPD 0.5%; 1.0%; 2.0%	2
Gender	
male/female	2/23
Age in years, mean (range)	50.2 yrs (18-71 yrs)

Table 2. Individual results of all 25 PPD allergic individuals to 45 min open use test exposure to hair dyes containing 2.0% of either PPD or ME-PPD and patch test results with increasing concentrations ME-PPD in pet.

Nr.	PPD patch test	Open use test hair dye		ME-PPD patch test				
		PPD	ME-PPD					
		2.0%	2.0%	0.1%	0.25%	0.5%	1.0%	2.0%
1	+	+++	++	++	+++	+++	+++	+++
2	+	?+	?+	-	-	+	++	++
3	+	+	-	?+	?+	-	-	-
4	+	+++	-	-	-	-	+	++
5	+	?+	-	-	-	?+	?+	?+
6	++	++	+	+	+	+	+	+
7	++	++	+	++	++	++	+++	++
8	++	+++	+	NT	NT	++	++	+++
9	++	++	-	++	+++	++	+++	+++
10	++	++	+	++	++	++	++	+++
11	++	++	+	?	+	+	++	+++
12	++	++	-	-	-	-	-	+
13	++	?+	-	-	-	-	-	-
14	++	+	-	?+	?+	?+	+	++
15	++	++	+	+	++	++	++	++
16	++	++	?+	-	-	+	+	+
17	++	-	-	-	-	-	-	-
18	+++	+++	+++	NT	NT	+++	+++	+++
19	+++	++	-	-	?+	+	+	+
20	+++	+++	++	+++	+++	+++	+++	+++
21	+++	++	-	+	++	++	++	++
22	+++	+++	-	+	++	++	+++	+++
23	+++	+++	++	++	+++	+++	+++	+++
24	+++	+++	++	+++	+++	+++	+++	+++
25	+++	+++	++	++	++	+++	+++	+++

'-', negative patch test reaction; '?+', doubtful patch test reaction, considered negative in statistical analyses; 'NT', not tested.

Figure 1A shows the distribution of the non-responsive subjects and cross-reacting subjects in ME-PPD open use testing against the strengths of the original diagnostic patch test reactions to PPD. The percentage of subjects cross-reacting to the ME-PPD open use test increased with the strength of the original PPD patch test reaction: 20% (1/5) with +, 50% (6/12) with ++, and 62.5% (5/8) with +++. In contrast, elicitation responses to identical open use tests with 2.0% PPD hair dye were as follows: 60% (3/5) with +, 83.3% (10/12) with ++, and 100% (8/8) with +++ (Figure 1B), giving an elicitation response rate of 84% (21/25) PPD-allergic subjects.

Furthermore, the reaction strength of the cross-elicitation response was reduced in 11 of the 12 PPD-allergic subjects who reacted to both PPD and ME-PPD open use tests; when plotted against their PPD patch test reaction strengths, reduced cross-elicitation reaction strengths occurred in all subgroups: 1 of 1 with +, 6 of 6 with ++, and 4 of 5 with +++ (Table 2).

None of the subjects showed immediate reactivity to either ME-PPD or PPD hair dye open use tests, as no positive reactions were seen 60 minutes after application. No positive reactions to the open use test with the vehicle (negative control) were seen. Eight of the 25 subjects had previously undergone an open use test with ME-PPD.⁷ None of these 8 subjects had been exposed to ME-PPD hair dye in any other manner prior to participation in the present study. The 2 who did not react to the ME-PPD open use test in the previous study did not react to ME-PPD in the present study, suggesting that they were not actively sensitized in the previous study.

Figure 1.

(A), Percentages of p-phenylenediamine (PPD)-sensitized subjects with either negative or positive reactions to 2.0% 2-methoxymethyl-p-phenylenediamine (ME-PPD) following 45 minutes of exposure in hair dye open use testing, plotted against the strengths of their patch test reactions to PPD. **(B)**, Percentages of PPD-sensitized subjects with either negative or positive reactions to 2.0% PPD following 45 minutes of exposure in hair dye open use testing, plotted against the strengths of their patch test reactions to PPD

Dose-dependent cross-elicitation reactions to ME-PPD in diagnostic patch testing

Figure 2 provides a summary of the relative frequencies of cross-elicitation reactions to increasing ME-PPD doses in diagnostic patch testing as compared with the rate of open use cross-elicitation reactions to 2.0% ME-PPD and the rate of open use elicitation reactions to 2.0% PPD. Cross-elicitation responsiveness to increasing concentrations of ME-PPD (from 0.1% to 2.0% pet.) was tested in PPD-allergic subjects by patch testing (23 were tested with all concentrations, and 2 were only available for the 3 highest concentrations). The percentage of subjects who showed a positive cross-elicitation reaction to ME-PPD 0.1% pet. was 52% (12/23, 95%CI: 32%-72%), and increased to 84% (21/25, 95%CI: 70%-98%) when they were exposed to ME-PPD 2.0% pet. A percentage of 84% also reacted to the open use tests with 2.0% PPD, although these were not exactly the same subjects; of the 21 subjects with an elicitation response to 2.0% ME-PPD, 20 showed a reaction to the open use test with 2.0% PPD, and vice versa (Table 2). No irritant patch test reactions were seen.

Figure 2.

Proportions of response of p-phenylenediamine (PPD)-sensitized subjects (n = 25) to 45 minutes of open use test exposure to hair dye containing 2% of either PPD (green bar) or 2-methoxymethyl-p-phenylenediamine (ME-PPD) (dark violet bar), and to 48 hours of patch test exposure to increasing ME-PPD concentrations (violet bars)

Figure 3 shows the distribution of the PPD-allergic subjects with positive cross-elicitation reactions to the increasing concentrations of ME-PPD against the strength of the original PPD diagnostic patch test reactions. The data indicate a clear correlation of increasing cross-reactivity to ME-PPD and increasing strength of PPD reactivity; that is, all subjects with an extreme (+++) positive reaction to PPD had a positive cross-elicitation reaction to ME-PPD at a concentration of 0.5% and above.

Figure 3.

Cross-elicitation reactions of p-phenylenediamine (PPD)-allergic subjects to patch testing with increasing 2-methoxymethyl-p-phenylenediamine (ME-PPD) concentrations plotted against the strengths of their patch test reactions to PPD

On the basis of the patch test results with ME-PPD, a cross-elicitation dose-response curve was plotted for the 25 PPD-allergic subjects (Figure 4, blue dashed line) and compared with a previously published elicitation dose-response curve for PPD in 15 PPD-allergic patients (Figure 4, grey line) from Sørensen et al.⁵ The curve for ME-PPD was shifted further towards higher concentrations, indicating that, over the entire concentration range, higher doses are needed to generate a response rate comparable to that of PPD. The expected patch test doses (ED₅₀ values) at which 50%, 75% and 90% of PPD-allergic subjects are estimated to develop a positive cross-reaction to

ME-PPD are shown in Table 3. The ED₅₀ for ME-PPD was calculated to be ~0.11%. When we used the data from Søstved et al in our calculation, the ED₅₀ was 0.03% for PPD, in line with the ED₅₀ value reported by Søstved et al.(5)

Figure 4. Cross-elicitation dose-response curve for 2-methoxymethyl-*p*-phenylenediamine (ME-PPD) patch testing. The blue line and circles represent the dose response to ME-PPD in 25 *p*-phenylenediamine (PPD)-allergic patients; the grey line and squares represent the dose response to PPD in the 15 PPD-allergic patients (taken from Søstved et al (5)). Each symbol corresponds to the number of subjects with a positive reaction at the concentration indicated

Table 3: Calculated values of Effective Dose (ED)_y for ME-PPD cross-elicitation reactions observed in PPD allergic subjects (n=25) with 95% confidence intervals compared to PPD elicitation dose response

	ME-PPD			PPD (Sosted <i>et al.</i>) ¹		
	Dose (%)	95% CI lower limit (%)	95% CI upper limit (%)	Dose (%)	95% CI lower limit (%)	95% CI upper limit (%)
ED ₅₀	0.11	0.03	1.49	0.03	0.01	0.10
ED ₇₅	0.72	0.32	1.63	0.10	0.04	0.51
ED ₉₀	4.94	0.75	32.36	0.28	0.10	3.40

¹ Values for ED_y for PPD 2.0% pet. patch test were taken from Sosted *et al.*, 2006. Values were transferred from ppm to %.

DISCUSSION

In this study, we have analysed cross-elicitation responses to ME-PPD in 25 PPD-allergic individuals by comparing their responses to open use tests simulating hair dyeing, and to diagnostic patch tests with increasing ME-PPD concentrations.

Open use testing with 2.0% ME-PPD in a hair dye showed cross-elicitation in 12 of the 25 PPD-allergic subjects, that is, in 48% (Figure 1A). In contrast, open use testing with 2.0% PPD (Figure 1B) showed an elicitation response in 84% (21/25). Furthermore, the reaction strength of the cross-elicitation response was reduced in 11 of the 12 (92%) PPD-allergic subjects who reacted to both PPD and ME-PPD open use tests (Table 2). This is in line with data from previous cross-elicitation studies,(7, 10) indicating partial cross-reactivity to ME-PPD under hair dye use conditions. In those previous studies, the response was ~30% with an exposure time of 30 minutes. The observed higher response of 48% to the 45-minute ME-PPD hair dye open use test in the present study confirms previous findings with PPD showing that an increase in the exposure time from 30 to 60 minutes increased the amount available for (cross-)elicitation by >2-fold. (11)

We also investigated whether non-responsiveness to ME-PPD open use tests in PPD-allergic subjects is dependent on the limited 45-minute exposure time. Exposure to increasing ME-PPD patch test doses for 48 hours under occlusion showed a dose-

dependent increase in the cross-elicitation response: at the lowest patch test dose of 0.1%, the response of 52% was similar to that with hair dye open use testing with 2.0% ME-PPD (48%), indicating that a comparable immune stimulus can be provided with either short high-dose exposure or longer low-dose exposure. This interpretation is further supported by similar cross-elicitation reaction strengths in 5 of the 9 subjects (55.0%) who showed elicitation reactions to both 0.1% patch tests and 2.0% open use tests.(12)

With increasing patch test doses up to 2.0%, an increasing number of PPD-allergic subjects cross-reacted to ME-PPD, with 84% showing positive reactions at the maximum tested dose of 2.0% (Figure 1). These findings indicate that the majority of PPD-allergic patients are probably able to cross-react to ME-PPD, provided that the dose is high enough and/or the exposure time is long enough. In line with the responses to open use testing (Figure 1A), a higher percentage of the extreme PPD responders than of the weaker PPD responders cross-reacted at lower ME-PPD patch test doses. A possible explanation might be a higher number of PPD-specific memory T cells being present in the extreme PPD responders.

On comparison of the ME-PPD cross-elicitation patch test dose response obtained in the present study with the patch test dose response to PPD obtained in 15 PPD-allergic subjects,(5) the curve for ME-PPD is shifted further towards higher concentrations (Figure 3). In other words, over the entire dose range, higher ME-PPD concentrations are needed to generate a response rate comparable to that seen with PPD. This is also indicated by the 4-fold increased ED₅₀ value for ME-PPD vs PPD, and is in line with the hypothesis that ME-PPD is a less potent allergen.(3, 5)

The measured exposure level (MEL) (representing the concentration that is available to the skin) of the applied PPD patch dose of 1.0% for 48 hours is 200 µg/cm². This is an order of magnitude higher than the MEL for PPD in hair dye open use conditions for 30 minutes, which ranges from 6.8 to 21 µg/cm².(11) This PPD concentration was found to be sufficient to elicit a reaction in 84% of PPD-allergic patients. For ME-PPD, the MELs for 1.8% applied under hair dye use conditions were 8.75 and 10.21 µg/cm² for 30 minutes and 60 minutes, respectively,(3) and were thus similar to the MEL for PPD. Consequently, it is reasonable to assume that the MEL for patch test conditions with exposure to 2% ME-PPD is at least >10 fold higher and in line with the higher response observed in patch testing than in open use testing.

In agreement with the present results, a study from Kock et al investigating cross-elicitation reactions to repeated hair dyeing with ME-PPD in PPD-allergic and/or TDA-allergic individuals showed that 29 of 43 were able to tolerate repeated hair colouring with an average of 9 treatments per year.⁽¹³⁾ In that study, 9 subjects did not react to the initial 45-minute open use test on the forearm, and 7 of them developed allergic contact dermatitis symptoms during the first hair colouration with ME-PPD. Together with our findings, these data suggest that an individual threshold for cross-elicitation to ME-PPD exists in PPD-allergic subjects. We assume that the skin site-specific occurrence of the cross-elicitation responses to ME-PPD between forearm and back (in the present study) or scalp (in the study from Kock et al ⁽¹³⁾) is related to the presence of (sufficient) residual memory T cells. Similar findings have been reported for patients with allergy to nickel: a rapid response to nickel only occurred after re-exposure to nickel on the exact body site that had been previously exposed, and the magnitude of the elicitation responses correlated with local recruitment of epidermal CD8+ T cells.⁽¹⁴⁾

In line with previous studies on ME-PPD, our hair dye open use and patch test data further support the recommendation for hair dye-allergic individuals to avoid hair colouring, because cross-elicitation responses to ME-PPD cannot be excluded.^(7, 10, 13)

Despite intensive investigations, the precise hapten responsible for PPD allergy has not been identified.⁽¹⁵⁾ Considering that induction of sensitization with PPD has been shown to be dependent on duration of exposure,⁽¹⁶⁾ PPD oxidation to protein reactive auto-oxidation products such as Bandrowski's base may be involved in elicitation.⁽¹⁷⁾ Furthermore, PPD undergoes N-acetylation when entering the epidermis, and thus becomes deactivated.⁽¹⁸⁾ In line with the findings for PPD, both oxidative activation of ME-PPD and deactivation by N-acetylation have been described in vitro, and human skin has been shown to actively N-acetylate ME-PPD.⁽³⁾ Correspondingly, the structural similarity to PPD and the consistent activation and deactivation mechanisms suggest that, in PPD-allergic individuals, the concentration-dependent immune response to ME-PPD is based on the partial inability of T cells to differentiate between PPD-derived and ME-PPD-derived haptens.⁽¹⁰⁾

CONCLUSION

Cross-elicitation analysis in PPD-allergic individuals indicates that ME-PPD is a less potent allergen than PPD under simulated hair dye use conditions and under diagnostic patch test conditions. The cross-reactivity shows a clear dose dependency, with increasing cross-reactivity to ME-PPD being seen at higher patch test doses. ME-PPD can only be considered to be an alternative hair dye for primary prevention of sensitization.

Conflict of interest

We declare that Procter & Gamble Professional Beauty (now represented by Coty) provided funding support for conducting the study and provided the hair dyes. The hair dye ingredient investigated in this study is currently used in commercial products marketed by Coty. C. Goebel is an employee of Coty. The authors alone are responsible for the content and writing of the article.

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CHAPTER

4

Persistence of contact allergy: a retrospective analysis

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ABSTRACT

Background

Studies have shown that not all positive patch test reactions are reproducible upon retesting, that is, persistent. Non-persistent reactions might represent initial false-positive reactions, meaning that patients might unnecessarily avoid allergens.

Objectives

To investigate the occurrence of both persistent and non-persistent patch test reactions, to explore possible explanations, and to investigate whether allergen-specific differences exist.

Methods

A retrospective analysis was performed on patients who were patch tested at least twice between 1 January 1995 and 31 October 2016, with at least one positive patch test reaction to an allergen that had been retested. Both univariable and multivariable analyses were performed to investigate the influence of several factors on persistence.

Results

Of 274 retested positive reactions in 119 patients, 183 (66.8%) reactions remained positive. The strongest predictor for non-persistence in both univariable and multivariable analyses was strength of the first patch test, with weak positive reactions being significantly less persistent. Regarding allergen groups, metals and fragrances were less persistent than other allergens.

Conclusion

Weak positive reactions have a low persistence rate, and the dermatologist should be conservative in advising the patient on avoidance of these allergens, especially if clinical relevance is uncertain.

There is an ongoing debate within the contact dermatitis research community on whether contact allergy, once acquired, is persistent or can be lost. Multiple studies spanning decades have investigated the persistence of positive patch test reactions and the loss of sensitization, and still no conclusion has been reached (1–11). Diagnosing a patient with allergic contact dermatitis is based on patch testing, which is the gold standard for diagnosing contact allergy. The most common methods with which patch test reactions are evaluated are according to the criteria of the ICDRG and those of the ESCD, respectively (12). Every positive patch test reaction has to be evaluated with regard to whether it is currently relevant, that is, the patient has current exposure to the allergen at the site of the dermatitis, or of past relevance. Patients receive oral information regarding their positive patch test reactions and what to avoid, and are given written information. Studies have shown that patients' ability to remember the patch test results is mediocre at best, and that they might be inclined to avoid every allergen to which they had a positive patch test reaction, which can have a major impact on a patient's job or lifestyle (13, 14). A positive patch test reaction can therefore have substantial consequences, and it is important to know (i) the odds of a positive reaction being either a false positive or not clinically relevant, (ii) whether a patient with a contact allergy can become tolerant, and (iii) whether any allergen-specific differences exist. To explore the issue of non-persistent patch test reactions, we investigated patients who have been patch tested twice or more at our department.

METHODS

We performed a retrospective search on our patch test database from 1 January 1995 to 31 October 2016 for patients who underwent patch testing at least twice, with at least one positive patch test reaction to an allergen that had been retested in the same concentration and the same vehicle, with no restriction on patch test series/allergen or age. If a patient had been patch tested more than twice, only the results of the last two patch tests were evaluated. All patients were tested with at least a baseline series, consisting of TRUE Test® (SmartPractice Europe, Reinbek, Germany) with additional investigator-loaded allergens (Chemotechnique, Vellinge, Sweden), tested in Van der Bend square chambers (Van der Bend, Brielle, The Netherlands). The composition of this baseline series has changed over time, having been adjusted to trends detected in epidemiological data. All patch tests were read by experienced dermatologists according to the ICDRG/ESCD criteria, with the possible outcomes being: negative, irritant, doubtful (?+), weak positive (+), strong positive (++), and extreme positive (+++).

Readings were performed on day (D) 3 and D7 by one of two trained dermatologists. For the present analysis, the maximum patch test reactions of these two readings were aggregated as the patch test outcome. Furthermore, no distinction was made between doubtful and irritant reactions, and these were counted as negative when persistence of contact allergy was calculated. As a general rule, patch testing is not performed at our department in patients receiving oral immunosuppressive therapy, and a wash-out period of at least five half-lives is adhered to before patch testing is performed.

Besides patch test reactions, other variables that were analysed were: age at second patch test, sex, history of atopic dermatitis, season in which the patient was patch tested ('warm season', ranging from April to September; and 'cold season', from October to March), years elapsed between the two patch tests, and the patch test system (TRUE Test® or investigator-loaded). Persistence is reported for the 12 most frequently tested allergens and for groups of allergens as reported in Table 1.

Table 1. Overview of the allergen groups used for logistic regression analysis, and composition of each group.

Allergen group	
Metals	Cobalt chloride, mercury, nickel sulfate, and potassium dichromate
Preservatives	Bronopol, Bioban P1487, MCI/MI, diazolidinyl urea, dichlorophene, formaldehyde, imidiazolidinyl urea, MDBGN, n-methylol chloroacetamide, quaternium 15, and sodium omadine
Fragrances	FM 1, FM 2, myroxylon pereirae (Balsam of Peru), lemon grass oil, HICC, orange oil (citrus dulcis)
Rubbers	Black rubber mix, carba mix, MBTS, dipentamethylenethiuram disulfide, mercapto mix, MBT, MOR, N-Cyclohexyl-2-benzothiazolesulfenamide, TMTD, TMTM, thiuram mix
Dyes/colours	4-Aminoazobenzene, disperse blue 124, disperse orange 3, PPD, TDA
Topical medicaments	Amerchol L 101, caine mix, compositae mix, oleamidopropyl dimethylamine, neomycin sulfate, parthenolide, propylene glycol, quinoline mix, sesquiterpene lactone mix
Corticosteroids	Budesonide, hydrocortisone-17-butyrate, methyl prednisolone, tixocortol-pivalate, triamcinolone acetonide

MCI/MI, methylchloroisothiazolinone/methylisothiazolinone; MDBGN, methyldibromoglutaronitrile; FM 1, fragrance mix 1; FM 2, fragrance mix 2; HICC, hydroxyisohexyl 3-cyclohexene carboxaldehyde (lyral); MBTS, dibenzothiazyl disulfide; MBT, mercaptobenzothiazole; MOR, morpholinyl mercaptobenzothiazole; TMTD, tetramethylthiuram disulfide; TMTM, tetramethylthiuram monosulfide; PPD, *para*-phenylenediamine; TDA, 2,5-toluenediamine

Statistics

Persistence is given as relative frequencies together with their 95% confidence interval. The chi²-test or Fisher's exact test was used to analyse differences between persistence proportions. Logistic regression was performed to analyse the influence of the documented independent variables on the persistence of a positive patch test reaction. Both univariable and multivariable backward regression analyses were performed. The influence of type of allergen was also included in these analyses, and allergens were grouped on the basis of chemical structure, cross-reactivity, and co-sensitization, resulting in seven groups: metals, preservatives, fragrances, rubbers, dyes/colours, topical medicaments, and corticosteroids (Table 1). Statistical analyses were performed with SPSS v.23 (IBM) and Excel 2013 (Microsoft).

RESULTS

Overall, 119 patients (66.4% female) were included in the analysis, with 274 initial positive reactions to an allergen that was retested in a second patch test. The mean age at the first patch test was 38.9±13.7 years, and that at the second patch test was 46.2±14.6 years. Of the 274 positive reactions, 183 (66.8%) remained positive upon retesting, 14 (5.1%) became doubtful, 1 irritant, and 76 (27.7%) became negative (Table 2). Of these 76 initially positive reactions that became negative, 64 (84.2%) were weak positive (+) reactions, 10 (13.2%) were strong positive (++) reactions [3 to nickel sulfate, 3 to *p*-phenylenediamine (PPD), and 1 each to mercaptobenzothiazole, neomycin sulfate, cobalt chloride, and Disperse Orange 3], and 2 (2.6%) were extreme positive (+++) reactions (to zinc dimethyl dithiocarbamate 1% pet. and tixocortol 21-pivalate 0.1% pet., resp.). All 15 reactions that became either doubtful or irritant reactions upon retesting were initially weak positive (+) reactions. Conversely, of the 199 initially weak positive reactions (+), 120 (60.3%) remained positive, of the 56 initially strong positive reactions (++) , 46 (82.1%) remained positive, and of the 19 initially extreme positive reactions (+++) , 17 (89.5%) remained positive.

Table 2. Reaction pattern of the second patch test stratified by strength of the original patch test.

		Patch test 2						Total
		negative	+	++	+++	doubtful	irritant	
Patch test 1	+	64	70	48	2	14	1	199
	++	10	16	23	7	0	0	56
	+++	2	1	7	9	0	0	19
Total		76	87	78	18	14	1	274

The persistence rates for the different variables that were analysed are shown in Table 3. Some of the findings are highlighted here. Of the 274 retested positive reactions, 195 were to allergens in TRUE Test®, and the remaining 79 were to investigator-loaded allergens. The persistence rates were 69.2% for TRUE Test® allergens and 60.8% for investigator-loaded allergens ($p=0.18$). Of the 119 patients, 83 (69.7%) had (a history of) atopic dermatitis. Of the 274 retested reactions, 195 (71.2%) were in patients with past or current atopic dermatitis. The persistence rate of positive patch test reactions in atopic dermatitis patients was 64.6%, as compared with 72.2% in patients without (a history of) atopic dermatitis ($p=0.26$). The influence of time elapsed between the two patch tests was also assessed, and three groups of patients were compared: retest within 3 years, retest between 3 and 8 years, and retest after >8 years. Persistence rates for these three groups were, respectively, 64.4%, 73.3%, and 61.5% ($p=0.20$). Moreover, seasonal influence on persistence was examined. The highest persistence rate was found in patients who were tested both times in a warm season (71.9%), and the second highest was found in patients tested both times in a cold season (67.5%). Patients who were tested in a different season had lower persistence rates: 64.5% for warm to cold, and 63.9% for cold to warm. The influence on persistence of the season during which patch testing took place was, however, not significant ($p=0.77$).

Table 3. Persistence rates for all analyzed variables

		Persistent reactions			
		Total (n)	N (%)		95% CI (%)
Total		274	183	(66.8)	60.9-72.3
Sex	male	69	45	(65.2)	52.8-76.3
	Female	205	138	(67.3)	60.4-73.7
Age at second patch test	<40 years	95	59	(62.1)	51.6-71.9
	≥40 years	179	124	(69.3)	62.0-75.9
TRUE Test® vs Chamber Loaded	TT	195	135	(69.2)	62.2-75.6
	CL	79	48	(60.8)	49.1-71.6
Atopic dermatitis	non atopic	79	57	(72.2)	60.9-81.7
	Atopic	195	126	(64.6)	57.5-71.3
Strength of the first patch test	+	199	120	(60.3)	53.1-67.2
	++	56	46	(82.1)	69.6-91.1
	+++	19	17	(89.5)	66.9-98.7
Metals	other allergens	201	138	(68.7)	61.8-75.0
	Metals	73	45	(61.6)	49.5-72.8
Preservatives	other allergens	233	151	(64.8)	58.3-70.9
	Preservatives	41	32	(78.0)	62.4-89.4
Fragrances	other allergens	246	168	(68.3)	62.1-74.1
	Fragrance	28	15	(53.6)	33.9-72.5
Rubbers	other allergens	230	149	(64.8)	58.2-70.9
	Rubbers	44	34	(77.3)	62.2-88.5
Dyes	other allergens	241	161	(66.8)	60.5-72.7
	Dyes	33	22	(66.7)	48.2-82.0
Topicals	other allergens	252	169	(67.1)	60.9-72.8
	Topicals	22	14	(63.6)	40.7-82.8
Corticosteroids	other allergens	265	179	(67.5)	61.5-73.1
	Corticosteroids	9	4	(44.4)	13.7-78.8
Years between patch test	retest within 3 years	82	53	(64.6)	53.3-74.9
	retest >3 years up to 8 years	101	74	(73.3)	63.5-81.6
	retest >8 years	91	56	(61.5)	50.8-71.6
Season	warm to warm	57	41	(71.9)	58.5-83.0
	cold to cold	83	56	(67.5)	56.3-77.4
	warm to cold	62	40	(64.5)	51.3-76.3
	cold to warm	72	46	(63.9)	51.7-74.9

To assess whether there was variation between allergens, persistence was calculated for individual allergens and for grouped allergens (Tables 3 and 4). Corticosteroids had the lowest persistence rate (44.4%; n=9), and preservatives had the highest persistence rate (78.0%, n=41). Fragrances also had a relatively low persistence rate (53.6%; n=28). The persistence rate for individual allergens ranged from 0.0% to 100.0%, although this was mostly a consequence of many allergens having been retested only once. However, large variation was seen in the top 12 most frequently retested allergens (Table 4), ranging from 35.3% for cobalt (n=17) to 100.0% for potassium dichromate (n=11), also indicating strong variation within allergen groups (in this case, metals).

Table 4. Top 12 of allergens most frequently re-tested

	Total	N	Persistent reactions	
			(%)	95% CI (%)
Nickel sulfate 200 µg/cm ²	44	27	(61.4)	45.5-75.6
Cobalt chloride 20 µg/cm ²	17	6	(35.3)	14.2-61.7
Fragrance mix I 430 µg/cm ²	14	9	(64.3)	35.1-87.2
p-Phenylene diamine (free base) µg/cm ²	13	9	(69.2)	38.6-90.9
Quaternium-15 100 µg/cm ²	12	11	(91.7)	61.5-99.8
Potassium dichromate 23 µg/cm ²	11	11	(100.0)	71.5-100.0
Carba mix 250 µg/cm ²	10	8	(80.0)	44.4-97.5
Colophony 850 (Rosin) µg/cm ²	9	7	(77.8)	40.0-97.1
Mercaptobenzothiazole(MBT) 75 µg/cm ²	9	5	(55.6)	21.2-86.3
Balsam of peru 800 µg/cm ²	8	5	(62.5)	24.5-91.5
Formaldehyde 180 µg/cm ²	8	7	(87.5)	47.3-99.7
Thiuram mix 25 µg/cm ²	8	7	(87.5)	47.3-99.7

In the univariable logistic regression analysis, only the strength of the first patch test reaction reached significance, confirming that strong (++) and extreme (+++) positive patch test reactions have higher persistence rates than weak (+) positive patch test reactions, with odds ratios (ORs) of, respectively, 3.03 and 5.60. A multivariable model was built with all variables included, with backwards elimination, resulting in model 1 (Table 5). In this model, the strongest significant positive predictor for persistence of a positive patch test reaction remained strength of the patch test reaction (++ reaction, OR 3.09; +++ reaction, OR 6.55). Atopic dermatitis, metals and the investigator-loaded patch test technique were negative predictors, with ORs of, respectively, 0.6, 0.55, and 0.53 (significant). Because we were interested in the predictive power of specific

allergen groups, a second model was built with the same technique but excluding strength of the patch test reaction, as this might overpower the influence of allergen groups on persistence. This resulted in a model with three significant negative predictors for persistence: the investigator-loaded technique (OR 0.5), metals (OR 0.48), and fragrances (OR 0.40).

Table 5. Uni- and multivariable log-binomial regression analysis with persistence as outcome.

Factor	Univariable analysis		Multivariable analysis	
		OR (95% CI)	Model 1 ^a OR (95% CI)	Model 2 ^b OR (95% CI)
Sex	Female	1.00		
	Male	0.91 (0.51 – 1.62)		
Age at second patch test	<40 years	1.00		
	≥40 years	1.38 (0.82 – 2.32)		
TRUE Test® vs. Chamber Loaded	TRUE Test	1.00	1.00	1.00
	Chamber loaded	0.69 (0.40 – 1.19)	0.53 (0.28 – 0.98)	0.50 (0.27 – 0.93)
Atopic dermatitis (AD)	No AD	1.00	1.00	
	AD	0.71 (0.40 – 1.25)	0.60 (0.33 – 1.09)	
Strength of first patch test reaction	+	1.00	1.00	
	++	3.03 (1.44 – 6.35)	3.09 (1.46 – 6.55)	
	+++	5.60 (1.26 – 24.89)	6.55 (1.44 – 29.79)	
Metals	Other	1.00	1.00	1.00
	Metals	0.73 (0.42 – 1.28)	0.55 (0.29 – 1.04)	0.48 (0.25 – 0.92)
Preservatives	Other	1.00		
	Preservatives	1.93 (0.88 – 4.24)		
Fragrances	Other	1.00		1.00
	Fragrances	0.54 (0.24 – 1.18)		0.40 (0.17 – 0.92)
Rubber	Other	1.00		
	Rubber	1.85 (0.87 – 3.93)		

Table 5. Continued

Factor	Univariable analysis		Multivariable analysis	
		OR (95% CI)	Model 1 ^a OR (95% CI)	Model 2 ^b OR (95% CI)
Dyes	Other	1.00		
	Dyes	0.99 (0.46 – 2.15)		
Topicals	Other	1.00		
	Topicals	0.86 (0.35 – 2.13)		
Corticosteroids	Other	1.00		
	corticosteroids	0.38 (0.10 – 1.47)		
Years between patch tests	<3 years	1.00		
	3 – 8 years	1.50 (0.80 – 2.82)		
	> 8 years	0.88 (0.47 – 1.63)		
Season	Warm to warm	1.00		
	Warm to cold	0.71 (0.33 – 1.54)		
	Cold to warm	0.69 (0.33 – 1.46)		
	Cold to cold	0.81 (0.39 – 1.69)		

^aModel 1; multivariable regression analysis with all variables entered, then backward elimination according to Wald.

^bModel 2; multivariable regression analysis with all variables entered except for strength of first patch test reaction, then backward elimination according to Wald.

Bold values indicate significant effects (p<0.05)

DISCUSSION

A retrospective analysis of our contact allergy database showed that, when positive patch test reactions were later retested, 66.8% remained positive, meaning that >30% became negative. This is in line with previous literature (4, 5, 7, 8). As expected, the large majority (almost 85%) of these non-persistent reactions were initially weak positive (+). One might argue that many or most of these had been false positives, as many allergens also have an irritant potential, even though the concentration in which allergens are tested is such that the risk of an irritant reaction should be minimal. Other possibilities must be considered; the patient could have become tolerant, or the threshold for elicitation could have changed. One study by Katsarou et al. investigated differences in T cell subsets expressed in persistent reactions as compared with diminished or lost reactions, and found that, in persistent reactions, a proliferation of CD45RO+/memory cells was seen, whereas in non-persistent reactions CD45RA+/suppressor-inducer cell proliferation was observed (15). These results support the theory that certain patients can become tolerant.

Another explanation for a non-persistent positive patch test reaction could be that the skin was momentarily more (or less) reactive. One known example of this is the excited skin syndrome or angry back (16, 17); however, when this is observed in our department, we report these reactions as negative. Experimental studies have also shown that the area of skin around a strong or extreme positive reaction has higher reactivity, possibly explaining false-positive reactions (18). Neither the amount of positive reactions that a patient had during a patch test nor the proximity of reactions to other strong/extreme positive reactions were taken into account in the current analysis.

There are known factors that can explain variation in individual skin reactivity during patch testing, for example hormones and ultraviolet radiation (19–22). Although there have been conflicting reports, in general it is believed that the immune response of the skin diminishes with ageing (23). Our data, however, show that the persistence rate is higher in patients aged ≥ 40 years at the second patch test than in patients aged < 40 years (69.3% versus 62.1%). This might result from chance, as patients aged ≥ 40 years are over-represented. Age at second patch test was not significant in the univariable regression analysis. The effect of age might be overshadowed by the fact that the most

likely reason for retesting is persistent dermatitis, suggesting that senescence of the immune system is not evident in these patients.

The persistence rate in patients with atopic dermatitis was lower than that in patients without (a history of) atopic dermatitis. Atopic dermatitis was also included in the first multivariable regression model, but it was not significant. It is interesting to note that atopic dermatitis was no longer included in the second model, in which strength of the first patch test reaction was not entered, suggesting that patients with (a history of) atopic dermatitis have more stronger patch test reactions that are not persistent than patients without atopic dermatitis. Of the 12 initially strong/extreme positive (++) reactions that became negative, 11 were in patients with atopic dermatitis (data not shown). It has been suggested that strong inflammatory activity of atopic dermatitis can decrease the elicitation response in patch testing, leading to a higher chance of false-negative reactions (24).

Persistence of a patch test reaction can also depend on the patch test technique used. A previous retrospective study focusing solely on patients tested with TRUE Test® found a persistence of 66%, which is similar to our results, in which 69.2% of positive TRUE Test® reactions were persistent, as compared with 60.8% of investigator-loaded reactions (7). Reactions to investigator-loaded allergens were significantly less persistent in the first multivariable regression model than reactions to TRUE Test® allergens, meaning that this difference was independent of the strength of patch test reaction. Gollhausen et al. investigated the reproducibility of TRUE Test® as compared with allergens tested in Finn Chambers®, and found that, in Finn Chambers®, reproducibility was half that in TRUE Test® (25). Ale and Maibach tested 491 patients with TRUE Test® on both sides of the back, and found a concordance of 95% (26). This is most likely a direct consequence of the fact that TRUE Test® is more standardized, whereas the investigator-loaded technique depends on the investigator, and the amount of vehicle containing allergen applied to each chamber might vary, except when a micropipette is used for aqueous solutions.

It has been suggested by previous studies that the chance of an irritant or doubtful reaction increases in cold and dry weather conditions, therefore increasing the risk of a false-positive reading (27, 28). This is more likely for allergens that are also marginally irritant, for example formaldehyde, for which the most evidence exists of increased odds of questionable and irritant reactions in cold/dry weather (28, 29). Other allergens for which this effect might exist are methylchloroisothiazolinone (MCI)/

methylisothiazolinone (MI) and PPD (29). The effect of weather conditions on patch test outcome might be stronger for hydrophilic allergens tested in aq. as the vehicle, as is the case for formaldehyde and MCI/MI (29). In our results, no influence of season on persistence was seen, and the persistence rates for formaldehyde, MCI/MI and PPD were all relatively high for cold to warm (81.8%, n=9/11) and relatively low for warm to cold (62.5%, n=5/8), which is in contrast to what one would expect on the basis of previous studies (28, 29). One possible explanation might be that formaldehyde and MCI/MI were tested in a povidone gel instead of aq. A more likely explanation is that, for all three allergens, only a few of the reactions were initially tested in a cold season and subsequently retested in the summer. It must also be considered that weather conditions in central Europe vary, and that the dichotomous distribution of 'cold' and 'warm' seasons is a very crude and possibly inaccurate approximation of actual weather conditions (28). This is, to our best knowledge, the first time that separate allergen groups have been compared with respect to the persistence of positive patch test reactions. In the second multivariable regression model, excluding strength of the first patch test reaction, metals and fragrances were significantly less persistent than other allergens. For metals, this effect appears to be mostly determined by nickel (persistence rate of 61.4%), as this gave the majority (60.3%) of all metal reactions. In previous studies, the persistence rate of nickel allergy ranged from 54% to 87% (1, 5, 30, 31). Cobalt had a very low persistence rate (35.3%), which is lower than seen in previous reports, in which the persistence rate was also on the low side, ranging from 47% to 57% (15, 31). Potassium dichromate, on the other hand, had a 100% persistence rate (n=11), as compared with a previously reported persistence rate of 63–79% (15, 32). An easy explanation for the low persistence rate of cobalt would be the high proportion of weak positive reactions (15/17): however, 7 of the 11 retested reactions to potassium dichromate were also weak positive (data not shown). There is no obvious explanation for why metals would have a low persistence rate as compared with other allergens. A possible explanation could be tolerization through oral exposure, which has been observed in both experimental animal studies and retrospectively in humans, especially for nickel (33, 34). One caveat, however, was that oral exposure had to take place prior to cutaneous exposure, although it could still be possible that oral exposure (at an appropriate dose) after cutaneous exposure might result in suppression of contact allergy (35).

The persistence rate of fragrances was mostly determined by fragrance mix (FM) I and *Myroxylon pereirae* (balsam of Peru), which have the highest sensitization prevalence in

Europe of all fragrance markers (36). The persistence rate of FM I (64.3%) was similar to that in a previous report, that is, 62% (7). The low persistence rate of fragrances as a group might be merely an artefact resulting from the small sample size. As a retrospective database study, the current study had some inherent limitations. In order to achieve an as large as possible sample size, we enforced no limitation with regard to which allergen was retested, which led to a heterogeneous sample consisting of a wide range of allergens. To assess whether there was any variability in persistence rates between allergen groups, they were pooled together. However, even larger sample sizes are required for any statements to be made on differences in persistence rates between allergens. Another limitation was that, for most patch test readings, data on the relevance of a patch test reaction were lacking. This could help to differentiate false-positive reactions from true-positive reactions.

In conclusion, our study once again confirms that the persistence of a patch test reaction is not 100%, and that non-persistence is particularly an issue for weak positive reactions. It is therefore important to be conservative in advising a patient in the case of a weak positive reaction, especially if the clinical relevance of the reaction is uncertain, as future avoidance of the specific allergen might be unnecessary. A repeated open application test might help to assess clinical relevance. Differences in the results of two consecutive patch tests in a patient might be explained by changing reactivity of a patient's skin, owing to both internal factors (atopic dermatitis activity and age) and external factors (meteorological conditions and ultraviolet radiation). Differences in persistence rates between specific allergens and allergen groups appear to exist, but further investigation is required.

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CHAPTER

5

Comparing patch test results of
methylchloroisothiazolinone/
methylisothiazolinone tested with
both TRUE Test® and 100 ppm
using investigator-loaded chambers

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Methylchloroisothiazolinone (MCI)/methylisothiazolinone (MI), a widely used preservative/biocide, was included in the European baseline series in 1988 at a concentration of 100 ppm (0.01%) in a ratio of 3:1 (1). Although recently it has been recommended to be tested at 200 ppm (0.02%), many centres still test it at 100 ppm (2). MCI/MI is also included in the TRUE Test®, offering an alternative patch test technique. The objective of the current investigation was to compare the diagnostic performance of MCI/MI 0.01% aq. with MCI/MI in the TRUE Test®.

METHODS

Between April 2013 and August 2016, a total of 1122 consecutive patients were patch tested with our departmental baseline series, of whom 1115 (99.4%) were tested simultaneously with both MCI/MI 4 µg/cm² (TRUE Test®; Mekos, Hillerød, Denmark) and MCI/MI 0.01% aq. (Trolab; Almirall Hermal, Reinbek, Germany), corresponding to a dose per unit area of 3 µg/cm², in Van der Bend® square chambers (van der Bend, Brielle, The Netherlands), attached to the back with Fixomull stretch® (BSN Medical, Hamburg, Germany). Twenty microlitres of the aqueous solution of MCI/MI was applied to the chambers with a micropipette. The patch tests were applied on the back for 48 h under occlusion, and readings were performed on day (D) 3 and D7 according to ESCD guidelines (3). The maximum patch test reactions were aggregated as the patch test outcome. In case of a positive reaction (+, ++, or +++), clinical relevance was determined on basis of patient history, clinical examination, and exposure patterns, with possible outcomes being unlikely/not, possible, probable, and certain. Statistical analyses were performed with SPSS (version 23.0; SPSS, Chicago, IL, USA), and guidelines for contact allergy data were followed (4). The McNemar test was used to compare the strength of reactions of both patch test preparations.

RESULTS

The MOAHLFA index for the investigated patient group was as follows: male, 32.6%; occupational dermatitis, 24.1%; atopic dermatitis, 40.9%; hand, 34.0%; leg, 22.4%; face, 3.7%; and age ≥40 years, 57.5%. A total of 14.4% (n=161) of patients had a positive reaction to one of the MCI/MI preparations; 13.6% (95%CI: 11.6–15.6%) had positive reactions to MCI/MI TRUE Test®, and 7.2% (95%CI: 5.7–8.7%) had positive reactions to MCI/MI 3 µg/cm² aq. The patch test reactions to MCI/MI TRUE Test® were significantly stronger (p<0.001). Table 1 shows the patterns of reactions to both patch

test preparations in greater detail. Of all patients with positive reactions to MCI/MI TRUE Test® (n=152), 81 (53.3%) did not have positive reactions to MCI/MI 3 µg/cm² aq. Furthermore, 35 of these 81 reactions were strong to extreme positive reactions. Conversely, of the 80 patients who had positive reactions to MCI/MI 3 µg/cm² aq., 9 (11.3%) did not react to MCI/MI TRUE Test®.

Table 1. Relationship between patch test reactions of MCI/MI TRUE Test® and MCI/MI 0.01% aq.

		MCI/MI 0.01% aq.						Total
		Negative	Irritant	Doubtful	+	++	+++	
MCI/MI TRUE Test	Negative	931	2	14	8	1	0	956
	Irritant	1	0	0	0	0	0	1
	Doubtful	5	0	1	0	0	0	6
	+	37	0	9	15	1	0	62
	++	27	0	2	29	16	0	74
	+++	6	0	0	4	5	1	16
Total		1007	2	26	56	23	1	1115

The clinical relevance of all positive reactions was determined, and is shown in Table 2. Of all positive reactions to MCI/MI TRUE Test®, 88.8% (135/152) had some degree of clinical relevance (ranging from possible to certain), as compared with 87.5% (70/80) of positive reactions to MCI/MI 3 µg/cm² aq. For the 9 patients with positive reactions to MCI/MI 3 µg/cm² aq. but without positive reactions to MCI/MI TRUE Test®, six reactions were considered to be of possible clinical relevance, two of probable clinical relevance, and one of no clinical relevance. Conversely, for the 81 patients with positive reactions to MCI/MI TRUE Test® but without positive reactions to MCI/MI 3 µg/cm² aq., 90.1% (n=73) of reactions had some degree of clinical relevance (39 possible, 24 probable, and 10 certain), three reactions were of unknown clinical relevance, and the remaining five were of no clinical relevance. This means that, if this cohort had been patch tested solely with MCI/MI 3 µg/cm² aq., there would have been 73 missed reactions, constituting 6.5% of all consecutively patch tested patients.

Table 2. The clinical relevance of all positive reactions to either MCI/MI TRUE Test or MCI/MI 0.01% aq.

Clinical relevance	% (n) of patients with a positive reaction to MCI/MI TRUE Test (n=152)	% (n) of patients with a positive reaction to MCI/MI 0.01% aq. (n=80)
Unlikely/not	7.2 (11)	11.3 (9)
Unknown	4.0 (6)	1.3 (1)
Possible	38.8 (59)	32.5 (26)
Probable	27.0 (41)	23.8 (19)
Certain	23.0 (35)	31.3 (25)

DISCUSSION

Although the prevalence of MCI/MI contact allergy in patch test populations remained relatively stable at 2.5% for a long period of time, recent publications have shown it to be rapidly rising (5–8). The most recent publication of the European Surveillance System on Contact Allergies (ESSCA) showed a standardized prevalence of 7.3% for contact allergy to MCI/MI 0.01% in 2013–2014, which is similar to our results (9). More surprising in the current results is the high proportion of positive reactions to the TRUE Test® preparation, which was almost twice as high, at 13.6%. A large majority of these positive reactions were deemed to have at least some degree of clinical relevance, making the possibility of false positives less likely. The concentration of MCI/MI in the TRUE Test®, at 4 µg/cm², is slightly higher than the 3 µg/cm² of MCI/MI 0.01% aq., which explains part of this discrepancy, as MCI/MI has a steep dose–response curve (7). This also illustrates that, besides dose, other factors, such as vehicle, also affect elicitation responses, as MCI/MI is tested in povidone in the TRUE Test® (10). A major limitation of the current study is that MCI/MI was not tested at a concentration of 0.02% (200 ppm or 6 µg/cm²), which would have allowed an even better comparison of the different patch test techniques, but this might be addressed by future studies.

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CHAPTER

6

European Surveillance System on Contact Allergies (ESSCA): polysensitization, 2009–2014

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ABSTRACT

Background

Polysensitization, defined as being allergic to three or more haptens from the European baseline series, is considered to reflect increased susceptibility to developing a contact allergy, and is likely to be associated with an impaired quality of life.

Objectives

To evaluate the prevalences of polysensitization across Europe and to analyse factors associated with polysensitization.

Methods

Patch test data collected by the European Surveillance System on Contact Allergies (ESSCA; www.essca-dc.org) in consecutively patch tested patients from January 2009 to December 2014, comprising 11 countries and 57 departments, were retrospectively analysed.

Results

A total of 86 416 patients were available for analysis, showing a standardized prevalence of polysensitization of 7.02%, ranging from 12.7% (Austria) to 4.6% (Italy). Allergen pairs with the strongest association are reported for the total population, for South Europe, and for North/Central Europe. Overall, polysensitized patients showed a higher percentage of extreme (+++) positive patch test reactions than oligosensitized patients. Female sex, occupational dermatitis and age > 40 years were risk factors for polysensitization.

Conclusions

The varying prevalences of polysensitization across Europe most likely reflect differences in patient characteristics and referral patterns between departments. Known risk factors for polysensitization are confirmed in a European dermatitis population.

Polysensitization, in terms of multiple contact allergies, is currently defined as sensitization to three or more non-related allergens of the European baseline series (EBS) (1). Although arbitrarily defined, this concept is used both to investigate risk factors for developing multiple contact allergies, and to address the topic of increased susceptibility, for example caused by genetic variants (2). For example, polysensitization was shown to be associated with certain genetic markers with links to pathogenetic steps of allergic contact dermatitis (ACD) (3, 4), reflecting increased susceptibility independently from actual haptens 5, which implies that allergen-specific susceptibilities should be considered as well (5, 6). Polysensitization has been extensively reviewed by Carlsen et al. and Schnuch et al. (7, 8). The main cause of acquiring multiple sensitivities is apparently high exposure to environmental allergens, for example by occupational exposure, or pre-existing inflammatory dermatoses conveying 'danger signals' [e.g. in patients with leg (stasis) dermatitis or leg ulcers] (9). Studies have shown, however, that polysensitized individuals, when sensitized in an experimental setting, have a lower elicitation threshold and generally show stronger elicitation reactions than single/double-sensitized individuals, providing arguments for different susceptibilities between these groups (10, 11).

Although quality of life has not been thoroughly investigated in polysensitized patients, one case–control study investigating quality of life in fragrance-allergic dermatitis patients as compared with dermatitis patients with no fragrance allergy found an increase in impairment in quality of life with an increasing number of positive patch test reactions to fragrance allergens (12, 13). Polysensitized individuals have a higher probability of relevant exposure, and therefore it is not difficult to imagine that they suffer more from persistent dermatitis and frequent relapses of their dermatitis.

The reported prevalences of polysensitization in patch test populations depends greatly on the tested population and the length of the tested baseline series, yielding prevalences ranging from 5.0% to almost 20.0% (this last result was based on a baseline series consisting of 73–80 allergens) (1, 11, 14). The prevalence of polysensitization in the largest reported group, that of the Information Network of Departments of Dermatology (IVDK) in Germany, has been found to be stable around 10.0% (15, 16). In one study, the prevalence of polysensitization found in a small general population sample was 0.7% (17).

The objective of this study was to evaluate prevalences of polysensitization in different European countries. We also aimed to evaluate factors associated with polysensitization in patients who were patch tested at departments of the European Surveillance System on Contact Allergies (ESSCA) network in the period from 2009 to 2014.

METHODS

The analysis is based on clinical data collected by the ESSCA network, as described in previous publications (18, 19). Briefly, clinical and demographic data, along with patch test results, of all patients patch tested in the departments participating in the ESSCA for suspected ACD caused by various potential exposures are documented electronically in the local departments. These use diverse data capture software and, partly, the multilingual software WINALLDAT/ESSCA provided by the ESSCA (20). Standardized patch testing follows international recommendations (21). The anonymized data delivered by the participants are pooled in the ESSCA data centre in Erlangen for further analysis, (22) with R (version 3.2.3) software (www.r-project.org, last accessed 22 March 2017). Pertinent guidelines for the statistical analysis of patch test data were considered (23, 24).

For analysis, the maximum patch test reactions between day (D) 3 and D5 (inclusive) were aggregated as the patch test outcome. Reactions designated as either +, ++ or +++ were classified as positive (allergic), and the remainder were classified as non-allergic. The study period was January 2009 to December 2014, and included, for the present analysis, 11 European countries and, in total, 57 departments.

Test results obtained with the EBS valid in the study period, during which methylisothiazolinone (MI) 2000 ppm aq. had been added, and the recommended test concentration of methylchloroisothiazolinone (MCI)/MI had been increased from 100 to 200 ppm aq., and that of formaldehyde had been increased from 1% to 2% aq., were analysed (25). Altogether, 86 416 patients were registered who were tested with the EBS and whose tests were read at least between D3 and D5. The TRUE Test® employing a hydrocellulose matrix for the haptens instead of pet. or aq. was used in a relatively small number of consultations, namely 3649, with the vast majority of patients being tested with pet.-based and aq.-based haptens and investigator-loaded chamber systems, respectively. Moreover, two German departments used a 1-day patch test exposure, applied to 2870 patients. As in previous analyses, the impact of

these variations of the standard technique was found to be limited, so the results have all been pooled (26).

Concerning the allergens of the EBS, which form the basis of the present analysis of polysensitization, the following rules were applied for counting of the number of individual positive patch test reactions:

- Positive reactions to fragrance mix II and hydroxyisohexyl 3-cyclohexene carboxaldehyde (HICC) were pooled, that is, counted as one reaction if one or the other, or both, were positive.
- The same rule was applied to reactions to mercapto mix 1% and 2% pet. (which were never both tested in one patient) and reactions to 2-mercaptobenzothiazole tested additionally.
- As a marker concerning local anaesthetics, benzocaine is tested in the current EBS, whereas caine mix III is tested by a large number of ESSCA members because of its better diagnostic yield; the positive reaction(s) were counted as one.
- Likewise, sesquiterpene lactone mix is included in the current EBS, but (partly differing versions of) Compositae mix were considered to be equivalent, and positive reaction(s) were counted as one.
- Partly, the mixture of methyldibromo glutaronitrile (MDBGN) and 2-phenoxyethanol 1:4, for example Euxyl® K 400, was tested instead of, or in addition to, MDBGN. All positive reactions were pooled into one.
- In view of corresponding dithiocarbamates and thiurams being redox pairs (27), positive reactions both to zinc diethyldithiocarbamate (ZDEC) or carba mix – which were never both tested in one patient – and thiuram mix were counted as one.
- Although it could be debated whether positive reactions to formaldehyde and various formaldehyde releasers are truly equivalent, as isolated reactions to a releaser might be attributable to the donor molecule, these were also pooled, which concerned quaternium-15, which is presently the only formaldehyde releaser contained in the EBS.

Besides polysensitization, prevalences are also provided for oligosensitization, defined as having one or two positive patch test reactions, but not more, and for 'at least one positive', also known as the 'P' measure (28). For the polysensitized group and for all patients with no positive patch test reactions to the EBS, the MOAHLFA (Male,

Occupational dermatitis, Atopic dermatitis, Hand dermatitis, Face dermatitis, Leg dermatitis, Age ≥ 40 years) index is given (29).

To evaluate regional differences for concomitant reactions, that is, pairs of positive reactions contributing to polysensitization (30), or 'oligosensitization', countries were classified into two regions, as previously done by Schuttelaar et al. (31). Two regions were defined: North/Central Europe, comprising Finland, Lithuania, Poland, Germany, Austria, Switzerland, The Netherlands, and the United Kingdom; and South Europe, comprising Spain, Italy, and Slovenia.

Besides descriptive statistics, adjusted multifactorial analyses were employed to quantify the independent association of potentially relevant explanatory factors and oligosensitization and polysensitization, respectively. To this end, log-binomial regression analysis was utilized, which quantifies the association by means of a prevalence ratio (PR), which can be interpreted as a representation of relative risk. Point estimates were accompanied by 95% confidence intervals (CIs) obtained with the profile likelihood method. The set of explanatory variables considered included the country, sex, age (dichotomized as < 40 years versus ≥ 40 years), occupational aetiology of contact dermatitis ('yes' or 'partly' versus 'no' or 'unknown'), atopic dermatitis, past or present, versus no atopic dermatitis, and the anatomical sites hand, leg, face, trunk (as reference), and 'other'. Moreover, the number of allergens in the baseline series tested was used as an adjustment factor, as the likelihood of one (or more) positive reactions depends also on the number of allergens tested (32).

RESULTS

In the years 2009–2014, altogether 86 416 patients were patch tested in the 57 European departments. The characteristics of the patients, and univariate results obtained with the EBS allergens, have been published in a number of publications covering the 2009–2012 study period and the 2013–2014 study period, respectively. For further information, the reader is referred to these (26, 33–38).

Prevalences of polysensitization across Europe

Crude and age- and sex-standardized prevalences for polysensitization and oligosensitization, stratified per country, are presented together with 95%CIs in Table 1. In order to put these results into perspective, prevalences for at least one

positive reaction ('P' measure) and for negative patch test results are also given (28). No large differences were seen between crude and standardized prevalences. Overall, the standardized prevalence of polysensitization was 7.05%, with the highest standardized prevalences being seen in Austria (12.7%) and The Netherlands (12.4%), and the lowest in Italy (4.6%) and Lithuania (5.2%). The standardized 'P' measure, presented as 'at least one positive reaction' to the EBS allergens, was 43.0% for the total population, with considerable variation between countries; from 52.9% in The Netherlands to 38.6% in the United Kingdom.

Table 1. Crude and age- and sex-standardized prevalences of patch tested patients in the European Surveillance System on Contact Allergies (ESSCA) database between January 2009 to December 2014 with negative patch tests, at least one positive patch test reaction ('P' measure), oligosensitisation, and polysensitisation, given per country.

Country	n (total)	Negative		at least 1 positive ('P' measure)		Oligosensitisation		Polysensitisation	
		n (%)	std. % (95% CI)	n (%)	std. % (95% CI)	n (%)	std. % (95% CI)	n (%)	std. % (95% CI)
Austria	1622	784 (48.34)	49.92 (47.32 - 52.52)	838 (51.66)	50.08 (47.48 - 52.68)	625 (38.53)	37.81 (35.29 - 40.33)	213 (13.13)	12.27 (10.61 - 13.93)
Switzerland	7508	3955 (52.68)	52.71 (51.53 - 53.89)	3553 (47.32)	47.29 (46.11 - 48.47)	2842 (37.85)	38.23 (37.07 - 39.38)	711 (9.47)	9.06 (8.39 - 9.74)
Germany	11243	6785 (60.35)	60.55 (59.56 - 61.54)	4458 (39.65)	39.45 (38.46 - 40.44)	3696 (32.87)	33.21 (32.25 - 34.16)	762 (6.78)	6.24 (5.77 - 6.71)
Spain	6567	3626 (55.22)	56.2 (54.95 - 57.44)	2941 (44.78)	43.8 (42.56 - 45.05)	2512 (38.25)	37.46 (36.25 - 38.68)	429 (6.53)	6.34 (5.73 - 6.95)
Finland	1289	661 (51.28)	50.83 (48.09 - 53.57)	628 (48.72)	49.17 (46.43 - 51.91)	513 (39.8)	40.22 (37.51 - 42.93)	115 (8.92)	8.94 (7.37 - 10.52)
Italy	12893	7483 (58.04)	58.38 (57.54 - 59.23)	5410 (41.96)	41.62 (40.77 - 42.46)	4840 (37.54)	37.24 (36.41 - 38.06)	570 (4.42)	4.38 (4.03 - 4.73)
Lithuania	1424	784 (55.06)	56.84 (54.08 - 59.6)	640 (44.94)	43.16 (40.4 - 45.92)	554 (38.9)	37.91 (35.18 - 40.64)	86 (6.04)	5.25 (4.11 - 6.39)
Netherlands	7001	3268 (46.68)	47.49 (46.31 - 48.66)	3733 (53.32)	52.51 (51.34 - 53.69)	2848 (40.68)	40.35 (39.19 - 41.51)	885 (12.64)	12.17 (11.41 - 12.92)
Poland	5527	2686 (48.6)	49.38 (48.05 - 50.7)	2841 (51.4)	50.62 (49.3 - 51.95)	2265 (40.98)	40.52 (39.21 - 41.82)	576 (10.42)	10.11 (9.31 - 10.9)
Slovenia	8640	5230 (60.53)	61.27 (60.24 - 62.3)	3410 (39.47)	38.73 (37.7 - 39.76)	2846 (32.94)	32.48 (31.49 - 33.48)	564 (6.53)	6.25 (5.74 - 6.75)
United Kingdom	22702	13745 (60.55)	61.85 (61.23 - 62.48)	8957 (39.45)	38.15 (37.52 - 38.77)	7677 (33.82)	32.84 (32.23 - 33.44)	1280 (5.64)	5.31 (5.03 - 5.6)
Total	86416	49007 (56.7)	57.41 (57.08 - 57.74)	37409 (43.3)	42.59 (42.26 - 42.92)	31218 (36.1)	35.75 (35.43 - 36.08)	6191 (7.2)	6.84 (6.67 - 7.01)

'P' measure, the proportion of patch tested patients with at least one positive patch test reaction; Oligosensitisation, the proportion of patch tested patients with one or two positive patch test reactions; Polysensitisation, the proportion of patch tested patients with three or more positive patch test reactions to unrelated allergens.

Of the total polysensitized population, the proportion of males aged <40 years was 6.5%, and the proportion of females aged <40 years was 24.3% (Table 2). The proportion aged ≥ 40 years and male was 19.9%, and the majority were female and aged ≥ 40 years (49.3%). These proportions were largely similar in the oligosensitized population, except that this population was younger. The MOAHLFA index of the polysensitized population is shown in Table 3, together with the total patch tested population as a reference 29. For most of the categories of the MOAHLFA index, the polysensitized group was similar to the total patch tested population, except that the percentage of females was slightly higher for the polysensitized group, and a higher percentage were aged ≥ 40 years. This difference in age distribution was most pronounced in the United Kingdom, whereas, in Spain and Poland, the percentages were almost the same for the polysensitized and the total patch tested population. The distribution of individuals with (a history of) atopic dermatitis was equal between the totals of both groups; however, country-specific differences could be seen. The primary site of dermatitis seemed to be distributed similarly in both groups overall, as well as for all of the individual countries.

Table 2. Age and Sex of patch tested patients in the European Surveillance System on Contact Allergies (ESSCA) database between January 2009 to December 2014 per country per polysensitised group; n (%). Results for patients without positive patch test reactions (negative) and for oligosensitisation are also presented as reference. M, male; F, female.

Country	n(total)	Negative			Oligosensitisation			Polysensitisation		
		M <4.0	M ≥4.0	F <4.0	F ≥4.0	M <4.0	M ≥4.0	F <4.0	M ≥4.0	F <4.0
Austria	1622	82 (10.5)	165 (21)	197 (25.1)	340 (43.4)	42 (6.7)	97 (15.5)	176 (28.2)	310 (49.6)	11 (5.2)
Switzerland	7508	694 (17.5)	1085 (27.4)	722 (18.3)	1454 (36.8)	270 (9.5)	613 (21.6)	683 (2.4)	1276 (44.9)	46 (6.5)
Germany	11243	934 (13.8)	2063 (30.4)	1171 (17.3)	2617 (38.6)	370 (10)	971 (26.3)	725 (19.6)	1630 (44.1)	48 (6.3)
Spain	6567	469 (12.9)	891 (24.6)	855 (23.6)	1411 (38.9)	197 (7.8)	413 (16.4)	651 (25.9)	1251 (49.8)	28 (6.5)
Finland	1289	152 (23)	162 (24.5)	162 (24.5)	185 (28)	78 (15.2)	98 (19.1)	141 (27.5)	196 (38.2)	10 (8.7)
Italy	12893	1465 (19.6)	1417 (18.9)	2163 (28.9)	2438 (32.6)	615 (12.7)	573 (11.8)	1728 (35.7)	1924 (39.8)	51 (8.9)
Lithuania	1424	76 (9.7)	118 (15.1)	177 (22.6)	413 (52.7)	27 (4.9)	56 (10.1)	189 (34.1)	282 (50.9)	0 (0)
Netherlands	7001	561 (17.2)	692 (21.2)	952 (29.1)	1063 (32.5)	312 (11)	520 (18.3)	866 (30.4)	1150 (40.4)	57 (6.4)
Poland	5527	414 (15.4)	526 (19.6)	718 (26.7)	1028 (38.3)	237 (10.5)	286 (12.6)	812 (35.8)	930 (41.1)	48 (8.3)
Slovenia	8640	817 (15.6)	1057 (20.2)	1430 (27.3)	1926 (36.8)	285 (10)	396 (13.9)	900 (31.6)	1265 (44.4)	35 (6.2)
United Kingdom	22702	2218 (16.1)	2800 (20.4)	4218 (30.7)	4509 (32.8)	560 (7.3)	1215 (15.8)	2416 (31.5)	3486 (45.4)	58 (4.5)
Total	86416	7882 (16.1)	10976 (22.4)	12765 (26)	17384 (35.5)	11635 (9.6)	13112 (16.8)	16021 (29.7)	18899 (43.9)	392 (6.3)

11635 (9.6) 13112 (16.8) 16021 (29.7) 18899 (43.9) 392 (6.3) 1219 (19.7) 1516 (24.5) 3064 (49.5)

Table 3. MOAHLFA index per country of patch tested patients in the European Surveillance System on Contact Allergies (ESSCA) database between January 2009 to December 2014 with polysensitisation (first row per country) and all patients tested (second row per country).

Country	(Sub-)group	n(total)	% M	% O	% A	% H	% L	% F	% A
Austria	Polysens.	213	20.2	18.8	29.1	9.4	2.8	7.5	70.9
	All tested	1622	26.4	16.8	21.3	10.1	1.8	5.4	65.5
Switzerland	Polysens.	711	28.7	15	24.6	8.3	2.3	5.5	74.1
	All tested	7508	38.2	12.9	19.6	9.6	2.2	6.5	66
Germany	Polysens.	762	32.8	33.9	26.5	36	6.3	9.7	80.2
	All tested	11243	40.8	32.4	26.4	36.2	5.1	9.3	70.2
Spain	Polysens.	429	30.3	18.9	12.1	31.7	7.2	11.9	67.8
	All tested	6567	32	13.1	14.4	27.5	7.5	13.9	64.8
Finland	Polysens.	115	27.8	34.8	33	57.4	4.3	0.9	67
	All tested	1289	40.5	47.6	30.3	64.2	2.9	4.7	55.7
Italy	Polysens.	570	26.8	7.7	13.7	25.6	4.7	11.9	60.2
	All tested	12893	32.8	5.3	16.8	21.6	6.6	11.6	51.9
Lithuania	Polysens.	86	11.6	14	10.5	18.6	23.3	32.6	70.9
	All tested	1424	20.2	12.7	11.5	25.5	11.4	24.9	65.3
Netherlands	Polysens.	885	26.4	22.5	38.6	22.3	4.4	18.3	67
	All tested	7001	33.1	17.8	34.6	19.7	4.4	18.9	57.4
Poland	Polysens.	576	20.3	25.7	17.4	22.2	3.5	11.1	57.8
	All tested	5527	28.6	17.7	16	22.3	3.4	11.2	56.1
Slovenia	Polysens.	564	22.7	—	—	—	—	—	66.5
	All tested	8640	31.1	—	—	—	—	—	58.1
United Kingdom	Polysens.	1280	24.2	9.1	31.6	29.5	4.5	26.8	72

Table 3. Continued

Country	(Sub-)group	n(total)	% M	% O	% A	% H	% L	% F	% A
Total	All tested	22702	31.3	8.7	36.1	26	5.4	27.8	57
	Polysens.	6191	26	20.8	26	30.5	5.8	18.2	69.2
	All tested	86416	33.2	16.3	25.7	29.2	6.1	19.2	59.7

M, Male; O, Occupational aetiology; A, Atopic dermatitis (history of); H, Hand dermatitis; L, leg dermatitis; F, face dermatitis; A, age over 40

The most common allergen pairs in polysensitized individuals and the strengths of patch test reactions

Within the polysensitized group, odds ratios (ORs) were calculated for all possible allergen pairs to evaluate concomitant positive patch test reactions. This was performed for the total population, and separately for South Europe and North/Central Europe as defined above, in order to observe any differences based on geographical location. The top 10 allergen pairs per region ranked by the highest OR are shown in Table 4. Overall, associations were stronger in South Europe than in North/Central Europe. For both regions, the top two concomitant reactions were those to quaternium-15/formaldehyde and carba mix/thiuram mix. The most probable reason for the concomitant reactions is also suggested in the footnotes to Table 4.

Table 4. Top 10 allergen pairs defined by the highest odds ratios (OR) for the total population and per 'European region', accompanied by a 95% confidence interval (CI), in patch tested patients in the European Surveillance System on Contact Allergies (ESSCA) database between January 2009 to December 2014 who were polysensitised. South Europe; Spain, Italy, and Slovenia. North/Central Europe; Finland, Lithuania, Poland, Germany, Austria, Switzerland, The Netherlands, and the United Kingdom.

Total population			South Europe			North/Central Europe		
Allergen pair	OR	(95% CI)	Allergen pair	OR	(95% CI)	Allergen pair	OR	(95% CI)
Quaternium15 - formaldehyde ^a	56.31	(46.55 - 68.11)	Quaternium15 - formaldehyde ^a	71.62	(48.36 - 106.07)	Quaternium15 - formaldehyde ^a	49.75	(40.01 - 61.86)
Carbamix - Thiuram mix ^a	30.84	(26.44 - 35.97)	Carbamix - Thiuram mix ^a	33.77	(22.42 - 50.86)	Carbamix - Thiuram mix ^a	32.44	(27.4 - 38.4)
mercaptopbenzothiazole/mercaptop mix - Thiuram mix ^c	13.15	(10.91 - 15.86)	Primin - Clioquinol ^d	24.05	(5.51 - 104.95)	Primin - Tixocortol-21-pivalate ^d	12.66	(7 - 22.91)
Tixocortol-21-pivalate - Budesonide ^b	12.77	(8.91 - 18.31)	Tixocortol-21-pivalate - Clioquinol ^b	23.43	(3.03 - 181.34)	mercaptopbenzothiazole/mercaptop mix - Thiuram mix ^c	11.81	(9.59 - 14.55)
Fragrance mix II/llyral - Fragrance mix ^b	12.21	(11.33 - 13.16)	Tixocortol-21-pivalate - Budesonide ^b	23.29	(8.98 - 60.39)	Tixocortol-21-pivalate - Budesonide ^b	11.38	(7.69 - 16.84)
Primin - Clioquinol ^d	12.12	(4.35 - 33.75)	Benzocaine/Caine mix - para-phenylenediamine ^a	20.6	(15.27 - 27.79)	Potassium dichromate - Cobalt ^b	11.15	(10.11 - 12.3)
Benzocaine/Caine mix - para-phenylenediamine ^a	11.97	(9.92 - 14.44)	Fragrance mix II/llyral - Fragrance mix ^b	18.11	(15.26 - 21.49)	Fragrance mix II/llyral - Fragrance mix ^b	11.04	(10.16 - 12)
Potassium dichromate - Cobalt ^b	11.80	(10.91 - 12.76)	mercaptopbenzothiazole/mercaptop mix - Thiuram mix ^c	17.17	(11.24 - 26.24)	Primin - mercaptopbenzothiazole/mercaptop mix ^b	10.78	(5.56 - 20.89)
Tixocortol-21-pivalate - Clioquinol ^b	10.95	(4.70 - 25.53)	N-Isopropyl-N-phenyl-4-phenylenediamine - para-phenylenediamine ^a	16.53	(11.26 - 24.26)	Clioquinol - Paraben mix ^b	10.4	(3.67 - 29.46)

Table 4. Continued

Allergen pair	Total population		South Europe		North/Central Europe	
	OR	(95% CI)	Allergen pair	OR	Allergen pair	OR
N-Isopropyl-N-phenyl-4-phenylenediamine - para-phenylenediamine ^a	10.87	(8.55 - 13.82)	Clioquinol - Balsam of Peru ^b	14.94	Neomycin - Tixocortol-21-pivalate ^b	9.77

^a cross-reaction; ^b co-sensitisation; ^c false cross-reaction; ^d neither cross-reaction or exposure related

The most commonly positive EBS allergens in polysensitized patients are shown and compared with those in oligosensitized patients in Table S1. There were no large differences regarding which allergens were the most commonly positive between the two groups.

The distribution of grades of positive reactions, from weak positive to extreme positive, in oligosensitized and polysensitized individuals stratified per country is shown in Table 5. Overall, for almost all countries, the polysensitized population had a slightly higher percentage of extreme positive (+++) reactions than the oligosensitized population. The percentage of strong positive (++) reactions was comparable between both populations, except for Finland, where the oligosensitized population had a higher percentage of strong positive reactions than the polysensitized population. Large differences between countries can also be observed; for example, the percentage of weak positive reactions in the polysensitized population in The Netherlands was 81.6%, as compared with only 21.0% in Spain.

Table 5. The distribution of grades of positive reactions (+, ++ vs. +++) in oligo- and polysensitized patients in the European Surveillance System on Contact Allergies (ESSCA) database between January 2009 to December 2014, respectively, stratified for country. In this analysis, Slovenia is not included as the departments only contributed data on reactions being positive, or negative, i.e., no reaction grades. N(pat.), number of patients oligo-/polysensitized; N(rea.), total number of reactions of any grade, i.e. multiple occurrences possible (up to 2 identical or differing reaction grades in oligosensitized, up to the observed maximum of 15, and minimum 3, in polysensitized).

Country	Oligosensitisation					Polysensitisation				
	N(pat.)	N(rea.)	% +	% ++	% +++	N(pat.)	N(rea.)	% +	% ++	% +++
Austria	625	860	79	20.5	0.6	213	824	81.6	16	2.4
Switzerland	2842	3760	57.8	31.1	11.1	711	2648	57.6	28	14.4
Germany	3696	4824	67	25.9	7.1	762	2860	62.4	26.8	10.7
Spain	2512	3357	21.7	60.6	17.6	429	1555	21	57.9	21.2
Finland	513	671	25.6	68.3	6.1	115	404	40.1	54.2	5.7
Italy	4840	6142	53.2	37.8	9	570	1919	54.9	34.7	10.4
Lithuania	554	728	48.1	36.8	15.1	86	317	51.4	33.1	15.5
Netherlands	2848	3866	81.6	16.4	2	885	3442	81.2	16.1	2.7
Poland	2265	3153	41.5	38.9	19.6	576	2103	40.8	38.1	21.2
United Kingdom	7677	9968	60.1	36.6	3.4	1280	4536	60.6	35.6	3.8
Total	28372	37329	56.4	35.3	8.3	5627	20608	58.7	31.6	9.8

The distribution of grades of positive reactions, per number of positive reactions, for the total population is shown as a stacked bar plot in Fig. 1. A clear positive correlation can be seen between the number of positive reactions and the percentage of extreme positive (+++) reactions.

Figure 1.

The distribution of reaction grades according to the number of positive reactions to allergens of the departmental baseline series, each summed up to 100%. The number of patients having one, two and more positive reactions is indicated on the top x-axis. One patient each with 15 and 12 positive reactions, 3 patients with 11 positive reactions and 6 patients with 10 positive reactions are omitted for clarity.

Analysis of risk factors for polysensitization

To assess the influence of country, sex, occupational contact dermatitis (OCD), atopic dermatitis, age ≥ 40 years and primary site of dermatitis on the risk of being polysensitized, a log-binomial regression analysis was performed (Table 6). The same

regression analysis was performed on the oligosensitized group for comparison. For quantification of the risk of being either oligo- or polysensitized associated with the country an individual has been patch tested in, Switzerland was chosen as reference, because it is situated between the 2 chosen European regions. The association between country and being oligosensitized was minimal; it was lower in Germany and the United Kingdom (PR = 0.79 and PR = 0.82, respectively) than in the reference country, Switzerland. In contrast, the association between country and being polysensitized was more pronounced. The risk of being polysensitized appeared to be significantly lower for most countries than for Switzerland, and was especially low in Germany (PR = 0.64), Italy (PR = 0.59), and the United Kingdom (PR = 0.54). Conversely, this risk was significantly higher for Austria (PR = 1.71) and The Netherlands (PR = 1.27).

Table 6. Log-binomial regression analysis with Oligosensitisation (left) and polysensitisation (right), respectively, as outcome. As Slovenian departments could not contribute information on anatomical site, these were omitted from the analysis. CI, confidence interval.

Factor	%(total)	Oligosens.		Polysens.	
		PR	95% CI	PR	95% CI
Switzerland	8.7	1.00	(reference)	1.00	(reference)
Austria	1.9	1.1	(0.99-1.22)	1.82	(1.44-2.27)
Germany	13	0.79	(0.74-0.83)	0.63	(0.54-0.74)
Spain	7.6	0.94	(0.89-1)	0.77	(0.66-0.91)
Finland	1.5	0.95	(0.88-1.03)	0.9	(0.73-1.12)
Italy	14.9	0.95	(0.9-1)	0.6	(0.51-0.7)
Lithuania	1.6	0.98	(0.9-1.06)	0.77	(0.59-0.99)
Netherlands	8.1	1.04	(0.98-1.1)	1.3	(1.12-1.51)
Poland	6.4	1	(0.93-1.06)	1.28	(1.09-1.52)
United Kingdom	26.3	0.82	(0.77-0.86)	0.55	(0.47-0.64)
Male Sex	33.2	0.69	(0.68-0.71)	0.58	(0.55-0.62)
Occupational Dermatitis	16.3	1.23	(1.19-1.27)	1.51	(1.4-1.64)
Atopic Dermatitis	25.7	0.92	(0.9-0.95)	1.08	(1.01-1.16)
Age 40+	59.7	1.1	(1.08-1.12)	1.69	(1.59-1.81)
Site: Trunk	9.7	1.00	(reference)	1.00	(reference)
Site: Hand	29.2	1.02	(0.99-1.07)	1.12	(1-1.26)
Site: Leg	6.1	0.91	(0.86-0.96)	1	(0.86-1.16)
Site: Face	19.2	1.03	(0.99-1.07)	1.03	(0.92-1.15)
Site: Other	35.8	0.98	(0.95-1.02)	1.03	(0.92-1.14)

Low PRs for male sex in both the oligosensitized and polysensitized groups (0.7 and 0.59, respectively) indicated that females had a significantly increased risk of being sensitized overall, and of being polysensitized in particular. Also, OCD was significantly associated with both oligosensitization and polysensitization, and again this association was even stronger for polysensitization. For atopic dermatitis, a small, significant association was seen for polysensitization, but not for oligosensitization. The highest PR for polysensitization was observed for age ≥ 40 years (1.71). Of the primary sites of dermatitis, only hand dermatitis (PR = 1.15) was found to be significantly associated with polysensitization when compared with trunk dermatitis as the reference. Leg dermatitis was inversely significantly associated with oligosensitization (PR = 0.92).

DISCUSSION

Prevalences of polysensitization

For the first time, prevalences of polysensitization are presented for different countries across Europe. Overall, the standardized prevalence of polysensitization was 7.1%, and the standardized prevalence of oligosensitization was 36.3%. The 'P' measure in this dataset was 43.0%, ie., the same as the 43% seen in the ESSCA population patch tested in 2005–2006 (39). Note that the 'P' measure includes, by definition, both oligosensitized and polysensitized patients, so similarities in association patterns are to be expected. There is considerable variation in polysensitization and oligosensitization between countries; besides reflecting actual sensitization frequencies and the differences thereof between countries, it can also mainly be explained by the varying eligibility criteria for patch testing between departments. Differences between countries and departments in relation to selection processes and health systems have been discussed in a previous ESSCA publication (26). Another explanatory variable is the length of the baseline series tested in a department; 'a bigger net catches more fish'. However, in the present analysis, this effect has a ceiling, in the sense that only allergens of the EBS as currently recommended were considered (25), together with possible substitutes or additions as detailed above, but not any length of departmental baseline series. Nevertheless, as some national groups or departments systematically omit haptens from the EBS, some adjustment for, basically, a lower number of allergens tested in patients (from one country and department, respectively) was necessary, and was employed in the regression analysis. However, the putatively lower sensitivity (and thus lower prevalence yielded) in those departments still using 100 ppm MCI/MI

and 1% formaldehyde, respectively, has not been separately addressed, as the effect is regarded as being relatively minor.

The high prevalence of polysensitization in Austria and The Netherlands may be explained by more restrictive patch testing (i.e. more selective) and different patterns of referral, for example in The Netherlands. It seems unlikely that these countries actually have a higher prevalence of polysensitization, as it does not seem obvious that individuals in these countries have higher exposure to baseline allergens than, for example, individuals in Italy or the United Kingdom. Additional explanations for variation in polysensitization prevalences seen between countries are variations in the factors described in the MOAHLFA index, that is, variations in proportions of atopic dermatitis, site of dermatitis and OCD in the patch tested population between countries. Many of these factors are positively or inversely related to polysensitization, as discussed below. The effects of age and sex, which certainly have an influence on polysensitization, on differences in prevalences between countries have been eliminated by using age- and sex-standardized prevalences, and by adjusting for these factors in the multifactorial analysis, respectively.

Taking these findings together, it appears that differences in polysensitization prevalences between countries are most likely explained by differences in patch tested populations, possibly patch test methodology, and the length of the baseline series (within the limit of the actual EBS set by the present analysis; see above). Therefore, studies investigating this topic have to take into account these factors when comparing their results with those of studies performed in a different department.

Similarly to what has been found in previous studies, the polysensitized population has a higher proportion of elderly individuals and females than patients with negative patch test results (1, 40). This is further supported by the present regression analysis, which shows that being male is a significant risk-lowering factor for polysensitization, and being aged > 39 years is a significant risk-increasing factor, most likely because of the effect of cumulative lifetime exposure. Furthermore, the present analysis was based on allergens of the EBS for better comparison, and the EBS may include some allergens of old relevance (such as clioquinol, primin, and neomycin) and does not rapidly adopt new allergens. Because of this, the overall yield of the EBS may be higher in the older population than in the younger population, and so will the odds of being polysensitized.

Occupational dermatitis

Occupational dermatitis is a known risk factor for polysensitization; this is most likely attributable to increased (work-related) exposure to multiple allergens (15, 41). Another risk factor for occupational dermatitis is wet work, which increases the risk of acquiring contact allergy. After age, OCD was most strongly associated with polysensitization, with a PR of 1.57. Certain countries contributed a higher proportion of OCD than others, for example Germany, Finland, and Poland, all three of which have contributions from departments specialized in occupational dermatology (33). A higher percentage of occupational aetiology of dermatitis in the polysensitized population than in the total patch tested population can be seen in all countries, except for Finland, where only 34.8% of the polysensitized group had OCD, as compared with 47.6% of all tested individuals. It is unclear whether this is simply an anomaly, but, without further investigation, there appears to be no easy explanation.

Atopic dermatitis

In a case–control questionnaire study focusing on polysensitization, 45.1% of polysensitized individuals had a history of atopic dermatitis, as compared with 31.0% of oligosensitized individuals (40). Another study, however, found similar proportions of atopic dermatitis in polysensitized and oligosensitized populations, which we also found (42). Atopic dermatitis has been reported to be a risk factor for polysensitization (15) Whether atopic dermatitis patients are at higher or lower risk of developing contact allergy has not been completely elucidated yet; because of immunological differences, atopic dermatitis patients are less easy to sensitize in an experimental setting; however, this appears to be mitigated by easier penetration of allergens, owing to an impaired skin barrier (43), and increased exposure, at least to those haptens encountered in the treatment of atopic eczema. Regarding geographical differences, both Italy and Spain (South Europe) have relatively low percentages of atopic dermatitis. Also of interest is that, in these countries, a lower percentage of atopic dermatitis is seen in the polysensitized population than in all individuals tested. The only other country with this observation is the United Kingdom; in all other countries, the opposite is found.

Sites of dermatitis

Given the findings of previous studies investigating the relationship between primary site of dermatitis and polysensitization, it is perhaps surprising that only one of the sites analysed in the present study was related to polysensitization. One possible explanation might be that only five sites were analysed, as opposed to previous studies,

in which between 12 and 19 sites were investigated (15, 44). Some sites previously found to be associated with polysensitization might have been combined with other areas in the present analysis, averaging out the previously found associations. One site of dermatitis that is widely accepted to be a risk factor for polysensitization is the lower leg, because of increased exposure to topical medicaments in patients with leg stasis dermatitis and/or chronic leg ulcers (9). In the present analysis, however, the site 'legs' also included the upper legs, knees, and popliteal fold. Another reason for the lack of association between the legs and polysensitization might be the decline in frequency of contact allergy in patients suffering from chronic leg ulcer/stasis dermatitis (45). Alternatively, or additionally, it might be that polysensitized patients suffer from more generalized dermatitis, as suggested by one study, but not confirmed by later studies (44, 46). Dermatitis of the hands is a well-known risk factor for polysensitization, and this was once again confirmed in the present analysis (15, 30, 44). More interesting, perhaps, are the country-specific differences; especially in Finland and Lithuania, the proportion of patients with hand dermatitis was lower in the polysensitized group than in all patch tested patients in those countries. This might be related to the difference in the proportion of OCD between the polysensitized and oligosensitized patients in Finland (discussed above), as hand dermatitis often has an occupational cause (41).

Concomitant reactions in polysensitized individuals

Concomitant patch test reactions can be explained by various mechanisms; co-sensitization, resulting from concomitant exposure to unrelated allergens, or cross-reactivity, resulting from structural similarities between allergens. Several studies have investigated associations between allergens, most recently in a publication by the IVDK (16, 18, 30, 36). Most pairs can be explained by either co-sensitization through simultaneous exposure (for example, at least historically, cobalt and potassium dichromate in cement), or by cross-reactivity [*p*-phenylenediamine (PPD)–N-isopropyl-N'-phenyl-4-phenylenediamine and PPD–benzocaine], or both (8, 47). Co-sensitization can result from either exposure to a product containing different non-related allergens (e.g. topicals containing fragrances or preservatives), or concomitant exposure to different products, for example in specific occupations. The two most common pairs in our analysis, in both North/Central and South Europe, were quaternium-15–formaldehyde and carba mix–thiuram mix, both with high ORs, providing further justification for counting positive reactions to these pairs as one in the analysis of polysensitization. In a previous study, 73% of patients with contact allergy to formaldehyde also reacted to quaternium-15, and, conversely, 59% of

quaternium-15-positive patients also reacted to formaldehyde (48). Concomitant reactions between dithiocarbamates and thiurams is a common finding; the OR of reactions to zinc diethyldithiocarbamate being coupled with reactions to thiuram mix was 166.43 (i.e., extremely high) in a previous study (16).

Ingredients of topical medicaments were often seen in the top 10 of both regions. One study showed that a larger proportion of polysensitized patients than of oligosensitized patients suffer from dermatitis, and that the dermatitis is of longer duration (for the group without atopic dermatitis) (1). Therefore, this group might have more intense and longer exposure to several topical medicaments than oligosensitized patients. An interesting observation is that three pairs contain primin, twice together with a topical medicament, and once with mercaptobenzothiazole–mercapto mix. Primin is the main allergen of *Primula obconica* H., and primin allergy occurs mainly in florists and gardeners (49). The relationships with clioquinol, an antiseptic agent used in both topicals and eye drops, and with tixocortol-21-pivalate, a corticosteroid, are not easily explained. The two might both be present together in topicals, or used concomitantly/successively to treat dermatitis. Its relationship with mercaptobenzothiazole–mercapto mix is most likely explained by the use of rubber gloves in florists and gardeners, or by the presence of mercapto compounds in fungicides.

There are no large differences between North/Central and South Europe, and five pairs are present in the top 10 of both regions; quaternium-15–formaldehyde, carba mix–thiuram mix, tixocortol-21-pivalate–budesonide, fragrance mix I–fragrance mix II (together with HICC), and thiuram mix–mercapto mix (together with mercaptobenzothiazole). Some of the variation between the two regions might be explained by the fact that some departments do not test certain allergens in the EBS; for example, clioquinol is only tested in Spain, Lithuania, The Netherlands, Poland, and Slovenia.

It is important to note, however, that although the allergen pairs presented here are often diagnosed together in 1 patient, this does not reflect actual sensitization prevalences of the different allergens. For this, the reader is referred to previous ESSCA publications. For example, the sensitization prevalence of primin in Europe has considerably decreased in the last decade, most likely as a result of the introduction of primin-free cultivars of *P. obconica* H. in 2000 (33, 50). The same is true for clioquinol, with stable low prevalences in the last decade (<0.5%), so its inclusion in the EBS seems no longer warranted (33, 35, 51).

Strengths of patch test reactions

In the present analysis, a positive correlation was seen between the number of positive patch test reactions and the proportion of extreme positive reactions. Similarly, one study found that individuals with strong or extreme positive patch test reactions were more likely to have additional positive reactions than individuals with weak positive reactions (52). Another study found that an extreme patch test reaction to fragrance mix I was associated with additional positive reactions (16). A possible explanation for our findings could be the often mentioned 'angry back syndrome', or 'excited skin syndrome', although the actual existence of these phenomena has been disputed, and the occurrence is thought to be rare at best (53, 54). The best explanation for more strong/extreme positive reactions in polysensitized individuals would be that polysensitization is a sign of heightened susceptibility, which is also expressed by increased reactivity. Differences between countries in the distribution of grades of positive reactions are not easily explained. As the distribution within each country was more or less comparable for oligosensitization and polysensitization, a possible explanation could be differences in how reactions were read, or differences in the patch test populations, or differences in the degree of contact sensitization, for example because of varying exposure conditions, as noted before concerning PPD (31).

LIMITATIONS

The present analysis is based on only the EBS. As the chance of observing polysensitization increases with the number of allergens tested, the oligosensitized subgroup might include polysensitized individuals, if these had been tested with a more extended baseline series. Comparison of prevalences between countries is also made more difficult by variations in the length of the tested baseline series, as discussed above, particularly in terms of omissions. For instance, the Austrian, German and Swiss departments mostly use a baseline series that does not include, for example, the two corticosteroid markers budesonide and tixocortol-21-pivalate, as well as benzocaine and neomycin, but includes other allergens considered to be important, although these have been disregarded in the present analysis. In addition, the EBS is prone to contain old allergens, and is in need for an update. The present analysis also does not include D7 readings, possibly leading to an underestimation of single contact allergies (particularly to corticosteroids) (55), and thereby of polysensitization. Another limitation of this study is that inherent differences between departments

and countries, for example the mode of reading a patch test and different patterns of referral, make interpretation of the results difficult.

CONCLUSION

Differences in polysensitization prevalences are seen between different countries in Europe, and while many reasons can be discussed, these differences are not easy to explain. Along with the variation in contact allergy prevalences between countries or even departments, polysensitization varies with the setting, and cannot be regarded as a fixed characteristic of patch tested patients. Known risk factors for polysensitization, such high age and occupational aetiology of dermatitis, were confirmed. Concomitant reactions to allergen pairs were evaluated for both South and North/Central Europe, showing no evidence of strong differences.

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Online supplemental table S1: The most commonly positive EBS allergens in polysensitised ($n=6383$) versus oligosensitised ($n=31403$) patients. CI, confidence interval; stand., age- and sex-standardised.

Allergen	Oligosens.		Polysens.	
	% pos. (95% CI)	% stand. pos (95% CI)	% pos. (95% CI)	% stand. pos (95% CI)
Nickel	42.6 (42–43.1)	40.9 (40.4–41.4)	52.6 (51.3–53.8)	52 (50.6–53.3)
Cobalt	10.1 (9.8–10.4)	10.7 (10.3–11.1)	35.4 (34.3–36.6)	37.4 (36.1–38.8)
Chromium	5.6 (5.3–5.8)	6 (5.7–6.3)	23.5 (22.4–24.5)	24.7 (23.4–25.9)
FM I	11 (10.7–11.4)	10.7 (10.3–11)	43.1 (41.9–44.3)	40.8 (39.5–42.2)
FM II	5 (4.7–5.2)	5 (4.7–5.2)	23.4 (22.4–24.4)	22.1 (21–23.2)
HICC	2.3 (2.1–2.4)	2.2 (2.1–2.4)	10.7 (10–11.5)	10.2 (9.4–11)
M. pereirae	9 (8.7–9.3)	8.7 (8.4–9)	33.1 (32–34.3)	31.3 (30–32.5)
Colophonium	4.1 (3.9–4.3)	4.2 (3.9–4.4)	16.3 (15.4–17.3)	15.7 (14.7–16.7)
Formaldehyde	2.7 (2.5–2.9)	2.8 (2.6–3)	9.3 (8.6–10.1)	9.3 (8.5–10.1)
Paraben Mix	0.8 (0.7–0.9)	0.9 (0.8–1)	4.4 (4–5)	4.9 (4.3–5.5)
Quaternium 15	1 (0.9–1.1)	1 (0.9–1.1)	3.2 (2.7–3.6)	3.1 (2.7–3.6)
MCI/MI	9.9 (9.6–10.2)	10.1 (9.7–10.4)	23.1 (22.1–24.2)	22.5 (21.4–23.6)
MI	4.6 (4.3–4.8)	4.5 (4.2–4.7)	9.6 (8.9–10.3)	9.3 (8.5–10)
MDBGN	3.1 (2.9–3.3)	3.1 (2.9–3.3)	13.4 (12.6–14.3)	13 (12.1–13.9)
PPD	5.7 (5.4–5.9)	5.5 (5.3–5.8)	11.2 (10.5–12)	11.2 (10.3–12)
Benzocaine	0.4 (0.3–0.5)	0.4 (0.3–0.5)	1.8 (1.5–2.2)	1.7 (1.4–2.1)
Clioquinol	0.1 (0.1–0.1)	0.1 (0.1–0.1)	0.7 (0.5–1)	0.7 (0.5–0.9)
Budesonide	0.8 (0.7–0.9)	0.8 (0.7–1)	2.4 (2–2.8)	2.5 (2–2.9)
Tixocortol pivalate	0.8 (0.7–0.9)	0.8 (0.7–0.9)	3.6 (3.2–4.1)	3.6 (3.1–4.1)
Neomycin sulfate	1.7 (1.6–1.9)	1.7 (1.6–1.9)	5.2 (4.7–5.8)	5.3 (4.7–6)
Thiuram mix	2.9 (2.7–3.1)	3 (2.8–3.2)	11.5 (10.8–12.3)	12.1 (11.2–13.1)
2-MBT	0.9 (0.8–1)	1.1 (0.9–1.2)	3.7 (3.3–4.2)	4.2 (3.6–4.8)
Mercapto mix	0.8 (0.7–1)	1 (0.9–1.1)	3.9 (3.5–4.4)	4.3 (3.7–4.9)
IPPD	0.8 (0.7–0.9)	0.9 (0.8–1)	3.5 (3.1–4)	3.9 (3.3–4.5)
Lanolin alcohol	2.5 (2.3–2.7)	2.6 (2.4–2.8)	10.6 (9.8–11.4)	10.7 (9.8–11.6)
SL mix	0.7 (0.7–0.8)	0.7 (0.6–0.8)	3 (2.6–3.4)	2.7 (2.3–3.2)
Primin	0.4 (0.3–0.4)	0.3 (0.3–0.4)	1.3 (1.1–1.7)	1.4 (1–1.7)
Epoxy resin	2 (1.9–2.2)	2.2 (2–2.4)	5.5 (5–6.1)	5.7 (5–6.3)
ptBFR	1.1 (0.9–1.2)	1.1 (1–1.2)	4.2 (3.7–4.7)	4.1 (3.6–4.7)

CHAPTER

7

Contact sensitization to hydroperoxides of limonene and linalool: Results of consecutive patch testing and clinical relevance

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ABSTRACT

Background

Hydroperoxides of limonene and linalool are potent sensitizers.

Objectives

To investigate the prevalence of contact allergy to both hydroperoxides of limonene and hydroperoxides of linalool, to report clinical relevance, and to investigate patient demographics.

Methods

A total of 821 patients (35.6% male, mean age 42.4 years $\pm$ 17.8 years) were consecutively patch tested with our departmental baseline series and our fragrance series, including hydroperoxides of limonene 0.3% pet. and hydroperoxides of linalool 1.0% pet. The clinical relevance was assessed for all positive reactions.

Results

Positive patch test reactions to hydroperoxides of limonene and to hydroperoxides of linalool were observed in 77 patients (9.4%, 95% confidence interval [CI]: 7.4%-11.4%) and in 96 patients (11.7%, 95%CI: 9.5%-13.9%), respectively; 38 of these patients (4.6%, 95%CI: 3.2%-6.0%) reacted to both. Most reactions were considered to be possibly or probably clinically relevant (66.3% and 68.8%, respectively), and a small proportion were deemed to be of certain clinical relevance (18.2% and 19.8%, respectively).

Conclusion

As compared with previous studies, high numbers of positive reactions to both hydroperoxides of limonene and hydroperoxides of linalool were observed, the majority of which were clinically relevant, supporting their inclusion in the European baseline series.

INTRODUCTION

Limonene (D-limonene) is the main ingredient of pressed oil from the peel of citrus fruits, and linalool is present in many herbs, flowers, woods, etc.(1, 2) Both are common ingredients in household products and cosmetics, such as hygiene products, perfumes, and detergents, as well as industrial products.(3, 4) Limonene and linalool are ubiquitous fragrance terpenes with low sensitizing potential.(1, 2, 5) However, upon air exposure, oxidation occurs, during which different oxidation products are formed. Of these oxidation products, the hydroperoxides are potent sensitizers. High prevalences of contact allergy to these hydroperoxides of limonene and linalool have been reported.(6-9) The aim of the current study was to investigate the prevalences of contact allergy to hydroperoxides of limonene and hydroperoxides of linalool, and to characterize patients allergic to either or both hydroperoxides of limonene and hydroperoxides of linalool, with respect to patient characteristics and concomitant fragrance contact allergies.

METHODS

A database study was performed on all patients who were at least patch tested with both hydroperoxides of limonene 0.3% pet. and hydroperoxides of linalool 1.0% pet. All patients referred to our tertiary referral centre with suspected allergic contact dermatitis (ACD) are consecutively patch tested with our departmental extended European baseline series (EBS), TRUE Test® panels 1 and 2 (SmartPractice Europe, Reinbek, Germany) supplemented with additional investigator-loaded allergens, and a fragrance series. When specific contact allergies are suspected, additional series are patch tested. All investigator-loaded allergens were tested in Van der Bend square chambers (Van der Bend, Brielle, The Netherlands), and all patch tests were attached to the back with Fixomull stretch (BSN Medical, Hamburg, Germany) for 2 days. Both hydroperoxides of limonene 0.3% pet. and hydroperoxides of linalool 1.0% pet. (Chemotechnique Diagnostics, Vellinge, Sweden) were included in our fragrance series from December 1, 2015.

Consecutively patch tested dermatitis patients from December 1, 2015 to December 15, 2017 were included in the current analysis. All patch tests were read and interpreted by the same dermatologist, with potential back-up from a dermatologist also trained in reading and interpreting patch test results, according to ICDRG/ESCD criteria, with

the possible outcomes being: negative, irritant, doubtful (?+), weak positive (+), strong positive (++) , and extreme positive (+++) reactions.⁽¹⁰⁾ Reactions were considered to be irritant if margins were sharply demarcated and the surface of the test area showed a silk paper structure or a shiny skin. Reactions were considered to be doubtful if erythema and infiltration did not cover the whole test area. Readings were performed on day (D) 3 and D7. For the present analysis, the maximum patch test reactions of these 2 readings were aggregated as the patch test outcome. The distribution of the strength of positive patch test reactions to the hydroperoxides are presented for different groups of patients: patients with positive reactions to either hydroperoxides of limonene or hydroperoxides of linalool but not to any other fragrance; patients with positive reactions to both hydroperoxides of limonene and hydroperoxides of linalool but not to any other fragrance; patients with positive reactions to hydroperoxides of limonene or hydroperoxides of linalool and to ≥ 1 other fragrances; and patients with positive reactions to both hydroperoxides of limonene and hydroperoxides of linalool and to ≥ 1 other fragrances.

Clinical relevance and additional contact allergies

For all positive patch test reactions, the current and/or past clinical relevance was determined based on patient history and exposure, with possible outcomes being unlikely/not, possible, probable, and certain. “Unlikely/not” suggested that there was no suspected ACD, “possible” suggested that there was some suspicion of a relationship between the allergen and the dermatitis (between 1% and 49% convinced), “probable” suggested that this suspicion was stronger (between 50% and 99% convinced), and “certain” meant that the relationship was proven (100% convinced) by the presence of allergen in a product to which there was exposure at the body site where there was dermatitis, with a clear temporal relationship. For hydroperoxides of limonene and hydroperoxides of linalool, patients were instructed to review the labelling of their products for either limonene (or D-limonene, also known as *R*-limonene, and its enantiomer *S*-limonene), or linalool, respectively, as ingredients in their products. Their findings were subsequently discussed at our outpatient clinic; if patients were unsure or unable to review their products, they were instructed to bring all of their suspected products for review by the dermatologist. Clinical relevance is presented for the same groups as described above for strength of patch test reaction. The types of product for which exposure caused ACD in patients with a contact allergy to hydroperoxides of limonene and/or hydroperoxides of linalool of certain clinical relevance are presented.

To evaluate concomitant reactions in patients with contact allergy to hydroperoxides of limonene, hydroperoxides of linalool, or both, the proportion of patients with at least ≥ 1 additional contact allergies apart from allergy to either hydroperoxides of limonene or hydroperoxides of linalool, and the proportion of patients with at least ≥ 1 additional non-fragrance allergies (excluding colophonium), are presented. Additional contact allergies were not limited to EBS allergens; that is, any contact allergy was considered.

Data analysis

For data analysis, different groups of patients were defined (Figure 1). Group A comprised all patients with at least positive patch test reactions to hydroperoxides of limonene and/or hydroperoxides of linalool. Group B and group C comprised all patients with positive patch test reactions to hydroperoxides of limonene and hydroperoxides of linalool, respectively. Group D comprised all patients with positive patch test reactions to both hydroperoxides of limonene and hydroperoxides of linalool. In other words, groups B-D are subsets of group A. Group E comprised all patients with at least 1 positive patch test reaction to a patch tested fragrance allergen but not to hydroperoxides of limonene/linalool, and group F comprised all other patch tested patients. Table S1 provides an overview of all fragrance allergens tested, including the tested concentration and vehicle, in the current cohort of patients. Patient demographics for these groups were described according to the MOAHLFA index.(11)

Figure 1.

Venn diagram illustrating the different groups of patients based on their patch test outcomes

Statistics

Statistical analyses were performed according to pertinent guidelines.⁽¹²⁾ Prevalences are provided in percentages together with 95% confidence intervals (CIs). The reaction index (RI) (showing the proportion of doubtful/irritant reactions relative to positive reactions, calculated with the formula $[\text{positive reactions} - (\text{doubtful reactions} + \text{irritant reactions})] / [\text{positive} + \text{doubtful} + \text{irritant reactions}]$, giving a value between -1 and 1) and positivity ratio (PR) (proportion of weak positive [+] reactions among all positive reactions) were calculated for both hydroperoxides of limonene and hydroperoxides of linalool.^(13, 14) For variables with a normal distribution, the mean and SD are given; for non-normally distributed variables, median and interquartile range are given. Logistic regression analysis was performed to assess the risk factors for being in 1 of the above-mentioned groups as compared with not being in that specific group. Both univariable and multivariable regression analyses were performed; for the multivariable regression analysis, all variables which reached a *P*-value of <0.1 in the univariable analysis were included. Variables analysed were: sex, age ≥ 40 years, (a history of) atopic dermatitis, occupational dermatitis, and primary site of dermatitis (generalized, trunk, hand, face, leg, and other). All *P*-values of <0.05 were regarded as being statistically significant. Statistical analyses were performed with SPSS v.23 (IBM) and Excel 2013 (Microsoft).

RESULTS

Hydroperoxides of limonene and linalool

A total of 821 patients (35.6% male, mean age 42.4 years $\pm$ 17.8 years) were tested with at least our departmental baseline series and our fragrance series including hydroperoxides of limonene and hydroperoxides of linalool. Altogether 77 patients (9.4%, 95%CI: 7.4%-11.4%) had positive patch test reactions to hydroperoxides of limonene, and 96 patients (11.7%, 95%CI: 9.5%-13.9%) had positive patch test reactions to hydroperoxides of linalool. The overlap between these 2 groups was 38 patients (4.6%, 95%CI: 3.2%-6.0%) who had positive reactions to both hydroperoxides of limonene and hydroperoxides of linalool. An overview of the patch test reactions to both hydroperoxides of limonene and hydroperoxides of linalool is shown in Table 1. A total of 141 doubtful (?+) reactions (17.2%, 95%CI: 14.6%-19.7%) and 7 irritant reactions (0.9%, 95%CI: 0.2%-1.5%) to hydroperoxides of limonene were observed, and 180 doubtful reactions (21.9% 95%CI: 19.1%-24.8%) and 16 irritant reactions (1.9%, 95%CI: 1.1%-2.8%) to hydroperoxides of linalool were observed. The RI and PR for hydroperoxides of limonene were -0.32 (95%CI: -0.41 to -0.24) and 85.7%

(95%CI: 77.9%-93.5%), respectively, and the RI and PR for hydroperoxides of linalool were -0.34 (95%CI: -0.42 to -0.26) and 88.5% (95%CI: 79.2%-94.9%), respectively. Of all positive reactions to hydroperoxides of limonene, 6 (7.8%) were either negative or doubtful on the D3 reading, and became positive on D7; for hydroperoxides of linalool, this was seen in 4 cases (4.2%). For both D-limonene 2.0% pet. and linalool 10.0% pet., 1 positive reaction per allergen was observed in 2 different patients, both of whom were also allergic to both hydroperoxides of limonene and hydroperoxides of linalool.

Table 1. Cross table of all patch test reactions (n=821) to hydroperoxides of limonene 0.3% pet. and hydroperoxides of linalool 1.0% pet.

		Hydroperoxides of linalool						Total
		Negative	Irritant	?+	+	++	+++	
Hydroperoxides of limonene	Negative	479	10	70	37	0	0	596
	Irritant	2	4	1	0	0	0	7
	?+	37	2	81	19	2	0	141
	+	9	0	26	26	5	0	66
	++	2	0	2	3	3	1	11
	+++	0	0	0	0	0	0	0
	Total	529	16	180	85	10	1	821

Strength of reactions

As can be deduced from the calculated PRs shown above, the majority of positive reactions to both hydroperoxides of limonene and hydroperoxides of linalool were weak (+) positive. Only 1 extreme (+++) positive reaction to hydroperoxides of linalool was observed. Table 2 shows the distribution of the strength of reactions for the different groups of patients. It can be observed that patients who reacted to only hydroperoxides of limonene or hydroperoxides of linalool had only weak (+) positive reactions, whereas patients who reacted to both hydroperoxides of limonene and hydroperoxides of linalool and at least 1 additional fragrance had a higher proportion of strong (++) positive reactions to hydroperoxides of limonene and/or hydroperoxides of linalool.

Table 2. The distribution of the varying degrees of positive reactions for both hydroperoxides of limonene and hydroperoxides of linalool, presented for different groups of patients; patients with only a positive reaction to one or the other hydroperoxides but not to any other fragrance; patients with a positive reaction to both hydroperoxides but not to any other fragrance; patients with a positive reaction to one or the other hydroperoxides and one or more other fragrances; and patients with a positive reaction to both hydroperoxides and one or more other fragrances.

Reaction profile		Strength of patch test reaction, n (%)			
		N(total)	Weak (+)	Strong (++)	Extreme (+++)
Only hydroperoxides of limonene	Limonene	26	26 (100.0)	0	0
Only hydroperoxides of linalool	Linalool	42	42 (100.0)	0	0
Both hydroperoxides of limonene/linalool	Limonene	16	15 (93.8)	1 (6.3)	0
	Linalool	16	13 (81.3)	3 (18.8)	0
Hydroperoxides of limonene + other fragrance	Limonene	13	9 (69.2)	4 (30.8)	0
Hydroperoxides of linalool + other fragrance	Linalool	16	14 (87.5)	2 (12.5)	0
Both hydroperoxides of limonene/linalool + other fragrance	Limonene	22	16 (72.7)	6 (27.3)	0
	Linalool	22	16 (72.2)	5 (22.7)	1 (4.5)
Total hydroperoxides of limonene	Limonene	77	66 (85.7)	11 (14.3)	0
Total hydroperoxides of linalool	Linalool	96	85 (88.5)	10 (10.4)	1 (1.0)

Clinical relevance

The clinical relevance of the positive reactions to both hydroperoxides of limonene and hydroperoxides of linalool were evaluated according to the groups described above (Table 3). Positive reactions to hydroperoxides of limonene/hydroperoxides of linalool in patients who were allergic to both, with or without additional fragrance allergies, were more frequently of certain clinical relevance (ranging from 27.3% to 37.5%, not statistically significant). The majority of reactions were evaluated as being of possible or probable clinical relevance. For patients with “certainly relevant” positive patch test

reactions, the product type responsible for the allergic contact dermatitis are shown in Table S2. In the majority of patients (15/21, 71.4%), the responsible product types were rinse-off products such as soap and shampoo, followed by leave-on products such as cosmetics and creams (9/21, 42.9%). Other product types included cleaning agents (detergents), deodorants, and perfumes.

Table 3. Clinical relevance for each positive reaction to hydroperoxides of limonene and hydroperoxides of linalool, presented for different groups of patients; patients with only a positive reaction to one or the other hydroperoxides but not to any other fragrance; patients with a positive reaction to both hydroperoxides but not to any other fragrance; patients with a positive reaction to one or the other hydroperoxides and one or more other fragrances; and patients with a positive reaction to both hydroperoxides and one or more other fragrances.

Reaction profile		Relevance, n (%)				
		N(total)	Unlikely/not	Possible	Probable	Certain
Only hydroperoxides of limonene	Limonene	26	5 (19.2)	14 (53.8)	4 (15.4)	3 (11.5)
Only hydroperoxides of linalool	Linalool	42	7 (16.7)	19 (45.2)	11 (26.2)	5 (11.9)
Both hydroperoxides of limonene/linalool	Limonene	16	1 (6.3)	6 (37.5)	4 (25.0)	5 (31.3)
	Linalool	16	0	6 (37.5)	4 (25.0)	6 (37.5)
Hydroperoxides of limonene + other fragrance	Limonene	13	3 (23.1)	7 (53.8)	3 (23.1)	0
Hydroperoxides of linalool + other fragrance	Linalool	16	1 (6.3)	8 (50.0)	5 (31.3)	2 (12.5)
Both hydroperoxides of limonene/linalool + other fragrance	Limonene	22	3 (13.6)	7 (31.8)	6 (27.3)	6 (27.3)
	Linalool	22	3 (13.6)	7 (31.8)	6 (27.3)	6 (27.3)
Total hydroperoxides of limonene	Limonene	77	12 (15.6)	34 (44.2)	17 (22.1)	14 (18.2)
Total hydroperoxides of linalool	Linalool	96	11 (11.5)	40 (41.7)	26 (27.1)	19 (19.8)

Patient characteristics

The patient characteristics according to the MOAHLFA index are shown in Table 4 for each of the defined groups (Figure 1). When group A (positive reactions to hydroperoxides of limonene and/or hydroperoxides of linalool) was compared with group F (no positive reactions to any fragrance), patients in group A were significantly less often male (24.4% vs 39.3%, $P=0.001$), significantly older (age >40 years; 67.4% vs 53.1%, $P=0.002$), suffer(ed) significantly less from atopic dermatitis (40.0% vs 50.7%, $P=0.028$), and less often had the face as the primary site of dermatitis (17.0% vs 21.8%, $P=0.24$). No notable differences were observed between patients in groups B, C, and D, except that patients in group D were almost exclusively female (10.5% males).

A regression analysis was performed for each of these groups (except for group F, all members of which were not allergic to a fragrance); the results are shown in Table 5. Data from group A showed that being female was a significant risk factor for contact allergy to hydroperoxides of limonene and/or hydroperoxides of linalool (odds ratio [OR] 1.91, 95%CI: 1.25-2.91). In group A, another significant association was found for patients aged ≥ 40 years (OR 1.86, 95%CI: 1.26-2.75). A decreased risk was found in patients with a current or past history of atopic dermatitis (OR 0.64, 95%CI: 0.44-0.93). In the multivariable analysis, atopic dermatitis was no longer a significant risk factor (OR 0.71, 95%CI: 0.48-1.06), whereas female sex became an even larger risk factor (OR 2.05, 95%CI: 1.33-3.13). In group A, females had significantly more atopic dermatitis than males (52.2% vs 43.8%, $P=0.019$), which might explain this finding. When group B (positive reactions to hydroperoxides of limonene) was compared with group C (positive reactions to hydroperoxides of linalool), the main difference was that age ≥ 40 years was a risk factor for patients in group C (OR 2.28, 95%CI: 1.43-3.65), but not significantly so in group B (OR 1.39, 95%CI: 0.86-2.26). Conversely, although female sex was a significant risk factor in both groups, it was a stronger risk factor in group B (OR 2.73) than in group C (OR 1.91). In the multivariable analysis, atopic dermatitis was significantly associated with being in group B (OR 0.59, 95%CI: 0.36-0.95), suggesting that the importance of atopic dermatitis in group A was mainly driven by the subset of patients allergic to hydroperoxides of limonene, whereas a larger proportion of the subset of patients allergic to hydroperoxides of linalool were aged ≥ 40 years. Occupational dermatitis and primary site of dermatitis were not significant risk factors for any of the groups.

Concomitant contact allergies

The proportions of patients with contact allergies other than to either hydroperoxides of limonene or hydroperoxides of linalool were 75.6% (95%CI: 68.4%-82.8%) for group A (patients allergic to hydroperoxides of limonene and/or hydroperoxides of linalool), and 84.2% (95%CI: 72.6%-95.8%) for group D (allergic to both hydroperoxides) (Table 6). The proportion of patients with additional contact allergies but not to fragrances and/or colophonium was 72.6% (95%CI: 65.1%-80.1%) for group A.

Including both hydroperoxides of limonene and hydroperoxides of linalool, a total of 233 patients (28.4%, 95%CI: 25.3%-31.4%) had at least 1 positive reaction to a fragrance. Of these 233 patients, 98 (11.9%, 9.7%-14.1%) had a positive reaction to a fragrance but not to either hydroperoxides of limonene or hydroperoxides of linalool, 53 patients (6.5%, 95%CI: 4.8%-8.2%) reacted to both at least 1 fragrance and hydroperoxides of limonene and/or hydroperoxides of linalool, and the remaining 82 patients (10.0%, 95%CI: 7.9%-12.0%) reacted only to hydroperoxides of limonene and/or hydroperoxides of linalool, but not to any other fragrance. Table 7 shows the numbers of concomitant fragrance reactions per patch tested fragrance. An important observation is that, regarding patients with contact allergy to hydroperoxides of limonene and/or hydroperoxides of linalool, a significantly larger proportion had a concomitant contact allergy to a fragrance and/or colophonium (39.3%, 95%CI: 31.3%-47.5%) than patients who were not allergic to hydroperoxides of limonene and/or hydroperoxides of linalool (14.3%, 95%CI: 11.7%-16.9%). On analysis of group A, patients who were allergic to both hydroperoxides of limonene and hydroperoxides of linalool more frequently reacted positively to additional fragrance allergens and/or colophonium (63.2%, 95%CI: 47.9%-78.5%) than patients who were allergic to hydroperoxides of limonene (33.3%, 95%CI: 18.5%-48.1%) and significantly more frequently than patients who were allergic to hydroperoxides of linalool alone (27.6%, 95%CI: 16.1%-39.1%).

Table 4. The MOAHLFA-index for the different groups of patients, as shown in figure 1.

	Group A; positive to either/both hydroperoxides of Linalool/limonene (n=135)	Group B; positive to hydroperoxides of limonene (n=77)	Group C; positive to hydroperoxides of linalool (n=96)	Group D; positive to both hydroperoxides of limonene/linalool (n=38)	Group E; positive to another fragrance (n=98)	Group F; not positive to a fragrance (n=588)
M	24.4	18.2	24.0	10.5	31.6	39.3
O	23.0	22.1	19.8	13.2	21.4	20.2
A	40.0	37.7	40.6	39.5	54.1	50.7
H	37.0	37.7	37.5	36.8	41.8	37.4
L	1.5	1.3	2.1	2.6	0	2.6
F	17.0	18.2	13.5	13.2	24.5	21.8
A	67.4	62.3	71.9	68.4	50.0	53.1

M, Male; O, Occupational dermatitis; A, a (history of) Atopic dermatitis; H, Hand as primary site of dermatitis; L, Leg as primary site of dermatitis; F, Face as primary site of dermatitis; A, Age over 40.

Table 5. Uni- and multivariable regression analysis performed for each group of patients as shown in figure 1, with the exception of group F, with the following variables: sex, age (<40 versus ≥40), a (history of) atopic dermatitis, occupational dermatitis, and primary site of dermatitis (generalized, trunk, hand, leg, face, and other). Variables were entered into the multivariable regression analysis if the p-value was <0.1 in the univariable regression analysis.

<i>Univariable regression</i>		Group A (either/both hydroperoxides of limonene/linalool) N=135		Group B (positive to hydroperoxides of limonene) N=77		Group C (positive to hydroperoxides of linalool) N=96		Group D (positive to both hydroperoxides) N=38		Group E (positive to another fragrance) N=98	
		OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
Sex	Male	1.00 (ref)		1.00 (ref)		1.00 (ref)		1.00 (ref)		1.00 (ref)	
	Female	1.91	(1.25-2.91)	2.73	(1.50-4.97)	1.91	(1.17-3.12)	5.03	(1.77-14.31)	1.24	(0.79-1.95)
Age	<40	1.00 (ref)		1.00 (ref)		1.00 (ref)		1.00 (ref)		1.00 (ref)	
	≥40	1.86	(1.26-2.75)	1.39	(0.86-2.26)	2.28	(1.43-3.65)	1.82	(0.90-3.65)	0.79	(0.52-1.21)
Atopic dermatitis	No	1.00 (ref)		1.00 (ref)		1.00 (ref)		1.00 (ref)		1.00 (ref)	
	Yes	0.64	(0.44-0.93)	0.63	(0.37-1.02)	0.67	(0.44-1.03)	0.66	(0.34-1.28)	1.24	(0.81-1.90)
Occupational dermatitis	No	1.00 (ref)		1.00 (ref)		1.00 (ref)		1.00 (ref)		1.00 (ref)	
	Yes	1.16	(0.79-1.81)	1.09	(0.62-1.91)	0.93	(0.55-1.59)	0.56	(0.22-1.47)	1.04	(0.62-1.74)
Site of dermatitis	Generalized	1.00 (ref)		1.00 (ref)		1.00 (ref)		1.00 (ref)		1.00 (ref)	
	Trunk	1.84	(0.61-5.53)	2.29	(0.37-14.35)	1.56	(0.50-3.26)	1.49	(0.20-11.00)	0.61	(0.18-2.14)
	Hand	1.45	(0.63-3.37)	2.87	(0.67-12.38)	0.99	(0.50-4.84)	1.37	(0.30-6.18)	0.86	(0.39-1.88)
	Leg	1.01	(0.19-5.38)	1.81	(0.15-21.29)	1.01	(0.19-5.38)	1.81	(0.15-21.30)	n.a.	
Face		1.15	(0.47-2.82)	2.72	(0.60-12.54)	0.61	(0.23-1.60)	0.85	(0.16-4.52)	0.90	(0.39-2.06)
	Other	1.98	(0.84-4.65)	4.30	(0.99-18.58)	1.56	(0.53-3.03)	2.00	(0.44-9.05)	0.58	(0.25-1.34)

Table 5. Continued

Multivariable regression (variables entered when <i>p</i> -value < 0.1 in univariable regression)		Group A		Group B		Group C		Group D	
		OR	95% CI	OR	95% CI	OR	95% CI		
Sex	Male	1.00 (ref)		1.00 (ref)		1.00 (ref)		1.00 (ref)	
	Female	2.05	(1.33-3.13)	2.89	(1.58-5.26)	2.04	(1.24-3.35)	5.21	(1.83-14.87)
Age	<40	1.00 (ref)				1.00 (ref)		1.00 (ref)	
	≥40	1.73	(1.14-2.62)			2.21	(1.35-3.62)	1.94	(0.96-3.91)
Atopic dermatitis	No	1.00 (ref)		1.00 (ref)		1.00 (ref)			
	Yes	0.71	(0.48-1.06)	0.58	(0.36-0.94)	0.81	(0.51-1.29)		

Table 6. The proportion of patients with at least one additional contact allergy other than hydroperoxides of limonene and hydroperoxides of linalool, and proportion of patients with at least one additional non-fragrance allergy, and the median additional (non-fragrance) reactions in group A (patients allergic to either/both hydroperoxides of limonene/linalool, see figure 1), and the subset of patients allergic to only hydroperoxides of limonene, patients allergic to only to hydroperoxides of linalool, and patients allergic to both hydroperoxides.

	Total, n	proportion with ≥ 1 additional contact allergy (other than hydroperoxide) N, (%)	proportion with ≥ 1 additional contact allergy (other than hydroperoxide or fragrance/colophonium) N, (%)	Additional reactions	Additional reactions (other than fragrance/ colophonium)	
					Median (IQR)	Median (IQR)
Group A (either/both hydroperoxides of limonene/linalool)	135	102 (75.6)	98 (72.6)	2 (1-6)		1 (0-4)
Only hydroperoxides of limonene	39	28 (73.7)	27 (70.7)	1 (0-7)		1 (0-5)
Only hydroperoxides of linalool	58	42 (72.4)	41 (70.7)	1 (0-5)		1 (0-3,25)
Both hydroperoxides of Limonene/linalool	38	32 (84.2)	30 (78.9)	3.5 (1-9)		2 (1-5)

Table 7. the number of patients with a positive reaction for each of the fragrance (markers) allergens and/or colophonium, presented for patients allergic to either or both hydroperoxides of limonene/linalool, the subset of patients allergic to only hydroperoxides of limonene, patients allergic to only to hydroperoxides of linalool, and patients allergic to both hydroperoxides, and for all other patients not allergic to either hydroperoxides.

Fragrance	Allergic to either or both hydroperoxides of limonene/linalool (n=135), n (%)			All other patients not allergic to either hydroperoxides (n=686), n(%)
	All (n=135)	Only Hydroperoxide of limonene (n=39), n (%)	Only Hydroperoxide of linalool (n=58), n (%)	Both hydroperoxides of limonene/linalool (n=38), n (%)
European Baseline series				
Fragrance mix I *	15 (11.1%)	3 (7.7%)	7 (12.1%)	5 (13.2%)
Fragrance mix II#	24 (17.8%)	3 (7.7%)	8 (13.8%)	13 (34.2%)
Hydroxyisohexyl 3-cyclohexenecarboxyaldehyde (Lyrall)#	10 (7.4%)	1 (2.6%)	3 (5.2%)	6 (15.8%)
Myroxylon Pereirae (Balsam of peru)	10 (7.4%)	3 (7.7%)	3 (5.2%)	4 (10.5%)
Colophonium (resin)	4 (3.0%)	1 (2.6%)	0	3 (7.9%)
Subtotal (≥10 of the above)	39 (28.9%)	9 (23.1%)	14 (24.1%)	16 (42.1%)
Fragrance series				
Amyl cinnamyl alcohol*	2 (1.5%)	0	0	2 (5.3%)
Anisyl alcohol (Anise Alcohol)	0	0	0	0

Table 7. Continued

Fragrance	Allergic to either or both hydroperoxides of limonene/linalool (n=135), n (%)				All other patients not allergic to either hydroperoxides (n=686), n(%)
	All (n=135)	Only Hydroperoxide of limonene (n=39), n (%)	Only Hydroperoxide of linalool (n=58), n (%)	Both hydroperoxides of limonene/linalool (n=38), n (%)	
Benzyl alcohol	0	0	0	0	3 (0.4%)
Benzyl benzoate	0	0	0	0	0
Benzyl cinnamate	0	0	0	0	0
Benzyl salicylate	1 (0.7%)	0	1 (1.7%)	0	0
Cinnamic alcohol	3 (2.2%)	1 (2.6%)	0	2 (5.3%)	5 (0.7%)
Cinnamic aldehyde*	6 (4.4%)	1 (2.6%)	1 (1.7%)	4 (10.5%)	10 (1.5%)
Citral [#]	8 (5.9%)	1 (2.6%)	4 (6.9%)	3 (7.9%)	5 (0.7%)
Citronellol [#]	4 (3.0%)	1 (2.6%)	2 (3.4%)	1 (2.6%)	3 (0.4%)
Coumarin [#]	2 (1.5%)	1 (2.6%)	0	1 (2.6%)	2 (0.3%)
Farnesol [#]	4 (3.0%)	0	1 (1.7%)	3 (7.9%)	1 (0.1%)
Geraniol*	6 (4.4%)	2 (5.1%)	2 (3.4%)	2 (5.3%)	2 (0.3%)
Hexyl cinnamic aldehyde (Hexyl Cinnamal) [#]	1 (0.7%)	0	0	1 (2.6%)	2 (0.3%)
Hydroxycitronellal*	7 (5.2%)	0	4 (6.9%)	3 (7.9%)	7 (1.0%)
Isoeugenol*	8 (5.9%)	3 (7.7%)	2 (3.4%)	3 (7.9%)	13 (1.9%)
Butylphenyl Methylpropional (Lilial)	4 (3.0%)	1 (2.6%)	1 (1.7%)	2 (5.3%)	2 (0.3%)

Table 7. Continued

Fragrance	Allergic to either or both hydroperoxides of limonene/linalool (n=135), n (%)				All other patients not allergic to either hydroperoxides (n=686), n(%)
	All (n=135)	Only Hydroperoxide of limonene (n=39), n (%)	Only Hydroperoxide of linalool (n=58), n (%)	Both hydroperoxides of limonene/linalool (n=38), n (%)	
Methyl 2-Octynoate (Methyl heptene carbonate)	1 (0.7%)	0	1 (1.7%)	0	1 (0.1%)
Alpha-isomethyl Ionone (gamma-Methylionone)	0	0	0	0	1 (0.1%)
Evernia Prunastri (Oakmoss absolute)*	11 (8.1%)	3 (7.7%)	3 (5.2%)	5 (13.2%)	6 (0.9%)
Evernia Furfuracea (Treemoss)	5 (3.7%)	0	2 (3.4%)	3 (7.9%)	7 (1.0%)
Amyl Cinnamal (alpha amyl Cinnamic aldehyde)*	1 (0.7%)	0	0	1 (2.6%)	1 (0.1%)
Eugenol*	7 (5.2%)	2 (5.1%)	2 (3.4%)	3 (7.9%)	1 (0.1%)
d-Limonene	1 (0.7%)	0	0	1 (2.6%)	0
Linalool	1 (0.7%)	0	0	1 (2.6%)	0
Subtotal (≥1 of the above)	36 (26.7%)	8 (20.5%)	12 (20.7%)	16 (42.1%)	86 (12.5%)
Total (≥1 fragrance/colophonium allergy)	53 (39.3%)	13 (33.3%)	16 (27.6%)	24 (63.2%)	98 (14.3%)

DISCUSSION

In our cohort of consecutively patch tested dermatitis patients, 9.4% and 11.7% showed positive patch test reactions to hydroperoxides of limonene and hydroperoxides of linalool, respectively, supporting the recent proposal to include them in the EBS. (15) This is a higher number than found in recent studies, in which prevalences of contact allergy to hydroperoxides of limonene ranged from 2.5% to 5.4%, and prevalences of contact allergy to hydroperoxides of linalool ranged from 3.9% to 7.7%. (6, 8, 9) However, in multicentre studies, a large variation in positive patch test reactions between centres was observed; for example, in one study, prevalences of contact allergy to hydroperoxides of limonene ranged from 0% to 24.8%. (8) A possible explanation for the large number of positive reactions to the hydroperoxides could be that our centre is a tertiary referral centre, so more patients with severe and/or persistent dermatitis are seen than in other centres. This could also explain the large proportion of additional positive reactions observed for patients with positive reactions to hydroperoxides of limonene and/or hydroperoxides of linalool, and the large number of fragrance-positive patients overall. Concerning doubtful and irritant reactions to both hydroperoxides of limonene and hydroperoxides of linalool—in our cohort, there were many doubtful (17.2% and 21.9%, respectively) and few irritant (0.9% and 1.9%, respectively) reactions—large variations in the numbers of doubtful and irritant reactions have been observed in different studies. For instance, Deza et al observed 0.4% doubtful and 1.5% irritant reactions to hydroperoxides of limonene 0.3% pet., whereas Bennike et al observed 13.7% doubtful and 5.8% irritant reactions. (6, 8) An even larger variation in the percentages of doubtful and irritant reactions has been reported for hydroperoxides of linalool. Our observation that the majority of positive reactions to either hydroperoxides of limonene or hydroperoxides of linalool were weak positive (+) is in line with the literature. (7, 16–18)

On the basis of our results, a low RI (<0) and a high PR (>80%) for both hydroperoxides of limonene and hydroperoxides of linalool were calculated, indicative of a problematic patch test concentration. (13, 14) Considering that the low RIs for both allergens are mostly caused by a high number of doubtful reactions, and only a small number of irritant reactions, a reasonable assumption would be that patch testing with higher concentrations might improve the diagnostic performance. Differentiation between doubtful reactions and irritant reactions can be difficult, as can be deduced from the variation in doubtful and irritant reactions in previous studies. It can therefore not

be excluded that, even though all visual readings were performed by an experienced dermatologist, some of the doubtful reactions were irritant. If this was the case, it might be prudent to test both allergens in a lower concentration. An additional concern regarding testing at higher concentrations is the higher risk of active sensitization, as studies have shown that an irritant effect can increase this risk.(19) Christensson et al patch tested dermatitis patients and healthy controls with sequentially diluted concentrations of oxidized limonene and oxidized linalool; for both, an increasing concentration led to more irritant reactions, although this effect was stronger for oxidized limonene than for oxidized linalool.(20) The highest tested concentration of oxidized linalool (20%, most likely containing 3.34% hydroperoxides of linalool, on the basis of the presence of 1.0% hydroperoxides of linalool in 6.0% oxidized linalool) (17) showed a mean irritation score of 1.63 points, and a maximum of 4 points, based on a scoring system ranging from 0 to 9 developed by Basketter et al,(21) in which an irritant reaction would be noted from 2 to 3 points.(20) Studies have been performed with lower patch test concentrations of hydroperoxides of limonene (0.1% and 0.2%) and hydroperoxides of linalool (0.25% and 0.5%), and have concluded that the current patch test concentrations are preferred over lower concentrations, as too many positive reactions might be missed.(8, 9) The observed high PR further supports the argument that the current patch test concentrations might be too low. Future studies should be performed to investigate the ideal patch test concentrations for hydroperoxides of limonene and hydroperoxides of linalool.

Clinical relevance is generally difficult to ascertain, as it depends on how well and how diligent a patient reads product labels and identifies the presence of contact allergens in the product. For hydroperoxides of limonene and hydroperoxides of linalool, an additional limitation is that these are not mentioned as such on labels. Therefore, patients have to look for limonene and/or linalool, as these are among the 26 fragrances for which labelling is required on cosmetic and detergent products in the EU.(22) Studies have shown that fine fragrances and essential oils, which often contain limonene and/or linalool, also contain hydroperoxides as a result of autoxidation. (23, 24) In the current study, the designation "certain" clinical relevance was reserved for patients who showed a clear temporal relationship between body site-specific exposure to a product containing limonene and/or linalool and dermatitis at that body site, even though the presence of actual hydroperoxides was not confirmed by analysis of the products, and no open use test or repeated open application test was performed. Notwithstanding these limitations, almost 20% of the reactions were evaluated as

being of “certain” clinical relevance, and at least another 20% were of “probable” clinical relevance.

Patients who are allergic to hydroperoxides of limonene and/or hydroperoxides of linalool were mostly female, were aged 40 years, and less frequently had (a history of) atopic dermatitis, in line with previous literature. The lower prevalence of atopic dermatitis in this patient group might explain the low number of irritant reactions to the hydroperoxides, as (a history of) atopic dermatitis is associated with increased susceptibility to irritants.⁽²⁵⁾ A recent study, however, did not find any differences in the prevalences of atopic dermatitis between patients with positive reactions to hydroperoxides of limonene and/or hydroperoxides of linalool, and patients with irritant reactions.⁽⁹⁾ A strength of the current investigation is that D7 readings were performed, as, without this late reading, approximately 8% and 4% of positive reactions to hydroperoxides of limonene and hydroperoxides of linalool, respectively, would have been missed.

In conclusion, high prevalences of contact allergy to hydroperoxides of limonene and hydroperoxides of linalool have once again been observed, supporting the proposed inclusion in the EBS. Furthermore, 40% of all reactions to hydroperoxides of limonene and hydroperoxides of linalool were of either “probable” or “certain” clinical relevance. Although varying proportions of doubtful and irritant reactions have been reported in the literature, the low RIs and high PRs calculated for both hydroperoxides of limonene and hydroperoxides of linalool indicate that the ideal patch test concentration might be higher than the currently tested concentrations, although the risk of active sensitization must be kept in mind. A large number of patients who are allergic to hydroperoxides of limonene and/or hydroperoxides of linalool have additional contact allergies, both to fragrances and to other non-fragrance contact allergens.

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Table S1. Overview of all fragrance allergens (and colophonium) patch tested in each consecutive patient at our patch test clinic. Fragrance mix I, myroxylon pereirae, and colophonium are tested with the TRUE Test®, all other fragrance allergens are tested in petrolatum in Van der Bend chambers.

Fragrance	Concentration and vehicle
<i>European Baseline series</i>	
Fragrance mix I	430 µg/cm ² (TRUE Test®)
Fragrance mix II	14.0% pet.
Hydroxyisohexyl 3-cyclohexenecarboxyaldehyde (Lyrall)	5.0% pet.
Myroxylon Pereirae (Balsam of peru)	800 µg/cm ² (TRUE Test®)
Colophonium (resin)	850 µg/cm ² (TRUE Test®)
<i>Fragrance series</i>	
Amyl cinnamyl alcohol	5.0% pet.
Anisyl alcohol (Anise Alcohol)	1.0% pet.
Benzyl alcohol	10.0% pet.
Benzyl benzoate	1.0% pet
Benzyl cinnamate	5.0% pet.
Benzyl salicylate	1.0% pet.
Cinnamic alcohol	2.0% pet
Cinnamic aldehyde	1.0% pet.
Citral	2.0% pet
Citronellol	1.0% pet.
Coumarin	5.0% pet.
Farnesol	5.0% pet.
Geraniol	2.0% pet
Hexyl cinnamic aldehyde (Hexyl Cinnamal)	10.0%
Hydroxycitronellal	2.0% pet
Isoeugenol	2.0% pet
Butylphenyl Methylpropional (Lilial)	10.0% pet.
D-Limonene	2.0% pet
Linalool	10.0% pet.
Methyl 2-Octynoate (Methyl heptene carbonate)	1.0% pet.
Alpha-isomethyl Ionone (gamma-Methylionone)	1.0% pet.
Evernia Prunastri (Oakmoss absolute)	2.0% pet
Evernia Furfuracea (Treemoss)	1.0% pet.
Amyl Cinnamal (alpha amyl Cinnamic aldehyde)	1.0% pet.
Eugenol	2.0% pet
Hydroperoxides of Linalool	1.0% pet.
Hydroperoxides of Limonene	0.3% pet.

Table S2. The product category which caused allergic contact dermatitis in subjects with a contact allergy of certain clinical relevance to either/both hydroperoxides of limonene and/or linalool. Presence of either or both limonene and/or linalool in the product was ascertained by the patients themselves, and by the dermatologist in case a patient brought their suspected products to the outpatient clinic.

subject	Contact allergy to hydroperoxides of:	<i>Product category</i>				
		soap, shampoo (rinse off products)	cosmetic, creams (stay-on products)	cleaning agents (detergents)	deodorant	perfumes
1	limonene	x				
2	limonene	x				
3	limonene	x	x			
4	limonene	x				x
5	limonene		x			
6	linalool	x				
7	linalool	x				
8	linalool		x			
9	linalool		x			
10	linalool		x			
11	both		x	x		
12	both	x		x		
13	both	x				
14	both	x	x		x	x
15	both	x				
16	both	x			x	x
17	both	x			x	
18	both	x	x	x		x
19	both				x	
20	both	x			x	
21	both	x	x			

CHAPTER

8a

Allergic contact dermatitis caused by acrylic acid used in transcutaneous electrical nervous stimulation

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Transcutaneous electrical nervous stimulation (TENS) is an effective therapy for chronic lower back pain, and has few side effects as compared with pharmacological treatment (1, 2). Electrodes are patched onto the skin, and an electrical current running between these inhibits the neural conduction of pain stimulation. The electrodes are self-adhesive and stay on the skin for up to several days, and can therefore cause both irritant and allergic contact dermatitis.

CASE REPORT

An 81-year-old man was referred to us with erythema, superficial erosions, haemorrhagic crusts and some petechiae on the lower back at the site where TENS had been applied continuously for the last 3 years to treat his chronic lower back pain (Fig. 1a). The symptoms first appeared 14 months after the start of treatment. Since then, a total of six different TENS electrodes have been prescribed, all of them causing the same symptoms. The TENS device was switched on continuously during the day but not at night, although the electrodes stayed on the skin. The TENS electrodes prescribed were: Flexibele Elektroden (Klinerva, Ankeveen, The Netherlands), Klinerva Silver Electrode, Klinerva Stimtrode (Model ST50505) (Klinerva), Dura-stick® plus (Ref. 42198), Dura-stick® (Ref. 42191), and Dura-stick® premium (Ref. 42209) (DJO Global, Guildford, UK). The patient was patch tested with our extended European baseline series (TRUE Test® panels 1 and 2; SmartPractice Europe, Barsbüttel, Germany), supplemented with additional chamber-loaded allergens and a (meth)acrylates series (Chemotechnique Diagnostics, Vellinge, Sweden), tested in Van der Bend chambers (Van der Bend, Brielle, The Netherlands) attached to the back with Fixomull stretch® (BSN Medical, Hamburg, Germany). Additionally, five of the six electrodes were patch tested 'as is', without an electrical current. One of the six, Klinerva Silver Electrode, was no longer in production, and could therefore not be patch tested. A 'use test' (with electrical current) was performed with four of the five electrodes on the lower back. The TENS devices were turned off during the night to reflect real-life conditions. The patch tests and electrodes were applied on the back for 2 days under occlusion, and readings were performed on day (D) 3 and D7, according to the guidelines of the ICDRG/ESCD (3). The results are shown in Table 1 and Figure S1. The patient had weak positive reactions to several (meth)acrylates, and strong positive reactions to four of the five tested TENS electrodes, but not to Flexibele Elektroden.

Figure 1.

(a) Itchy rash on the lower back with erythema, superficial erosions, haemorrhagic crusts, and some petechiae. (b) Skin lesions on the lower back disappeared after switching to the Dura-stick premium Blue transcutaneous electrical nervous stimulation electrodes.

Table 1: Patch test results of electrodes 'as is', electrodes 'use test' (with current), the positive reactions to the (meth)acrylates series, to the acetone extracts, the thin layer chromatograms (TLCs), and to glycerine (10.0% aq.) and acrylic acid (0.1% pet.), as assessed according to the guidelines of the ICDRG.

	Day 2	Day 3	Day 7
Electrodes 'as is'			
Flexibele Elektroden	NR	-	-
Klinerva Stimtrode	NR	+++	+++
Dura-stick plus	NR	++	++
Dura-stick	NR	+++	+++
Dura-stick premium	NR	++	++
Electrodes 'use test'			
Flexibele Elektroden	NR	-	-
Klinerva Stimtrode	NR	+++	+++
Dura-stick plus	NR	+++	+++
Dura-stick	NR	+++	+++
(Meth)acrylates series (positive reactions)			
2-Hydroxyethyl acrylate 0.1% pet.	NR	++	+
2-Hydroxypropyl acrylate 0.1% pet.	NR	+	+
2-Hydroxyethyl methacrylate (2-HEMA) 2.0% pet.	NR	+	+
Ethyleneglycol dimethacrylate (EGDMA) 2.0% pet.	NR	+	+
1,4-butanediol diacrylate 0.1% pet.	NR	+	+

Table 1. Continued

	Day 2	Day 3	Day 7
Diethyleneglycol diacrylate 0.1% pet.	NR	+	+
Acetone extracts, TLCs, and ingredients			
1. Dura-stick plus extract	-	-	-
2. Stimtrode extract	-	-	-
3. Dura-stick extract	-	-	-
4. Dura-stick premium extract	-	-	-
Mix electrodes 1-4, acetone extract, non-evaporated	?	++	+
Mix electrodes 1-4, acetone extract, evaporated	++	+++	++
TLC strip non-evaporated	-	-	-
TLC strip evaporated	-	-	-
Glycerine 10.0% aq.	-	-	-
Acrylic Acid 0.1% pet.	?	++	+++

NR = no reading performed; '-' = negative; '?' = doubtful reaction

Material data safety sheets (MSDSs) of all electrodes were retrieved, and showed that the same adhesive gel was used in Klinerva Stimtrode, Dura-stick®, Dura-stick® plus and Dura-stick® Premium: Multistick® Hydrogel. The composition was as follows: glycerol (glycerin) (non-tallow), water, and poly(acrylate) copolymer (CAS no. 28062-44-4; acrylic acid-*N*-vinylpyrrolidone copolymer). The data sheet did not mention the presence of any of the (meth)acrylates to which our patient was sensitized. Our hypothesis was that, despite this fact, there could still be traces of (meth)acrylates present in the adhesive layer. The MSDS for Flexibele Elektroden showed that no (meth)acrylates were present, and we postulated that the patient most likely had developed irritant contact dermatitis initially. Another TENS electrode without (meth)acrylates was prescribed: Dura-stick premium Blue (PALS® Platinum Blue Electrode with Blue Hydrogel). The ingredients according to the MSDS were: polyethylene glycol, water, magnesium acetate, and polyvinylpyrrolidone (CAS no. 9003-39-8). The skin lesions disappeared completely (Fig. 1b).

To ascertain whether there were any (meth)acrylates present in Multistick® Hydrogel, and to identify the true culprit allergen, additional patch testing was performed with acetone extracts of the electrodes, thin-layer chromatograms (TLCs), and the ingredients according to the MSDS. Non-evaporated extracts of each electrode containing Multistick® Hydrogel on its own and combined as a mix were prepared in

acetone, and an evaporated extract of all electrodes combined (mix) in acetone was also prepared. Extraction was performed with an ultrasonic bath, and the supernatant was concentrated with a vacuum evaporator (4). Two TLCs were prepared, one by application of 100 μ l of the non-evaporated mix, and the other by application of 60 μ l of the evaporated mix, and then separated on silica gel (5). The mobile phase was 50:50 heptane/ethyl acetate. Of the ingredients, acrylic acid (CAS no. 79-10-7) was patch tested 0.1% pet., and glycerol (CAS no. 56-81-5) was patch tested 10% aq. The extracts, acrylic acid, glycerol and TLCs were applied for 48 h, and read on D2, D3, and D7 (Table 1). Both extracts of the mixes and acrylic acid 0.1% pet. (Fig. 2) gave positive results, but the TLCs gave negative results. Subsequently, one Dura-stick® premium electrode was analysed for the presence of acrylic acid by the use of high-performance liquid chromatography coupled with ultraviolet detection (HPLC-UV). The electrode was extracted in the mobile phase used for the analysis of acrylic acid (20:80 acetonitrile/water with 0.1% phosphoric acid). HPLC-UV of this extract showed an undisturbed spectrum of acrylic acid, and quantification showed that there was ~ 57 μ g of acrylic acid per gram of electrode. When calculated for the electrode when it was attached to the back, the release of acrylic acid from the electrode was ~ 7.6 μ g/cm² (the patch test preparation of 0.1% acrylic acid corresponds to a dose of ~ 39 μ g/cm²).

Figure 2.

Patch test reactions to acrylic acid 0.1% pet. on day 2 (a), day 3 (b), and day 7 (c), showing a clear crescendo pattern.

DISCUSSION

Previously, 4 cases of contact allergy to (meth)acrylates used in TENS have been reported (6–8). Prior to that, there were reported cases of allergic contact dermatitis caused by propylene glycol in a conductive gel used for TENS application (9, 10), 1 case report of contact allergy to nickel in a TENS electrode (11), and 1 case report of TENS contact dermatitis in which the allergen was not identified (12). When (meth)acrylates were identified as the causative allergens, this was based on information from the MSDS or from the producer.

In the present case report, the initial patch test showed (weak) positive reactions to (meth)acrylates. Although the MSDS only showed the presence of a poly(acrylate) copolymer, it has been shown through analysis of products containing acrylate polymers that the MSDS often provides incomplete information regarding the acrylate monomers present in the product (13). Our initial hypothesis was that traces of (meth)acrylate monomers were the likely cause of the skin symptoms; however, further testing with acetone extracts and the ingredients according to the MSDS provided new information. The patient developed a +++ reaction to acrylic acid, showing a clear crescendo pattern. Positive reactions were also seen to the extracts of multiple electrodes combined, but not to extracts of the individual electrodes. This can be explained by the differences in concentrations of substances between the different extracts. It is interesting to note that the TLCs did not give positive reactions, even though they were prepared from the extracts that did give positive reactions. This might be explained by the volatile nature of (meth)acrylates (14). Another explanation might be a too low surface concentration of acrylic acid on the TLC plate, as it showed poor resolution on TLCs, without a well-defined spot for this substance.

HPLC-UV of a sample of the Dura-stick® premium electrode (containing Multistick® Hydrogel) showed that acrylic acid was indeed present in the adhesive, and we therefore believe that acrylic acid was the culprit allergen. The MSDS of Dura-stick® premium Blue, which the patient tolerated, showed that it did not contain any acrylic acid. Acrylic acid was also shown to be the culprit allergen in a recent case report of allergic contact dermatitis caused by the adhesive layer of electrocardiogram electrodes (15).

The release per cm^2 of acrylic acid in the electrode was lower than that of the concentration in pet. With which the patient was patch tested ($7.6 \mu\text{g}/\text{cm}^2$ as compared with $39 \mu\text{g}/\text{cm}^2$). However, given the fact that 0.1% acrylic acid gave a +++ reaction, it is not unlikely that the elicitation threshold might even be $<7.6 \mu\text{g}/\text{cm}^2$. Furthermore, the electrodes were attached to the lower back continuously, whereas the patch test is applied for only 48 h. The positive reactions to the various (meth)acrylates might be explained as cross-reactions, although a recent patch test study did not show any cross-reactions between acrylic acid and (meth)acrylates (16). Another explanation might be previous sensitization, even though a detailed history of the patient did not show exposure to products containing (meth)acrylates.

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Following submission of the case report, two more patients were referred to our outpatient clinic presenting with contact dermatitis to their TENS electrodes. A short overview is provided below in table S1.

Table S1: Patch test results to TENS electrodes, acrylic acid 0.1% pet., and positive reactions to the (meth)acrylates series (Chemotechnique Diagnostics, Vellinge, Sweden) in two additional cases presenting with allergic contact dermatitis to their TENS electrodes; a 81 year old, non-atopic, female who developed contact dermatitis after 6 months of TENS application, and a 70 year old, non-atopic, male who developed contact dermatitis to his TENS electrodes shortly after start of TENS application. TENS electrodes that were used by the patients were: Uni-Patch S Series (REF 696SS, Covidien, Dublin, Ireland)(cases 2 and 3), Dura-stick premium (REF 42209, DJO Global inc., Guildford Surrey, England)(cases 2 and 3), ValuTrobe (Model CFF153, Axelgaard Manufacturing CO, Ltd. Falbrook, USA) (case 2), Flexibele elektroden (Eurocept Homecare, Houten, the Netherlands, previously known as Klinerva BV, Ankeveen, The Netherlands)(case 3), and Elektroden rechthoekig (Eurocept Homecare, Houten, the Netherlands)(case 3).

Patch testing and readings were performed as described in the case report. Both cases developed a positive patch test reaction (strong positive and extreme positive, respectively) to acrylic acid, present in Multi-stick Hydrogel, used in the following TENS electrodes: Dura-stick, Dura-stick premium, ValuTrobe. Covidien was contacted to provide the material data safety sheet for the Uni-Patch S series TENS electrode, but none were provided.

	Case 2 (81-year old female)			Case 3 (70 year old male)	
	Day 3	Day 7	Day 11	Day 3	Day 7
Electrodes 'as is' + acrylic acid					
Uni-Patch S Series	-	-	-	-	-
Uni-Patch S Series*		Re-test	-(D ₄ reading)		
Dura-stick premium	?	+++	++	+++	++
ValuTrobe	-	++	+	NT	NT
Flexibele elektroden	NT	NT	NT	-	-
Zelfklevende elektroden rechthoekig	NT	NT	NT	++	+
Dura-stick	NT	NT	NT	+++	++

Table S1. Continued

	Case 2 (81-year old female)			Case 3 (70 year old male)	
	Day 3	Day 7	Day 11	Day 3	Day 7
Acrylic Acid 0.1% pet.	++	+	+	+++	++
(Meth)acrylates series (positive reactions)					
Ethyl acrylate 0.1% pet.	+++	+	NR	++	++
Butyl acrylate 0.1% pet.	++	+	NR	++	++
2-hydroxyethyl acrylate 0.1% pet.	++	++	NR	+++	++
2-hydroxypropyl acrylate 0.1% pet.	++	++	NR	+++	+++
Methyl methacrylate 2.0% pet.	-	-	NR	+	+
2-hydroxyethyl methacrylate (HEMA) 2% pet.	++	+	NR	++	++
2-hydroxypropyl methacrylate (HPMA) 2% pet.	++	+	NR	++	++
ethyleneglycol dimethacrylate (EGDMA) 2% pet.	++	+	NR	++	++
Triethylene glycol dimethacrylate (TEGDMA) 2% pet.	-	?	NR	?	+
1,4-Butanediol dimethacrylate 2.0% pet.	-	-	NR	+	++
1,4-butanediol diacrylate 0.1% pet.	+	++	NR	+++	++
1,6 hexanediol diacrylate 0.1% pet.	+	++	NR	+++	++
diethyleneglycol diacrylate 0.1% pet.	+++	+++	NR	+++	+++
tripropylene glycol diacrylate 0.1% pet.	-	+	NR	+++	++
trimethylolpropane triacrylate 0.1% pet.	-	?	NR	++	++
pentaerthritol triacrylate 0.1% pet.	-	+	NR	++	++
Oligotriacrylate 0.1% pet.	-	+	NR	+	+
Urethane diacrylate (aliphatic) 0.1% pet.	-	-	NR	?	-
Urethane diacrylate (aromatic) 0.05% pet.	-	-	NR	+	+
Epoxy acrylate 0.5% pet.	-	+	NR	++	++

Table S1. Continued

	Case 2 (81-year old female)			Case 3 (70 year old male)	
	Day 3	Day 7	Day 11	Day 3	Day 7
triethylene glycol diacrylate 0.1% pet.	+	+++	NR	+++	+++

‘-’, negative patch test reaction; NR, not read; NT, not tested (because patient did not use the TENS electrodes).
 * During application of the patch test material, case 2 revealed she had been using clobetasol ointment once per day on the whole back. For this reason, the Uni-Patch S Series TENS electrode was re-tested on the day 7 (D7) reading to confirm the negative reaction seen on the D3 and D7 reading, and subsequently read on D4, providing an opportunity for a D11 reading for the other TENS electrodes.

Figure S1.
 Patch test results of the electrodes at day 3. The top row shows the patch test results of the electrodes ‘as is’. The bottom row shows the patch test results of the ‘use test’; i.e., electrodes exposed with electrical current. A1/2, Flexibele Elektroden; B1/2, Klinerva Stimtrode; C1/2, Durastick® plus; D1/2, Dura-stick®; E, Dura-stick® premium.

8a

CHAPTER

8b

Allergic contact dermatitis in two employees of an ethylene amine-producing factory

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Ethylenediamine [EDA, $\text{C}_2\text{H}_4(\text{NH}_2)_2$, CAS no. 107-15-3] is an aliphatic amine with a broad range of applications in rubber, dyes, insecticides, and synthetic waxes. Historically, the most common reason for allergic contact dermatitis caused by EDA was its use as a stabilizer in topical medicaments (e.g. Mycolog® cream) (1, 2). This application of EDA has been discontinued. In 1995, EDA was removed from the European baseline series because of the low incidence of EDA sensitization (3). However, it is still present in the bronchodilator aminophylline, and is often found in occupational settings (4). In this article, we present two process operators with allergic contact dermatitis caused by EDA working in the same company producing ethylene amines.

CASE REPORTS

Case 1

A 55-year-old, healthy, non-atopic male was referred to our centre with complaints of periocular oedema, erythema, and pruritus for over 1 year (Fig. 1). In addition to skin complaints, he suffered from nocturnal nasal obstruction and sneezing. Both skin and nasal symptoms subsided during weekends and holidays, suggesting a relationship with work. The patient had been working for the company for ~5 years, and worked with safety goggles. The severity of his symptoms was apparently related to the presence and direction of the wind, and increased after he had been dealing with equipment malfunction or leakage.

Figure 1.

Case 1: oedema and erythema in the periocular region. Picture printed with full consent of the patient.

Case 2

A 27-year-old, healthy, non-atopic male was referred with pruritic hyperkeratosis, fissures and occasional vesicles of the hands (predominantly thumb and index fingers; Fig. 2). He had been working for the company for 8 months, and the first symptoms started 6 months ago. The symptoms persisted during weekends and holidays. During a previous internship at the same company ~7 years ago, he also experienced complaints of 'dry red hands' with pruritus. These symptoms resolved 4 months after the end of the internship.

Both patients denied previous use of EDA-containing (topical) medicaments or aminophylline. The production facility of this company is completely outdoors. The patients' work activities included controlling, connecting, and cleaning distillation columns; residual water from the distillation process is used for cleaning. Cleaning is performed with hoses, during which vapour of the processed water is in the air. Documentation from the factory showed that the residual water used for cleaning

contained traces of EDA, diethylenetriamine (DETA), and triethylenetetramine (TETA), among other substances.

Figure 2.

Case 2: hyperkeratosis, fissures and lichenification of predominantly the thumb and index finger of the left hand. The right hand showed hyperkeratosis and fissures on the palm (photograph not shown).

Patch testing was performed in both cases with the European baseline series, partly with the TRUE Test® (Mekos, Hillerød, Denmark), which included ethylenediamine dihydrochloride 50 µg/cm² in a hydrophilic matrix. Additional patch testing was performed with DETA 1.0% pet. and TETA 0.5% pet. (Chemotechnique Vellinge, Sweden) in Van der Bend® square chambers (van der Bend, Brielle, The Netherlands). In addition, all products of the distillation process were provided by the company as 1.0% aq., and subsequently patch tested. The tests were read according to ICDRG guidelines on day (D) 3 and D7 (Table 1). Both patients had positive patch test reactions to EDA included in the TRUE Test® and 1.0% aq., to DETA 1.0% pet. and 1.0% aq., and to TETA 0.5% pet., but not to TETA 1.0% aq. Both workers had to change jobs because exposure to EDA could not be avoided while they were working as process operators at the ethylene amine-producing factory. Cessation of exposure to ethylene amines led to resolution of all symptoms.

Table 1. Patch test results

Patch tests	Case 1		Case 2	
	D3	D7	D3	D7
Departmental baseline series^a				
Ethylenediamine (EDA) dihydrochloride 50 µg/cm ²	++	+	++	+
Additional patch tests				
Diethylenetriamine (DETA) 1.0% pet.	+	-	++	+
Triethylenetetramine (TETA) 0.5% pet.	?	+	++	+
Distillation products				
Ethylenediamine (EDA) 1.0% aq.	-	++	++	+
Diethylenetriamine (DETA) 1.0% aq.	-	+	++	+
Triethylenetetramine (TETA) 1.0% aq.	-	-	-	-
Tetraethylenepentamine (TEPA) 1.0% aq.	-	-	-	-
Pentaethylenhexamine (PEHA) 1.0% aq.	-	-	-	-
N-aminoethylpiperazine (n-AEP) 1.0% aq.	-	-	-	-
Higher ethylene polyamines (HEPA) 1.0% aq.	-	-	-	-
Higher ethylene polyamines (HEPA) s140 1.0% aq.	-	-	-	-
Piperazine anhydrous (PIP) 1.0% aq.	-	-	-	-
Aminoethylethanolamine (AEEA) 1.0% aq.	-	-	-	-
Ethylene dichloride (EDC) 1.0% aq.	-	-	-	-
Process water, undiluted	-	-	-	-

^a Only relevant patch tests results are shown.

DISCUSSION

Occupational allergic contact dermatitis caused by EDA has been described, with the cause being exposure to aminophylline, lubricants, metal polishers, detergents, floor polish removers, and epoxy resin hardeners, among other substances (5–13). One study investigated a cohort of employees of an ethylene amine-producing factory exposed to EDA over the course of 36 years, and found that many had developed skin and/or respiratory complaints (14). In this case report, we describe 2 patients with allergic contact dermatitis working at the same factory, the first of whom also suffered from rhinitis. Another process operator from the same company was also seen at our outpatient clinic, but with respiratory complaints only. The ethylene amines are manufactured by a process in which ammonia reacts with ethylene dichloride

under high pressure. Soda is added to the process, and multiple end-products (Table 1, distillation products) are subsequently isolated by distillation. Heated residual water remains and is used, among other purposes, for cleaning. This water contains small amounts of ethylene amines, including, and most prominently, EDA. Both workers were sensitized to EDA, DETA, and TETA, and, because those were present in varying concentrations in the water and in the air at the factory, most likely all three were responsible for their allergic contact dermatitis.

Case 1 presented with periocular airborne allergic contact dermatitis related to the direction of the wind, in spite of using safety goggles. He had positive patch test reactions to EDA, DETA, and TETA, although TETA only gave a positive reaction as 0.5% pet. and not as 1.0% aq. taken from the distillation process. This might be because pet. is a more appropriate vehicle for patch testing with TETA. Another explanation might be that the TETA aq. solution provided by the company actually contained <1.0% TETA. The localization of the patient's skin symptoms could be explained by trapping of the airborne ethylene amines behind his safety goggles. His rhinitis could be explained by either a type IV hypersensitivity reaction or an irritant reaction, although a type I hypersensitivity reaction cannot be excluded. An assay for specific IgE for EDA has not been developed yet, and could therefore not be utilized. Occupational rhinitis caused by ethylene amines has been described previously, although the exact mechanism remains unclear (15). Case 2 presented with hand eczema, with positive patch test reactions to the same substances as case 1. His nitrile gloves are impermeable to liquids, but we hypothesize that either the residual water might have entered at the edge of the glove and become trapped inside, or that contamination occurred during the taking off and putting on of already used gloves, or a combination of both. The resulting hand dermatitis was diagnosed as allergic contact dermatitis with an irritant component.

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CHAPTER
General Discussion

9

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MAIN FINDINGS

The focus of this thesis is on three different aspects of contact allergy: immunology, patch testing, and epidemiology, aspects that do not exist in a vacuum but inform one another. For example, in order to understand how patch testing works and how to correctly interpret its findings, some understanding is needed of immunology of contact allergy. In order to place epidemiological findings in the correct context, understanding is needed of how certain factors such as for example inter-individual variability and even meteorological conditions can influence patch test results.

First, a short overview of the main findings of this thesis will be presented, after which two themes will be explored; whether polysensitisation represents a valid phenotype for increased susceptibility to develop contact allergy, and quantitative risk assessment, a toxicological method that serves as primary prevention to prevent induction of contact allergy to occur.

Chapter 2 presents a review of the role of TNF in both the induction and elicitation phase of contact allergy, the role of genetic variants in the promoter region of TNF in increased susceptibility to become sensitised to contact allergens, and how best to perform future investigations into the association between specific genetic variations and susceptibility to contact allergy. By reviewing the role of TNF in contact allergy induction the role of the innate immune system is highlighted, indicating that irritancy of a chemical can facilitate induction of contact allergy by producing reactive oxygen species, eventually leading to activation of dendritic cells needed to transport the hapten to draining lymph nodes where it can be presented to naïve T cells, followed by a cascade of events resulting in a hapten-specific delayed type hypersensitivity. It has been hypothesized that specific single nucleotide variants in the promoter region of TNF, specifically TNF -308 G->A, leads to increased TNF production, possibly providing a clue to why some individuals are more susceptible, or, have a lower threshold, to develop contact allergy. Based on all available genetic association studies performed on this specific SNV and contact allergy, no evidence for such a relationship has been shown so far. An explanation for this is the small sample sizes. Moreover, future studies should focus on using an appropriate phenotype to represent susceptible individuals. Our suggestion that polysensitization might function as thus is explored below.

Cross-elicitation responses to the newly developed hair dye molecule 2-methylethoxy-para-phenylenediamine (ME-PPD) were investigated in 25 subjects with contact allergy

to para-phenylenediamine (PPD) in **chapter 3**. Quantitative risk assessments (see below) have indicated that although ME-PPD is a moderate sensitizer according to the LNNA, it is not expected to induce contact allergy for hair dye consumers at the concentration used in hair dye conditions. Our study showed that ME-PPD is not a safe alternative for individuals already sensitised to PPD, as 48% showed an elicitation response to an open use test with a hair dye preparation containing 2.0% ME-PPD and an exposure time of 45 minutes. In PPD-sensitised individuals, 84% showed a positive patch test response to ME-PPD 2.0% pet, the difference with the open use test being an exposure time of 48 hours and under occlusion. A dose-response relationship shows that higher doses of ME-PPD are needed to elicit an allergic contact dermatitis reaction compared to PPD. Another finding was that the dose of ME-PPD needed to elicit a reaction depends on the strength of PPD patch test reactions; individuals with an extreme (+++) positive PPD patch test reaction showed a positive patch test reaction to lower concentrations of ME-PPD on average compared to individuals with a weak (+) or strong (++) PPD patch test reaction.

A retrospective database study investigating the persistence of positive patch test reactions upon re-testing is performed in **chapter 4**, showing that 33.2% of all positive reactions were negative at a second patch test procedure. As expected, the vast majority of non-persistent reactions were initially weak (+) positive, although there were two reactions that had changed from extreme (+++) positive to negative. The only factors found to be associated with a more consistent level of persistence were allergens tested in the TRUE Test and strength of initial patch test reactions; no influence of patient specific factors was observed nor of time passed between two patch tests. Although retrospective and with a relatively small sample size, the findings do shed some light on the validity of patch test results and should be considered when drawing conclusions from epidemiological studies. The possibility of a sensitised individual to become tolerant is something that could be explored more in future studies. A possible explanation for non-persistence of a positive patch test is a decreased function of the immune system with increasing age, although evidence exists that this most likely is not relevant until 80 years and older.⁽¹⁾ Other explanations are that the first positive patch test reaction was a false positive or an irritant reaction wrongly interpreted as positive, or that the consequent negative patch test reaction was false negative.

Another aspect of patch testing – the concentration and vehicle in which a hapten is tested – is explored in **chapter 5** in which the diagnostic performance of methylchloroisothiazolinone/methylisothiazolinone (MCI/MI) tested in 0.01% aq. (corresponding to 3 mg/cm²) is compared to MCI/MI 4 mg/cm² as tested in the TRUE Test. In total, 14.4% of the patch tested population showed a positive reaction to one or both patch test preparations, indicating once again the extent of the MI contact allergy epidemic.(2, 3) A higher number of positive reactions were observed to the TRUE test preparation, almost twice as many, despite the concentration that is tested in the TRUE Test is only a 4:3 ratio higher. A steep dose-response relationship for MCI/MI could explain this finding, as well as the different vehicle. A major limitation of this study is that the current recommended patch test concentration for MCI/MI of 0.02% aq. was not included.(4)

Chapter 6 presents a large European multicenter study investigating polysensitization, defined as sensitizations to three or more non-related allergens of the European baseline series. An age- and sex-standardized prevalence of about 7% was observed, but with some variability observed between countries. Although it is tempting to assume this variability might be explained by differences in exposures between countries, a more likely explanation is the difference in patch tested populations between centers, especially eligibility criteria for patch testing. Previously described risk factors for polysensitization is confirmed in a larger population, namely female sex and age older than 40. An interesting observation was the increased proportions of strong and extreme positive reactions in polysensitised individuals compared to oligosensitised (1 or 2 positive reactions) individuals. The study presents a more refined definition of polysensitization as well by listing combinations of contact allergens that should be counted as one based on cross-reactivity and co-sensitization. One example of this is when a person has positive patch test reactions to both PPD and benzocaine; as these cross-react it is likely that this person is only sensitised to one of both, and therefore these should not be counted as two separate reactions when assessing polysensitization.

Recently the European baseline series with recommended additions 2019 were published (Wilkinson et al.).(5) Hydroperoxides of limonene and hydroperoxides of linalool were recommended to be included in the additional baseline series, while further information is needed to confirm or refute their importance.

The importance of this possible inclusion is underscored in **Chapter 7** because 9.4% and 11.7% positive reactions to hydroperoxides of limonene and hydroperoxides of linalool, respectively, were observed. An important observation was that the majority were weakly positive, and a relatively high proportion of doubtful and low proportion of irritant reactions were observed, supporting the argument for higher patch test concentrations. This study attempts to characterize patients allergic to either or both hydroperoxides and how they differ from patients allergic to other fragrances, patients allergic to other allergens, and patients without any positive patch test reactions, indicating the majority are female. Clinical relevance is also presented, indicating that 40.3% and 46.3% of positive reactions to hydroperoxides of limonene and hydroperoxides of linalool, respectively, were of probable to certain clinical relevance. A limitation of the clinical relevance assessment was that hydroperoxides are not labelled, only the unoxidized forms of limonene and linalool. Therefore clinical relevance was evaluated by the presence of limonene and linalool in the products and improvement of the symptoms after stopping exposure to these allergens.

Chapter 8 presents two case reports. The first is a case report of an elderly man who developed allergic contact dermatitis to his Transcutaneous Electric Nervous Stimulation (TENS) electrodes which he used to treat lower back pain. The search for the culprit allergen is presented and the use of thin layer chromatography is highlighted, resulting in the identification of acrylic acid being the responsible hapten. The second presents two patients with allergic contact dermatitis to ethylenediamine, both working at the same factory that produces this chemical. The first suffered from peri-ocular airborne allergic contact dermatitis, most likely caused by trapping of ethylene diamine behind his safety goggles, and concomitant rhinitis. The second patient suffered from hand dermatitis despite handling products with protective gloves, most likely as result of some excess ethylene diamine containing water getting inside the gloves.

POLYSENSITISATION AS A PHENOTYPE FOR INCREASED SUSCEPTIBILITY

When patch testing a dermatitis population it is not uncommon to find positive reactions to more than one sensitising chemical. Previous literature termed two or more such positive reactions polysensitization,(6) in more recent publications the term was defined as three or more positive reactions to contact allergens in the European

Baseline Series.(7) In our multicenter study (chapter 6), we formulated the additional requirement of counting concomitant sensitisations caused by either cross-reactions or co-sensitization as one positive reaction. However, different definitions are still used within the scientific community. A recent study investigating allergic contact dermatitis in atopic dermatitis patients also defined polysensitization as reacting to at least three different unrelated contact allergens, however these also included contact allergens tested in additional series other than the European Baseline series. (8) Examining the literature investigating polysensitization to food- and aeroallergens provides a similar pattern of varying definitions.(9, 10)

The importance of using one definition agreed upon by the scientific community as opposed to many different ones is the opportunity it presents for comparing data and performing meta-analyses. There are some challenges in finding the correct definition for polysensitization, mostly because it is not yet understood whether polysensitization represents true increased susceptibility or is simply the result of increased exposure.(6) Should positive reactions detected by additionally tested series be counted or not, and if yes, should the number of positive reactions required for polysensitisation increase with the amount of allergens tested?

There is some evidence that polysensitization is the result of increased susceptibility. A landmark study by Moss and Friedmann showed that polysensitised individuals, defined by three or more contact allergies to unrelated allergens, were more easily sensitised to 2,4-dinitrochlorobenzene (DNCB) at lower concentrations.(11) They further investigated monosensitised individuals and found they were also slightly easier to sensitize compared to non-sensitised individuals, but not significantly so.(11) This implies that susceptibility to develop contact allergies might be a graded phenomenon rather than a binary one. Our own results (chapter 6) show a positive correlation between number of positive reactions and proportion of extreme (+++) positive reactions, indicating increased reactivity and therefore increased susceptibility.

As mentioned above and in the introduction of this thesis, there are many factors influencing the outcome of a particular patch test; external influences, personal factors, and patch testing related factors. These factors can influence the probability of a false positive or a false negative reactions. An example of external influences are meteorological conditions.; one study found that for certain allergens the probability of doubtful positive and irritant patch test reactions increased during cold and arid weather.(12) Personal factors can include hormonal influences as discussed in the

introduction of the current thesis.(13-15) Another example is atopic dermatitis, which is a topic of some controversy in whether it increases or decreases the susceptibility to developing contact sensitization. In our large European multicenter study (chapter 6), atopic dermatitis was a small but significant risk factor for polysensitisation. This could however be because of increased exposure (e.g. to topicals) as opposed to increased susceptibility.

An example of a patch test related factor is the 'spill-over' effect; patch testing with a marginally irritating concentration of sodium lauryl sulfate at varying distances from an positive patch test reaction (to example nickel, epoxy resin, PPD, etc) results in to enhanced irritant reactions the closer the proximity.(16)

Another factor that should be considered is that an increasing number of positive reactions might enhance the probability of a false positive reaction. For example, one study showed that thiuram mix was significantly more likely to be positive with increasing concomitant positive reactions to allergens unrelated to thiuram mix.(17) Furthermore, our own results of chapter 4 suggest that a significant number of mostly weak positive patch test reactions are non-persistent upon re-testing. These studies provide some evidence for the existence and extent of false positive reactions, which should be taken into account when defining polysensitization. Another important confounding factor is of course the difference in exposure and exposure in general as the most logical explanation for becoming polysensitised.

These considerations lead us to a discussion on how polysensitization should be best defined when the aim is to use it as a phenotype for increased susceptibility. The possibility of false positives and the 'spill-over' effect indicate that choosing three or more positive patch test reactions as the cut-off is on the conservative side. A way to decrease the effect of these confounders is to only count strong (++) and extreme (+++) positive reactions, perhaps scoring weak (+) positive reactions as half a positive reactions, i.e. two weak positive reactions to be counted as one. If following this line of thought, another question is whether doubtful (?) patch test reactions should be considered as well, as these might well become positive at higher concentrations. It should however be noted that evaluation and interpretation of these type of questionable reactions might be too dependent on the experience of the investigator, notwithstanding inter-investigator variability. Deciding to use a more strict definition for polysensitization (more positive reactions, not counting weak positive reactions) can aid in the search for genetic variants associated with increased susceptibility to become sensitised. The other side of this argument is that choosing such a strict

definition makes it more difficult to find individuals meeting these criteria. To reiterate, the most important thing is that the scientific community should settle on one definitive definition taking the above in consideration.

RISK OF ALLERGY INDUCTION AND ATTEMPTS AT PRIMARY PREVENTION

Our skin is exposed to many chemicals in daily life that are potential sensitizers, both at home and in occupational settings. As explored in the current thesis, this leads to adverse health effects such as allergic contact dermatitis. In order to determine whether a chemical is a sensitizer different *in vivo* investigations have been developed throughout the decades. The Local Lymph Node Assay (LLNA), a murine assay in which proliferative responses of lymphocytes in local lymph nodes are following repeated topical exposure, has provided a sensitive method for this.⁽¹⁸⁾ Besides identification of sensitizing potential, the LLNA provides the additional value of being able to estimate sensitizing potency. The LLNA of a chemical results in an EC₃ value, from which sensitizing potency can be extrapolated.^(19, 20) The EC₃ represents the effective concentration required to obtain a three-fold increase in lymph node cell proliferative activity compared to vehicle tested controls. It is used to categorize a chemical into being a weak, moderate, strong, or extreme sensitizers.⁽¹⁾ Currently, the use of the LLNA cannot be used for cosmetic ingredients due to a ban on animal testing, necessitating *in vitro* alternatives to assess the sensitization potential of chemicals.⁽²¹⁾

Quantitative risk assessment

The reason for evaluating not just the ability to sensitize (hazard) but also the potency of how sensitizing a chemical is, is that this allows for determining the risk it poses in real life context. One of the main tenets of toxicology is that risk equals hazard times exposure, where in the case of induction of skin sensitization hazard is the intrinsic property of a chemical to cause harm. Risk is driven by exposure and translates to the probability of an adverse event or in this scenario induction of contact allergy with possible subsequent allergic contact dermatitis.

The Research Institute for Fragrance Materials (RIFM) introduced quantitative risk assessment (QRA) for fragrance ingredients following discussions on the burden of fragrance contact allergy.⁽²²⁾ One publication that followed from this discussion was the 7th amendment of the Cosmetic Directive in 2003 which introduced the requirement that of 26 fragrance substances their presence cannot exceed 10 ppm

(or, mg/L) for leave-on products and 100 ppm for rinse off products.(23) Dermal sensitization quantitative risk assessment was developed as a model to determine safe concentrations of fragrance ingredients in consumer products, i.e., concentrations that would not induce skin sensitization.(24) The three pillars of QRA are i) identification and quantification of hazard, ii) assessing both acceptable and actual exposure and iii) comparing actual to acceptable exposure to characterize the risk of induction of sensitization.

It is assumed that there is a threshold below which the dose of a sensitizer is unlikely to induce sensitisation. For hazard identification and quantification this threshold, termed the no expected sensitization induction level (NESIL), needs to be determined. The NESIL is derived from both animal studies (LLNA) and human data (human repeated insult patch test, see (25) for more detail). As the sensitising potency increases, the amount of the chemical needed to induce sensitization decreases, so it follows that the NESIL will also decrease. The NESIL is then adjusted using appropriate safety factors (termed sensitization assessment factors (SAF)). Examples of SAFs are inter-individual variability, differing exposure matrices, and possible exposure conditions or characteristics not included in the actual exposure assessment. Adjusting the NESIL by applying SAFs results in the acceptable exposure level (AEL), expressed in dose/unit daily. The AEL is derived by dividing the NESIL by the SAF, which are often in the range of a factor 10 to 300. Characterizing the real life risk is performed by comparing the resulting AEL to the consumer exposure level (CEL), where the AEL has to be equal or larger than CEL to avoid induction of sensitization.(24, 26)

Hair dye ingredients: does QRA address risk in consumers who experience an adverse reaction after hair dying

Although QRA was developed for fragrance ingredients, it has been used for other product groups and chemicals as well, such as PPD in hair dye, classified as a strong sensitizer based on LLNA data. (27, 28) After ME-PPD was developed by the industry as a less sensitizing hair dye ingredient (categorized as a moderate sensitizer) than the extreme sensitizers PPD and toluene-diamine (TDA), QRA was performed indicating a much lower allergy induction risk under consumer hair dye usage conditions.(27) ME-PPD was developed as a means of primary prevention of hair dye induced allergic contact dermatitis, and therefore its primary focus was not on consumers with a pre-existing contact allergy to important hair dye ingredients. QRA also does not address this as it only assesses induction risk and not risk of (cross-)elicitation. Different studies have investigated cross-elicitation responses to ME-PPD in PPD allergic individuals,

the latest of which is part of this thesis. An important conclusion is that while ME-PPD might fulfill its role as a hair dye ingredient with good hair dyeing performance with negligible risk of allergy induction, it does pose a risk of eliciting allergic contact dermatitis under hair dye use conditions in individuals previously sensitised to PPD, indicating cross-reactivity. A dose-response curve showing proportion of PPD allergic individuals responding to increasing ME-PPD patch test doses showed that while a higher concentration of ME-PPD is needed to elicit a similar proportion of elicitation reactions than to PPD, virtually all PPD allergic individuals are able to respond to ME-PPD as long as the exposure dose is high enough. This study is based on single exposure open use test to a hair dye preparation containing 2.0% ME-PPD for 45 minutes, not on multiple exposures as might occur in a real life context. A study by Kock et al. revealed that a proportion of PPD allergic individuals initially showing no reaction to an ME-PPD open use test, might still develop allergic contact dermatitis following repeated hair dyeing.(29)

This example of QRA in action in regards to the newly developed hair dye ingredient ME-PPD shows that although it can indicate the risk of induction of contact allergy, it cannot completely address the risk for adverse skin events in consumers as it does not consider cross-reactivity between chemicals.

Other concerns about QRA

The lack of focus on prevention of elicitation, and other concerns about QRA, were addressed in an opinion published by the European Commission Scientific Committee on Consumer Products (SCCP) in 2008. (30) Further criticism was focused on the lack of consideration of aggregated exposure (concomitant exposure to multiple fragrance ingredients in one product, and frequency of exposure). Many products containing fragrance ingredients contain multiple fragrance ingredients, and other potential skin sensitisers such as preservatives. This is supported by a recent study which found that of cosmetic products containing at least one of the 26 fragrance ingredients identified by the Cosmetic Directive, 85.5% and 73.9% contained at least more than two or more than three fragrance ingredients, respectively.(31) Occupational exposure was another area which deserved more attention. Other points of concern were the low estimated exposure levels in consumers, and the conservative use of SAF.

However, the main concern was that despite the introduction of QRA no reduction in sensitization trends to fragrances had been observed so far in epidemiological data. This was true in 2008 and is, unfortunately, still true.(32) Our investigation on polysensitization in patch tested patients in Europe between 2009 and 2014

showed that in this subgroup, 40.8% (sex- and age-standardized) had a positive patch test to fragrance mix I (containing eight fragrance allergens), as well as 10.7% of oligosensitised patients. Complicating interpretation of such epidemiological data is that an unknown proportion of patients were most likely sensitised prior to the introduction and implementation of QRA.

In response to this criticism, the fragrance industry set up International Dialogue on the Evaluation of Allergens (IDEA), developing QRA2.(32) A recent example of a fragrance allergen dermal sensitization QRA taking into account aggregated exposure is hand exposure to geraniol in personal care products and household cleaning agents. It indicated that aggregated exposure exceeded the AEL, thereby seemingly confirming the validity of this criticism.(33) Another recent dermal sensitization QRA focused on occupational exposure to hair dye ingredients in hair dressers, addressing another of the criticisms of the SCCP that QRA was focused solely on protecting consumers and not employees. In this specific QRA, other risk factors for induction of contact allergy in the setting of hair dressers were considered, for example wet work, an irritant factor decreasing the barrier function of the skin. (34)

Hydroperoxides of limonene and linalool: challenges of QRA and primary prevention

Two fragrance allergens that show especially high prevalences of contact allergy in patch tested populations are hydroperoxides of both limonene and linalool, as shown again in this thesis. Another observation of concern was that of all patients allergic to either or both hydroperoxides of limonene and hydroperoxides of linalool, 39.3% had at least one additional fragrance contact allergy. This increased to 63.2% of patients with a contact allergy to both hydroperoxides of limonene and hydroperoxides of linalool. A potential explanation for this high rate of additional fragrance allergies is concomitant exposure to multiple fragrances, as cosmetic products that contain either limonene or linalool also often contain other fragrances. Furthermore, terpenes (including limonene and linalool) function as skin penetration enhancers, possibly facilitating induction of contact allergy to other concomitantly present sensitisers. (35) Sensitisation rates to either hydroperoxides in the general population have not been investigated yet. Considering relatively high rates of fragrance contact allergy in the general population, it is not unlikely that a considerable proportion of the general population has a contact allergy to either hydroperoxide of limonene or linalool.(36,

37) Pending further epidemiological investigations, the need for primary prevention for these contact allergens is apparent.

In recent years, this need has been voiced. For example, the oxidized forms of limonene and linalool were identified as allergens of high concern in the European Commission Scientific Committee on Consumer Safety (SCCS) opinion on fragrance allergens in cosmetics in 2012.⁽³⁸⁾ These oxidized forms of limonene and linalool contain different oxidation products including multiple different hydroperoxides. It was proposed that content of the oxidized fraction represented by the (hydro)peroxide content (so not including secondary oxidation products such as aldehydes and epoxides) in cosmetics should not exceed 10 ppm, or, that the total oxidized fraction should not exceed 100 ppm. As limonene and linalool are pre-haptens (auto-oxidation has to take place in order for hydroperoxides, identified as the main contact allergens in oxidized limonene and linalool, to be formed), prevention of activation into haptens can help as a prevention measure, for example by preventing air exposure during handling and the addition of suitable antioxidants.

In 2015, the RIFM published a risk assessment of linalool which states that linalool itself does not present a concern for skin sensitization, acknowledging that the oxidation products are known sensitizers.⁽³⁹⁾ It recommended, in line with International Fragrance Association (IFRA) standards, to limit the peroxide levels to 20 mmol/l, and to add an antioxidant during production. LLNA investigation of oxidized limonene and linalool, and specific hydroperoxides, concluded that oxidized limonene and oxidized linalool were moderate sensitizers, and hydroperoxides more potent sensitizers.^(40, 41) A complicating factor is that although the hydroperoxides have been identified as the most important sensitizers in oxidized limonene, other oxidation products have also been shown to be potential sensitizers, for example carvone and limonene-1,2-oxide.⁽⁴²⁾ Karlberg et al. found that a certain proportion of guinea pigs sensitized to limonene-1,2-oxide, did not show elicitation responses to hydroperoxides of limonene, and vice versa, confirming that hydroperoxides are not the sole allergen in oxidized limonene.⁽⁴⁰⁾

Therefore, if primary prevention is to be implanted, it is necessary to perform QRA's on all potential sensitizers in oxidized limonene and oxidized linalool. However, in order to be able to do this, the exposure levels to each potential sensitizer in consumers would have to be known. It is currently unknown what proportion of limonene and linalool auto-oxidizes in consumer products, in particular the proportion of hydroperoxides and other oxidation products. This is most likely different per product category, and also

depends on how it is handled by consumers. Reasonable assumptions can be made as to which product categories pose an increased risk for sensitization to hydroperoxides of limonene and linalool and should therefore be of particular concern; any product for which auto-oxidation is able to take place, for example products which are frequently opened (or left open by accident) and products that are present on the skin for longer durations of time. In our investigation into contact allergy to hydroperoxides of limonene and linalool we assessed which product categories were the cause of allergic contact dermatitis in patients with a certain clinically relevant positive patch test reaction: rinse of products (soap, shampoo), stay on products (cosmetics creams), cleaning agents, deodorants, and perfumes. Whether these patients were sensitised by the same products that caused their allergic contact dermatitis cannot be said with complete certainty. A recent case study did identify hydroperoxides of linalool in shampoo as the most likely culprit of allergic contact dermatitis of the eyelids in a young girl.⁽⁴³⁾

Ultimately, although many challenges are still to be overcome in implementing a truly effective QRA and primary prevention, the importance of such work cannot be overstated. The prevalence of contact allergy is and remains high, with many implications for health, occupational consequences, and an impaired quality of life.

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APPENDIX

Summery
Samenvatting
Acknowledgements
List of publications
Curriculum vitae

SUMMARY

This thesis presents studies investigating different aspects of contact allergy: immunology, patch test methodology, and epidemiology. Contact allergy is a type IV hypersensitivity reaction that is frequent and impairs quality of life; it can lead to itchy and painful skin rashes, can cause problems in occupational settings, and can limit someone's use of products such as cosmetics. Sensitized individuals develop allergic contact dermatitis upon re-exposure of the skin to the responsible contact allergen. Patch testing diagnoses specific contact allergies by exposing individuals to a large range of contact allergens and forms the basis of epidemiological data. Epidemiological studies help identify groups at risk for developing contact allergy overall and to specific contact allergens. **Chapter 1** introduces these topics in detail and serves as a general framework within which the subsequent chapters can be understood.

In **chapter 2** we reviewed the role of tumor necrosis factor (TNF) in contact allergy and allergic contact dermatitis, and whether genetic variants in the TNF gene increases susceptibility to develop a contact allergy.

TNF is a pro-inflammatory cytokine and plays a role in many inflammatory diseases, including contact allergy. During the sensitization phase, TNF is produced by keratinocytes because of the innate immune system reaction caused by skin contact with a hapten. It helps the maturation of dendritic cells, which subsequently carry the hapten to local lymph nodes to present it to immature T lymphocytes. During the elicitation phase, TNF is produced by the hapten-specific T lymphocytes who, following sensitisation, reside in the skin. It helps produce dermatitis because of its cytotoxic effect and production of adhesion molecules that recruit circulating T lymphocytes. Although exposure is the main factor in sensitization – theoretically anyone can become sensitized as long as exposure is of high enough dose or long enough duration – inter-individual differences in susceptibility exist. Genetic differences might account for part of this variation. The most extensively researched genetic variant in the TNF gene is TNF -308 G->A, a single nucleotide variant in the promoter region of TNF. In vitro data suggests that this variant increases TNF production, possibly making carriers of this variant more susceptible to develop contact allergy. However, genetic association studies performed so far have failed to support this hypothesis. We propose two possibilities: or this variant is not functional to such an extent that it increases

susceptibility, or these genetic association studies lack power and suffer from other methodological flaws.

Para-phenylenediamine (PPD) is a hair dye ingredient with strong coloring properties and is a potent sensitizer responsible for the majority of hair-dye related contact allergies. 2-methoxyethoxy-para-phenylenediamine (ME-PPD) is a new hair dye ingredient with a weaker sensitising potential. Contrary to PPD, which is classified as a strong sensitizer, ME-PPD is a moderate sensitizer. In **chapter 3** we looked at whether ME-PPD is also safe to use for PPD allergic individuals. We investigated cross-elicitation responses in 25 PPD-allergic subjects with a history of hair dye related allergic contact dermatitis. We performed both open use testing with hair dye preparations containing either 2.0% PPD or ME-PPD and patch testing with increasing doses of ME-PPD in petrolatum. 48% of study participants showed an elicitation reaction to open use testing with ME-PPD, and 84% showed a positive patch test reaction to 2.0% ME-PPD. Dose-response curves indicated a lower reactivity of ME-PPD compared to PPD; higher doses of ME-PPD are needed to elicit a comparable response to PPD. We also found that the proportions of positive reactions to ME-PPD was correlated with the strength of the original PPD patch test response; 20% of individuals with a weak positive PPD patch test reaction reacted to ME-PPD as well, while 62.5% of individuals with an extreme positive PPD patch test reaction reacted to ME-PPD.

Chapter 4 investigates the persistence of positive patch test reactions. We found that 33.2% of all positive patch test reactions had turned negative upon re-testing. The majority of these non-persistent reactions were weak positive; strong and extreme positive patch test reactions showed a persistence of 82.1% and 89.5%, respectively. Patient characteristics did not influence persistence rates.

Considering the immunology of contact allergies it is unlikely that they can be transient. Other explanations are that the initial positive reaction was a false positive, or the consequent negative reaction a false negative. Possibilities of becoming tolerant to a contact allergy should be explored, including predictive factors.

Chapter 5 explores the impact of the concentration and vehicle in which an allergen is patch tested by comparing methylchloroisothiazolinone/methylisothiazolinone (MCI/MI) in 0.01% aq. (corresponding to 3 µg/cm²) to 4 µg/cm² in the TRUE Test, a ready-made patch test with povidone as its vehicle. Almost twice as many patients tested

positive to the higher concentration of 4 µg/cm² in the TRUE Test compared to 3 µg/cm² in water, which can be explained by the steep dose-response relationship of MCI/MI, or by the different vehicles. For the latter to be investigated, the same concentration should be compared in both vehicles.

Polysensitisation, defined as three or more contact allergies to non-related allergens of the European baseline series, is a phenotype used, among other purposes, to investigate susceptibility to acquiring contact allergies. It also serves as an epidemiological measurement that provides additional information over just a prevalence. We have improved this definition by indicating which contact allergen couples should be counted as one sensitisation, for example because of cross-reactivity or because of concomitant testing of a mix and one of its ingredients. **Chapter 6** found that about 7% of patients from patch testing centres across Europe and were polysensitized. Previously suggested risk factors – female sex and age over 40 years old – were confirmed. A new finding was that polysensitized patients showed a higher proportion of strong and extreme positive patch test reactions compared to oligosensitized patients (one or two unrelated positive patch test reactions).

Limonene and linalool are common fragrance terpenes present in many household products. They transform into allergenic hydroperoxides after adequate air exposure. In **chapter 7** we found that 9.4% and 11.7% of our patch tested population had a contact allergy to hydroperoxides of limonene and hydroperoxides of linalool, respectively. Clinical relevance is difficult to assess for both hydroperoxides as they are not mentioned on labels; only the presence of limonene or linalool is mentioned. Clinical relevance was therefore determined based on their presence in the product and improvement of symptoms following cessation of exposure. Because a high amount was clinically relevant, we can assume that most reactions were true positives. The optimal patch test concentration for hydroperoxides of limonene and hydroperoxides of linalool is a contested subject. We propose a higher concentration based on a high amount of weak positive and doubtful reactions and a low amount of irritant reactions.

In chapter 8 we present two case reports and highlight the at times complex journey to discover the allergen causing the allergic contact dermatitis. The first case report shows how using thin layer chromatography aided in identifying the culprit allergen in a case of allergic contact dermatitis to Transcutaneous Electric Nervous Stimulation

(TENS) electrodes. The second case report reports two employees from the same ethylene amine producing factory with occupational dermatitis and other complaints. Exposure to many by-products in the manufacturing of ethylenediamine required patch testing with a range of chemicals in order to diagnose allergic contact dermatitis. The challenge in testing with work-related substances is to find a patch test concentration that does not give irritant reactions but is high enough to diagnose a contact allergy.

NEDERLANDSE SAMENVATTING

Dit proefschrift beschrijft studies die verschillende aspecten van contactallergie onderzoeken: immunologie, de methodologie van plakproeven, en epidemiologie. Contactallergie is een type IV overgevoelighedsreactie welke frequent voorkomt en de kwaliteit van leven vermindert; het kan leiden tot een jeukende en pijnlijke huidaflslag, het kan problemen veroorzaken in de werkomgeving en kan het gebruik van producten zoals cosmetica beperken. Gesensibiliseerde individuen ontwikkelen allergisch contacteczeem bij hernieuwde blootstelling van de huid aan het verantwoordelijke contactallergeen. Plakproeven diagnosticeren specifieke contactallergieën door individuen bloot te stellen aan een groot aantal contactallergenen en vormt de basis voor epidemiologische data. Epidemiologische studies helpen bij het identificeren van risicogroepen voor het ontwikkelen van een contactallergie in het algemeen en voor specifieke contactallergenen. **Hoofdstuk 1** introduceert deze onderwerpen in detail en functioneert als een algemeen referentiekader waarbinnen de volgende hoofdstukken kunnen worden begrepen.

In **hoofdstuk 2** wordt de rol van tumornecrosefactor (TNF) in contactallergie en allergisch contacteczeem besproken, en wordt besproken of genetische varianten in het TNF-gen de gevoeligheid voor het ontwikkelen van een contactallergie verhogen. TNF is een pro-inflammatoire cytokine en speelt een rol in vele inflammatoire aandoeningen, waaronder contactallergie. Tijdens de sensibilisatie fase wordt TNF geproduceerd door keratinocyten nadat het aangeboren immuunsysteem is geactiveerd door huidcontact met een contactallergeen. Het helpt de rijping van dendritische cellen, die vervolgens het contactallergeen aan onrijpe T-lymfocyten presenteren in lokale lymfeklieren. Tijdens de eliciterings-fase wordt TNF geproduceerd door de contact-allergeen-specifieke T-lymfocyten, die zich na de sensibilisatie fase in de huid bevinden. TNF draagt bij aan de ontstaan van eczeem door een cytotoxische effect en door de productie van adhesiemoleculen die circulerende T-lymfocyten aantrekken.

Hoewel blootstelling de belangrijkste factor is bij sensibilisatie - theoretisch kan iedereen worden gesensibiliseerd zolang de blootstelling lang genoeg duurt en de concentratie hoog genoeg is – bestaan er interindividuele verschillen in gevoeligheid voor het ontwikkelen van contactallergie. Genetische verschillen kunnen een deel van deze variatie verklaren. De meest uitgebreid onderzochte genetische variant in het TNF-gen is TNF-308 G-> A, een enkel-nucleotide variant in het promotor gebied

van TNF. In-vitro gegevens suggereren dat deze variant de TNF-productie verhoogt, waardoor het mogelijk zou kunnen zijn dat dragers van deze variant vatbaarder zijn voor het ontwikkelen van contactallergie. Genetische associatiestudies die tot nu toe zijn uitgevoerd, hebben deze hypothese echter niet ondersteund. Dat kan worden op verschillende manieren worden verklaard: of deze variant is niet zodanig functioneel dat het de gevoeligheid verhoogd, of de steekproefgrootte van deze genetische associatie studies waren te klein, naast andere methodologische tekortkomingen.

Para-phenylenediamine (PPD) is een kleurstof in haarverf en is een potent allergeen dat verantwoordelijk is voor veel haarverf gerelateerde contactallergieën. 2-Methoxyethoxy-*para*-phenylenediamine (ME-PPD) is een nieuwe grondstof voor haarverf met minder sensibiliserend vermogen. In tegenstelling tot PPD, wat een sterk sensibiliserend vermogen heeft, heeft deze grondstof matige potentie tot sensibilisatie. In **hoofdstuk 3** hebben we gekeken of ME-PPD ook veilig te gebruiken is door PPD-allergische personen. Om dit te onderzoeken heb we gekeken naar kruisreactiviteit in 25 PPD-allergische personen met een voorgeschiedenis van haarverf-gerelateerde allergisch contacteczeem. We voerden zowel *open use test* uit met haarverf die óf 2,0% PPD óf 2,0% ME-PPD bevatte en plakproeven met toenemende concentraties ME-PPD. 48% van de PPD-allergische deelnemers toonde een reactie op de ME-PPD *open use test* en 84% toonde een positieve plakproefreactie op 2,0% ME-PPD. Dosis-respons curven toonden dat voor ME-PPD hogere doses nodig zijn om een vergelijkbaar percentage mensen te laten reageren in vergelijking met PPD. De resultaten lieten verder zien dat de proportie van positieve reacties op ME-PPD geassocieerd was met de sterkte van de oorspronkelijke PPD plakproefreactie; 20% van individuen met een zwak positieve PPD plakproefreactie reageerde ook op ME-PPD, terwijl 62,5% van individuen met een extreem positieve PPD plakproefreactie op ME-PPD reageerde.

In **hoofdstuk 4** wordt de reproduceerbaarheid van positieve plakproefreacties onderzocht. Wij vonden dat 33,2% van alle positieve plakproefreacties negatief waren geworden bij een tweede keer testen. De meerderheid van deze niet-persistente reacties waren zwak positief; sterke en extreem sterke positieve plakproefreacties toonden een reproduceerbaarheid van respectievelijk 82,1% en 89,5%. De reproduceerbaarheid van positieve plakproefreacties werd niet beïnvloed door patiëntkarakteristieken.

Het is immunologisch gezien onwaarschijnlijk dat contactallergieën van voorbijgaande aard zijn. Andere verklaringen zijn dat de aanvankelijk positieve reactie fout positief

was, of de daaruit voortvloeiende negatieve reactie fout negatief. Mogelijkheden om tolerant te worden voor een contactallergie moeten verder worden onderzocht, inclusief voorspellende factoren.

Hoofdstuk 5 onderzoekt de invloed van de plakproefconcentratie en het vehikel waarin een allergeen getest wordt door 0,01% methylchloroisothiazolinone/methylisothiazolinone (MCI/MI) in aqua (overeenkomend met 3 µg/cm²) te vergelijken met 4 µg/cm² in de TRUE-test, een kant-en-klare plakproeftest met povidon als vehikel. Bijna twee keer zoveel patiënten reageerden positief op de hogere concentratie van 4 µg/cm² in de TRUE-test vergeleken met 3 µg/cm² in aqua, wat kan worden verklaard door de steile dosis-responsrelatie van MCI/MI, of door de verschillende vehikels. Om dit laatste te onderzoeken, moet dezelfde concentratie in beide vehikels worden vergeleken.

Polysensibilisatie, gedefinieerd als drie of meer contactallergieën voor niet-gerelateerde allergenen van de Europese standaardreeks, is een fenotype dat onder andere wordt gebruikt om de gevoeligheid voor het verwerven van contactallergieën te onderzoeken. Epidemiologisch gezien geeft het aanvullende informatie naast enkel een prevalentie. We hebben deze definitie verbeterd door aan te geven welke contactallergeenkoppels als één sensibilisatie moeten worden geteld, bijvoorbeeld vanwege kruisreactiviteit of omdat zowel een mix van allergenen als een van de bestanddelen van de mix wordt getest. In **hoofdstuk 6** vonden wij dat bij ongeveer 7% van de patiënten van plakproefcentra in Europa sprake was van polysensibilisatie. Eerder aangetoonde risicofactoren - vrouwelijk geslacht en leeftijd ouder dan 40 jaar - werden bevestigd. Een nieuwe bevinding was dat patiënten met polysensibilisatie een hoger percentage sterke en extreem sterke positieve plakproefreacties vertoonden in vergelijking met patiënten met oligosensibilisatie (één of twee niet-gerelateerde positieve plakproefreacties).

Limonene en linalool zijn veel voorkomende terpenen met een karakteristieke geur die in veel huishoudelijke producten aanwezig zijn. Ze transformeren in allergene hydroperoxiden na voldoende luchtblootstelling. In **hoofdstuk 7** vonden we dat 9,4% en 11,7% van onze plakproef populatie een contactallergie hadden voor hydroperoxiden van limonene en hydroperoxiden van linalool. Voor beide hydroperoxiden is de klinische relevantie moeilijk vast te stellen omdat hydroperoxiden niet op labels worden aangegeven; enkel de aanwezigheid van limonene en/of linalool. De klinische relevantie

werd daarom bepaald op de aanwezigheid van limonene of linalool, en het verbeteren van het klinische beeld nadat blootstelling werd vermeden. Aangezien een aanzienlijk percentage klinisch relevant was, kunnen we veronderstellen dat de meeste reacties niet fout positief waren. De optimale plakproef concentratie voor hydroperoxiden van limoneen en hydroperoxiden van linalool is een veel besproken onderwerp. Wij beargumenteren voor een hogere concentratie op basis van een grote hoeveelheid zwak positieve en twijfelachtige reacties en een lage hoeveelheid irritatieve reacties.

In **hoofdstuk 8** presenteren we twee casuïstieken en benadrukken we de soms complexe reis om het allergeen te achterhalen dat het allergisch contacteczeem veroorzaakt. De eerste casus laat zien hoe het gebruik van dunnelaagchromatografie (thin layer chromatography (TLC)) geholpen heeft bij het identificeren van het verantwoordelijke allergeen bij allergisch contacteczeem op Transcutane Elektrische Neurostimulatie (TENS) elektroden. In het tweede artikel worden twee medewerkers beschreven met werk gerelateerde klachten van dezelfde ethyleen amine producerende fabriek. Blootstelling aan vele bijproducten bij de productie van ethyleendiamine vereiste plakproeven met een reeks van chemicaliën waarna de diagnose allergisch contacteczeem gesteld kon worden. De uitdaging bij het testen met werk gerelateerde producten is om te testen met een concentratie die geen irritatieve reactie geeft maar wel hoog genoeg is om een contactallergie te kunnen aantonen.

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LIST OF PUBLICATIONS

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CURRICULUM VITEA

Daan Dittmar was born in Alphen a/d Rijn on march 25th 1990. He graduated from the Scala College in Alphen a/d Rijn in 2008, after which he started studying medicine at the University of Groningen (Rijksuniversiteit Groningen). During his masters he did one year of internships in Oldenburg, Germany. His scientific internship was on developing a new classification for hand eczema, done at the department of Dermatology of the University Medical Center Groningen under the supervision of dr. M.L.A. Schuttelaar. Immediately following his graduation as medical doctor he started his PhD under his supervisors dr. M.L.A. Schuttelaar and prof. dr. M.F. Jonkman, followed by prof. Dr. P.J. Coenraads at a late stage due to the unfortunate passing of prof. dr. M.F. Jonkman. In May 2018 he started working as a Dermatology resident at the University Medical Center Groningen with Barbara Horváth as his tutor.

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