Immunological, chemical and clinical aspects of exposure to mixtures of contact allergens

Charlotte M. Bonefeld[1*]; Carsten Geisler[1]; Elena Gimenéz-Arnau[2]; Jean-Pierre Lepoittevin[2]; Wolfgang Uter[3]; Jeanne D. Johansen[4]

[1]Department of Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen, 2200 Copenhagen, Denmark.

[2] Laboratory of Dermatochemistry, University of Strasbourg, Institute of Chemistry-CNRS UMR 7177, 67081 Strasbourg, France.

[3]Department of Medical Informatics, Biometry and Epidemiology, University of Erlangen/Nürnberg, 91054 Erlangen, Germany

[4]National Allergy Research Centre, Department of Dermatology-Allergy, Copenhagen University Hospital Gentofte, 2900 Hellerup, Denmark.

[*]Corresponding author: Charlotte Menné Bonefeld, Department of Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen, Blegdamsvej 3, DK-2200, Copenhagen, Denmark. Phone: + 45 3532 7866, e-mail: cmenne@sund.ku.dk

Running head: Skin sensitisers in combination

Conflicts of interest: WU: Accepted travel reimbursement and partly honorarium for presentations given to cosmetic industry (associations) by them. Lecture fee from Almirall Hermal for educational lectures on contact allergy. The other authors declare none.

Summary (152 words, max 200 words)

Allergic contact dermatitis is one of the most frequent forms of skin inflammation. Very often, we are exposed to mixtures of allergens with varying potency, dose/area and exposure time. Therefore, improved knowledge about immune responses to combinations of contact allergens is highly relevant. In this article, we provide a general introduction to immune responses to contact allergens and discuss the literature concerning immune responses to mixtures of allergens. Normally, increased responses are induced following sensitization with combinations of allergens compared to single allergens. The response against a mixture of allergens can be both additive and synergistic depending on the dose and combination of allergens. Importantly, sensitization with combinations of either fragrance allergens or metal salts can result in increased challenge responses to specific allergens within the mixture. Taken together, the immune responses to mixtures of allergens are complex, and further studies are required to obtain the necessary knowledge to provide better consumer safety.

Key words: combinations of allergens; metals; fragrances; hair dyes;

Contact allergy is very frequent in the European population as demonstrated by Diepgen et al, who found that 27 % had a contact allergy to common allergens (1). This may lead to allergic contact dermatitis if the exposure exceeds the individual threshold of response.

The allergic response is frequently directed towards nickel, cobalt, fragrances, hair dye chemicals or preservatives. Many of these molecules are categorized as weak to moderate allergens – this term will be used throughout the paper globally for (pre-/pro-) haptens (2). Surprisingly, even 'weak to moderate' allergens do induce allergic contact dermatitis in many people. This indicates that exposure parameters are critical such as dose and exposure frequency. Interestingly, consumers are rarely exposed to one isolated allergen but more often to mixtures of allergens e.g. in the form of metal alloys, cosmetics and cleaners (3,4). The impact of being exposed to a mixture of allergens instead of a single allergen is not well studied. Studies from our group suggested that exposure to mixtures of allergens has a great impact on the immune response to single allergens within the mixture. In this review, we will discuss the current knowledge on how exposure to mixtures of contact allergens affects the immune system by 1) alterations of the chemical property of single allergens in the mixtures 2) changing the inflammatory response and 3) affecting T cell activation, proliferation and differentiation. Furthermore, we will discuss other factors like the presence of irritants, disrupted skin barrier, local skin inflammation and local skin memory that could increase the response to allergens. Finally, we will discuss the clinical impact of exposure to mixtures of contact allergens versus isolated contact allergens.

Immune response to contact allergens in general

Allergic contact dermatitis is a complex immunological response dominated by T cells and is induced following exposure of the skin to a contact allergen. Most of our knowledge about the immunological mechanisms mediating the allergic response comes from animal experiments, especially the mice models for allergic contact hypersensitivity (CHS). It has been shown that both $CD4^+$ and $CD8^+$ T cells are involved in mediating the inflammatory response, and which of the subset that dominates seems to be dependent of the allergen and the experimental set-up (5-8). In addition to $CD4^+$ and $CD8^+$ T cells, several other cell types are involved in the response including keratinocytes, Langerhans cells (LC), dermal dendritic cells (dDC), mast cells, γδ T cells, NKT cells, NK cells and B cells (9-19). The allergic response is divided into two phases: the sensitization phase and the challenge/elicitation phase. During the sensitization phase, the allergen will induce an inflammatory response leading to activation of antigen presenting cells (APC) (LC and dDC) in the skin (10,13,16). These cells then migrate to the draining lymph nodes (dLN), where they present naïve $CD4^+$ and $CD8^+$ T cells for the allergen. This will lead to T cell activation, and the T cells will start to differentiate and proliferate and some of them will become memory T cells (5,7,8,20). By the generation of memory T cells, the individual has become sensitized to the allergen. Re-exposure to a sufficient dose of the allergen, i.e., elicitation or challenge, causes reactivation of the memory T cells leading to a faster and stronger inflammatory response than seen during the primary response to the allergen (21).

The potency of an allergen could be determined by two features: 1) its ability to trigger activation of the innate immune response and 2) its ability to induce T cell activation. The epidermis is the first part of the body that comes in contact with the allergen and here keratinocytes and LC mediate the first response towards the allergen (11-13). The innate immune system particularly recognizes microbes and damaged self via the pattern recognition receptors; Toll-like receptors (TLR) and NOD-

like receptors (NLR) leading to the production of various cytokines and chemokines and the expression of co-stimulatory molecules on APC. During the last decade it has become clear that TLR are involved in the initiation of the inflammatory response towards contact allergens (22-25). It has been shown that both TLR2 and TLR4 play an important role in the response to contact allergens (22,23,25). Exposure to oxazolone induces a rapid up-regulation of the expression of TLR2 in mouse skin (22). Furthermore, a decreased ear-swelling was seen in mice lacking TLR2 following challenge with oxazolone which correlated with a decreased level of IFN-γ mRNA but normal levels of IL-4 mRNA in the skin (22). In addition, the authors found impaired Th1 polarization of naïve $CD4^+$ T cells stimulated with DC lacking TLR2 (22). Thus, this suggests that TLR2 signalling plays a role in allergen-induced inflammation and in the Th1 response induced by contact allergens. Recently it was shown that TLR3 also is involved in the skin inflammation induced by exposure to contact allergens (24). Challenge with trinitrochlorobenzene (TNCB) mediated a decreased response in TLR3 deficient mice and increased response in TLR3 transgenic mice showing that TLR3 can be an important regulator of the inflammatory response to contact allergens (24). Interestingly, it was shown that mice lacking either TLR2 or TLR4 only had a minor reduction in the response to TNCB whereas TLR2/TLR4 double-deficient mice were resistant to CHS (23). Taken together, TLR seem to play an important role in initiating of the inflammatory response to contact allergens, and at least for TLR2 and TLR4 there seem to be some redundancy.

Allergens have been shown to stimulate TLR both directly and indirectly by inducing the expression of endogenous TLR ligands (25-27). Both nickel and cobalt bind directly to human TLR4 thereby inducing stimulation (25,28). Allergen exposure of mice can lead to the degradation of hyaluronic acid (HA) in the skin leading to generation of HA degradation products that can function as TLR2 and TLR4 ligands (26). In addition, it has been shown that treatment of keratinocytes with

allergens induces the production of high-mobility group protein B1 (HMGB1), which can function as an endogenous TLR4 ligand (27). Furthermore, it is likely that RNA released from necrotic keratinocytes induced by exposure to contact allergens can serve as ligands for TLR3 (24). Interestingly, it appears that triggering of TLR by non-allergens can also modify the response to allergens (29,30). Pre-treatment with the TLR7 agonist Imiquimod, which is utilized to treat external genital warts, actinic keratosis, and basal cell carcinoma, induced increased ear-swelling in mice challenged with dinitrofulorobenzene (DNFB) and dinitrochlorobenzene (DNCB) compared to mice pre-treated with the control vehicle (29,30). These observations indicate that the simultaneous presence of non-allergens with the potential to stimulate TLR in consumer products will increase the risk of developing an allergic response to allergens in the products.

Contact allergens can also lead to activation of the NLRP3 inflammasome that activates caspase-1 leading to processing of pro-IL-1β and pro-IL-18 to IL-1β and IL-18, respectively (31,32). IL-1β is induced in the skin already 15 minutes after exposure to allergens and plays a central role for the allergic response (12,31-35). Activation of the NLRP3 inflammasome by contact allergens seems to be mediated by an indirect pathway via ATP (26,27,35). Thus, exposure to contact allergens leads to an increase in extracellular ATP that can bind to the transmembrane ATP receptor $P2X_7$ (35). Binding of ATP to $P2X_7$ induce the activation of NLRP3 inflammation (35). Interestingly, the response to TNCB is abrogated in mice lacking $P2X_7$ indicating that the ATP- $P2X_7$ pathway plays a central role in the response to contact allergens (35). Furthermore, it has been shown that some contact allergens like DNCB, 4-nitrobenzylbromide, diphenylcyclopropenone, oxazolone and TNCB can induce the production of reactive oxygen species (ROS) in both human and mice keratinocytes (26,27,36). Interestingly, it was recently shown that the allergen-induced ROS production leads to release of ATP though opening of pannexin hemichannels (36). As the allergen-induced ROS production correlates with IL-18 production it is likely that ROS-induced ATP binds to $P2X_7$ leading to

NLRP3 inflammasome activation (27). However, this way of inducing NLRP3 inflammasome activation seems to be allergen-dependent as treatment of keratinocytes with $NiCl_2$ did not induce ROS production and as it is uncertain if *p*-phenylenediamine (PPD) can induce ROS production in keratinocytes (26,27,36).

The activation of naïve T cells has a central role in sensitization to contact allergens. A naïve T cell requires three signals to be activated and to differentiate into effector and memory T cells: signal 1) recognition of the specific MHC-peptide complex, signal 2) stimulation by co-stimulatory molecules and signal 3) cytokine stimulation. The mechanisms mediating the two last requirements following allergen-exposure are described above. Contact allergens are small molecules (mostly <500 Daltons) which by themself are not immunogens. Instead, contact allergens react with and modify self-proteins which thereby can become an immunogen. The modification of self-proteins by contact allergens can be mediated by different mechanisms: 1) direct binding of the allergen to MHC:peptide complex, 2) modification of self that requires that the allergen is present during protein processing, 3) allergen-induced alterations of protein-processing, and 4) allergen can mediated binding between the T cell receptor and MHC molecule independent of the peptide bound to the MHC molecule (37-43). Upon recognition and if signal 2 is present, the specific naïve T cell becomes activated and will differentiate into effector and memory T cells. Depending on the local cytokine environment (signal 3) different types of T cells will be generated. For many years, IFN-γ producing T cells, both Th1 and $CD8^+$ T cells have been thought to be the main effector cells (8). However, it is now clear that both Th2 and Th17 cells play a role in the response depending on the allergen (44-47). Finally, it is clear that regulatory $CD4^+$ T cells play a critical role in controlling the response (48,49). A fraction of the T cells will differentiate into memory T cells that can rapidly mediate a response following re-exposure to the allergen. Interestingly, it has recently been shown

that exposure to DNFB leads to the generation of both circulating memory T cells and skin-resident memory T cells (20).

Effects of mixing contact allergens

Mixing different contact allergens can in theory result in different outcomes: 1) the response can be additive, 2) the mixed allergens can work in synergy, 3) one allergen can inhibit the effect of another allergen within the mixture (50,51). These effects can be mediated by different immunological mechanisms like induction of danger signals, cross-reactive T cells and induction of anti-inflammatory mechanisms. Moreover, the effect of an additional (or several additional) hapten(s) can be exerted during the induction phase and the elicitation phase.

The role of altered chemistry in the mixture

When mixing allergens one cannot exclude alteration to occur with either a neutralization of the reactivity and/or the formation of new sensitizers with a different sensitizing potential (either reduced or increased). However, as skin sensitizers are electrophilic substances, the probability for them to interact chemically is rather low but one should admit that this aspect has been very poorly studied. It has been recently published that terpene hydroperoxides from citrus oil could react with aldehydes (both are skin sensitizers) to form peroxyhemiacetals but of unknown reactivity (52).
Another indirect alternated chemistry could arise from metabolic competition of prohaptens to metabolic enzymes. Indeed when two prohaptens of similar structures are present in a mixture one could expect a competition to occur but here again these aspects in relation with skin sensitization have not been studied and reported in the literature.
Moreover, the deliberate addition of antioxidants to a mixture of prehaptens will delay the formation of oxidation products which are more sensitizing than the initial material, as has been shown for the addition of BHT and subsequent autoxidation of d-limonene (53). However, if antioxidants are added to decrease sensitisation risk, the risk associated with the antioxidants themselves needs to be considered.

The effect on the immune response

Immune response to mixtures of metals

Metal allergy is the most frequent type of allergic contact dermatitis with allergy to nickel and cobalt being the major forms affecting 14.5% and 2.2% of the European population, respectively (1). The way metals, especially nickel, activate both the innate and adaptive immune response has been extensively studied (25,28,38-40,43-49,54). Both nickel and cobalt facilitate dimerisation of human TLR4 thereby inducing production of various cytokines and chemokines required for initiating the allergic response (25,28,55). Furthermore, we have shown that nickel allergy can be induced in mice via a MyD88 and IL-1-dependent but TLR4-independent pathway, indicating that nickel can activate the innate immune response by various mechanisms (33). In agreement with this, it has been shown that $NiCl_2$ induce NLRP3-ASC-capase-1 inflammasome activation in APC resulting in IL-1β production via mechanisms involving lysosome rapture, mitochondrial ROS generation and cation flux (56). As stimulation of keratinocytes with hexavalent chromium also induces ROS production, we find it likely that hexavalent chromium also can induce NLRP3-ASC-capase-1 inflammasome activation in APC (57,58). In addition to activation of the innate immune system via different pathways, nickel can also activate T cells via different mechanisms (37-40,42,43). Nickel can be presented to T cells both via peptide-dependent and peptide-independent ways, where nickel bridges the TCR and MHC in a way similar to superantigens (37,40,43). Whether other metals can activate T cells by similar mechanisms needs further investigations.

People are mostly exposed to metals by contact with jewellery, tools and coins. Often these exposures are via contact with alloys like stainless steels. Interestingly, very little is known about how co-exposure to metals affects the immune response to an individual metal used in the alloy. In a study investigating how co-exposure to nickel and cobalt affected the challenge response to either

nickel or cobalt, we found that co-sensitization boosted the challenge response to both metals (59). Addition of 1% $NiCl_2$ during sensitization with 10% $CoCl_2$ led to a strong increase in the challenge response to 10% $CoCl_2$ compared to the challenge response in mice sensitized with 10% $CoCl_2$ alone as measured by ear-thickness and proliferation of B and T cells (59), i.e., a synergistic or – in this case – "adjuvant" effect was noted. In contrast, addition of 1% $CoCl_2$ to 10% $NiCl_2$ during the sensitization response only lead to a minor increase in $CD8^+$ T cell proliferation following challenge with 10% $NiCl_2$ compared to mice sensitized with 10% $NiCl_2$ alone (59). T cell cross-reactivity does not seems to explain these observations, as despite the high prevalence of concomitant nickel and cobalt allergy (60), it has been shown that human nickel-specific T cells do not cross-react with cobalt (39,54). We found that nickel induced more local inflammation than cobalt in mice only exposed to the individual metal during challenge, indicating that nickel is a more potent adjuvant than cobalt (59). Interestingly, it has been shown that chromium (Cr(VI)) is more cytotoxic and that it is accumulated more within keratinocytes than nickel and cobalt (61,62). This indicates that chromium (Cr(VI)) might be an even stronger adjuvant than nickel during co-exposures to cobalt and other metals. However, further investigations are needed to clarify this issue.

Immune responses to mixtures of fragrance allergens

Allergic contact dermatitis to fragrance allergens is common in Europe (1,63). Fragrances are complex mixtures that often contain between 10-100 components with several of these being contact allergens (3). However, the effect of mixtures of fragrance allergens on the immune system is still under debate. It has recently been suggested that mixing isoeugenol and cinnamal has an additive effect during sensitization (64). This was examined using a modified version of the local lymph node assay (LLNA) followed by complex mathematical analysis where the number of leukocytes in draining lymph nodes and IFN-γ production by these upon poly-clonal stimulation with conca-

navalin A were used (64). Surprisingly, it was suggested that isoeugenol and cinnamal followed the same dose response curve even though these are known to have different EC3 values, 1.3 and 2.0, respectively (64,65). High doses were used in the experiment (1.9%-30% for cinnamal and 1-16% for isoeugenol) (64), which may have affected the results. Cinnamal is known to be irritant to the skin and is for this reason patch tested maximally in 1% concentration in man, while the standard for isoeugenol is 2% (66). Experiments should be performed in the low dose range. Unfortunately, no raw data was included in this paper making it difficult to follow the suggested conclusions (64). We have shown that combining HICC, cinnamal and isoeugenol leads to an increased proliferation of both $CD4^+$ and $CD8^+$ T cells during sensitization compared to the response induce in mice exposed to one of the allergens (67). However, the effect on the response by the addition of the other allergens differed with the strongest effect seen for isoeugenol < HICC < cinnamal indicating that combing fragrance allergens can result in both additive and synergistic effects depending on the allergens (67). In addition to artificial, man-made fragrance allergens, several natural extracts exist including the mixtures oak moss (*Evernia prunastri*) and tree moss (*Evernia furfuracea*). When comparing the immune response during sensitization induced by oak moss with two of the major identified allergens within oak moss, namely atranol and chloroatranol, we found that oak moss induced a much stronger response compared to the response induced by either atranol or chloroatranol as measured by both B cell infiltration and T cell proliferation in the draining lymph nodes (68). However, whether the increased response was mediated by a synergistic effect mediated by co-exposure to atranol and chloroatranol or by additional allergens in oak moss could not be answered due to the experimental set-up (68). It was concluded by Kienhuis et al. that the additive effect on the sensitization response when mixing isoeugenol and cinnamal implies that quantitative risk assessment based on the response of single allergens in the products would be safe for the consumer (64). However, as we have shown that mice sensitized with mixtures of either HICC, cin-

namal, isoeugenol or oak moss, show an increased immune response upon challenge with either cinnamal or chloroatranol, respectively, than seen in mice sensitized only with the allergen used for challenge, in the same concentration as used in the mixture (67,68), we question this conclusion. There is no evidence that the levels identified in the current version of quantitative risk assessments based on LLNA results will be safe in the sense that no or few cases of sensitization will occur. Many individuals in the population are sensitized to cinnamal and isoeugenol already and will not be protected. The mechanisms mediating the combination effect in elicitation are not fully known but seem to be correlated with increased level of danger signal e.g. pro-inflammatory cytokines during sensitization in mice exposed to the mixture compared to the single allergen (68). Taken together, this shows that for safety (hazard) evaluation of mixtures of allergens the challenge response to single allergens within the mixture has to be evaluated (also) upon sensitization with a typical, relevant mixture, or mixtures.

Finally, is has been suggested that mixing fragrance allergens might have an inhibitory (antagonistic) effect on the response to single fragrance allergens in the mixture. In 1976, Opdyke suggested that addition of one fragrance allergen to another fragrance allergen could "quench" the ability of this to induce an allergic reaction (69). This quenching phenomenon was found when adding eugenol to cinnamal, limonene to citral and dipropylene glycol to phenylacetaldehyde (69). Subsequently, several attempts have been made to confirm the quenching phenomenon and to understand the mechanisms that mediated the quenching (70). Unfortunately, these attempts have not been successful and the ability of one fragrance allergen to quench another fragrance allergen is questionable and has never been proven (70).

Immune responses to hair dyes

Hair dyes are complex mixtures of chemicals including dyes, couplers, fragrances and a number of other types of ingredients of which several can induce allergic reactions. The best studied allergens in relation to allergic reactions induced by hair dyes are the dyes PPD and PPD-related allergens like toluene-2,5-diamine (PTD), all categorized as extremely strong allergens. In 1966, Kligman showed that patch testing with 10% PPD induced sensitization in 100% of 25 healthy volunteers (71). However, whether PPD directly activates the immune system or its products generated by au-toxidation like Bandrowski's base (BB), which is a potent allergen in animal experiments, is not fully understood (41,72-74). Oxidized PPD and BB seem to be more efficient activator of *in vitro* generated DC-like cells than freshly prepared PPD measured as CD86 expression and level of IL-1β and IL-8 (72). However, treatment with freshly prepared PPD induced DC-like cell activation (72). Both PPD- and BB-reacting T cell clones could be isolated from individuals with PPD allergy (41). It was suggested that PPD and BB required different pathways of processing to be recognized by T cells, as T cell proliferation could be induced following PPD presentation on fixed APC whereas BB presentation needed living APC (41). Interestingly, Coulter et al. showed that stimulation with BB could induce T cell proliferation in blood samples from both individual with PPD allergy and healthy controls whereas PPD only induced proliferation in blood samples from individuals with PPD allergy (73). This suggests that PPD induced T cell proliferation could be used to discriminate PPD allergic versus non-allergic individuals (73). Taken together, this shows the complexity of the immune response to PPD and its oxidative products and indicates that studying the real hair dyes are even more complex.

LLNA experiments where the response to PPD and methyldibromo glutaronitrile or mixtures of these were studied show that mixing these had a synergistic effect on the proliferative response at

induction, especially when low doses of the allergens were used (75). Despite these results, it was suggested by Aeby et al. that PPD-containing hair dyes are weak sensitizers due to excess of couplers, controlled oxidation and short exposure time (72). However, these suggestions were based on experiments studying the immune response to PPD, BB or acetylated forms of PPD and not on responses to the real mixture or mixtures of different allergens known to be used in hair dyes (72). We have studied the immune response to different hair dye products available for consumers, containing either PPD or PTD (76-78). We found that both PPD and PTD containing hair dyes are very potent inducers of local skin inflammation and T cell proliferation in the draining lymph nodes when using a modified version of LLNA (76,78). Interestingly, we found a stronger response in mice exposed to the mixture of colour gel and developer (oxidant) than to just the colour gel, showing that the final mixture of hair dyes can be a very potent immune activator (76). Furthermore, the level of the response seems to be correlated both with the concentration of the dye and number of additional allergens within the mixture (78). Finally, the exposure regime also seems to play a critical role for the immune response induced by hair dye. Using an experimental set-up where mice were repeatedly exposed to the hair dyes every second week for a total of 10 weeks, we found that both PPD- and PTD-containing hair dyes still induce an immune response yet to a lesser extent than seen using the modified version of LLNA (76-78). The explanation for this seems to be that in addition to pro-inflammatory mechanisms, repeated hair dye exposure also induced anti-inflammatory mechanisms as regulatory T cells and skin IL-10 production (77,78). Interestingly, we found that the hair dye is more potent in inducing regulatory T cells than pure PPD (77). Taken together, hair dyes are complex mixtures that can be very potent immune activators depending on the dose of dye allergens, presence of additional allergens and the exposure regime.

Other factors that increase responses to allergens

In 1966, Kligman showed that pre-treatment of human skin with sodium lauryl sulphate (SLS) increased the frequency of individuals who became sensitized to various contact allergens (79). The ability of an added irritant to enhance the response to contact allergens has been confirmed by others using mice models (80,81). The effect of pre-treatment with an irritant is especially seen when the responses to low concentrations of the allergen or weak allergens are studied (80,81), which is also observed in experimental human studies (see below). Interestingly, whereas exposure of the skin to the tolerogen dinitrothiocyanobenzene (DNTB) prevents skin inflammation induced by subsequent exposure to DNFB, addition of the irritant SLS to DNTB during sensitization inhibits the tolerogenic effect of DNTB (34). The ability of an irritant to lower the threshold for allergen sensitization is likely to be mediated by a combination of irritant induced skin inflammation and decreased skin barrier function (34,82). Since the discovery ten years ago that loss-of-function mutations in filaggrin is a major predisposing factor for atopic dermatitis, broad research has addressed the correlation between the skin barrier status, filaggrin mutations/content and skin inflammation (83). Associations between nickel allergy and loss-of-function mutations in filaggrin have been found. However, to understand the role of filaggrin for the sensitization to other contact allergens further research is needed (84,85). From a mouse model for filaggrin deficiency, the flaky tail mouse, it has been shown that filaggrin deficiency results in an increased response to oxazolone, likely mediated by a combination of increased allergen penetration and low-grade skin inflammation (86,87). Finally, it has recently been shown that skin exposure to a contact allergen results in the generation of skin-resident memory T cells (20,88). We have shown that re-exposure to the same skin areas result in a rapid increase in IL-1β compared to previously unexposed skin. The increase in IL-1β seems to be mediated by IL-17A and IFN-γ produced by skin-resident $CD8^+$ memory T cells (88). Taken together, the addition of irritants, a decreased skin barrier and the im-

mune status of the skin are all important factors that can change the activation threshold of a given allergen.

Clinical experience

In the clinic combination effects are well-known and referred to as compound allergy (89). This refers to observations that patients may react at patch testing or at normal use to products, which are mixtures, but not to any of the individual ingredients, when tested. Several patch testing preparations are mixtures in order to mimic exposures e.g. the fragrance mix I and II, which consists of 8 and 6 allergens, respectively (90). In 38.8% of cases positive to fragrance mix I the test is negative if the individual ingredients are tested in the same concentration as in the mix (91). For this reason the individual ingredients of FMII are routinely tested in the double concentration (63) and a similar recommendation exits for FM I (66) to compensate for the mixture effect, when testing the individual ingredients. These effects have also been subject to clinical experimental investigations (51,92). McLelland and Shuster showed that the threshold for a response to one allergen was lowered by the presence of another in patients sensitized to both allergens and that the response to the combined allergens was invariably greater than to the single allergens as measured by skin fold thickness corresponding to an additive effect (92). They concluded that the use of single allergens is inadequate for the investigation of contact dermatitis (92). In another clinical study it was found that the combination of two unrelated fragrance allergens in individuals allergic to both substances had a synergistic effect on the elicitation response evaluated by size of reaction, blood flow and clinical grading (51). It follows that the patch test stimulus with a single substance may be too weak to detect an allergic reaction elicited by mixtures of substances under natural exposure circumstances (51). The effect of mixtures is an integrated part of testing for contact allergy and understanding allergic contact dermatitis.

For good reasons no studies exist on how combined exposures to allergens effects induction thresholds in man, however in real life most cases of sensitization will be a result of exposures to mixtures of multiple allergens in different kind of products and patients with multiple allergies are commonly seen (93,94). Multiple sensitivities are among others seen in patients allergic to metals. The combination of contact allergy to nickel and cobalt as well as chromium and cobalt are more often seen than can be explained by chance (95). This may be due to the immunogenic effect of nickel and in particularly chromium ((Cr(VI)) which at least in theory may explain that construction workers get allergic to not only chromium but also to cobalt concomitantly, even though it is only present in minute amounts in cement.

Conclusion

In conclusion, we and others have shown that mixing metals, fragrances and hair dyes allergens can result in an increased immune response. Whether the combination of allergens results in an additive or a synergistic response seems to be both allergen-dependent and dependent on the dose of the different allergens in the mixture. We find it likely that the effect of mixing allergens is most profound during exposures to low doses of allergen, which is often the situation for consumers. As sensitization with a mixture of allergens can lead to an increased challenge response to specific allergens within the mixture, we question if the way risk assessment is done today is sufficient to protect the consumers. Finally, several other parameters seem to be important for the immune response to mixtures of contact allergens like additions of irritants and the status of the consumer skin, e.g. skin barrier function and/or low-grade inflammation.

Perspectives-unanswered questions

Further studies on the effect of mixing contact allergens are central for understanding the immunological mechanisms mediating allergic contact dermatitis and for understanding why some weak allergens can be a clinical problem. Genomic profiling of the response induced by nickel, fragrance allergens and rubber allergens shows that several common pathways seem to be induced. However, all three types of allergens also induce a unique profile suggesting that even though the responses look similar clinically they might be very different immunologically (96). It is therefore important to understand how different types of contact allergens induce activations of the innate immune response, e.g. whether different allergens induce different types of endogenous TLR-ligands and stimulate different TLRs and other innate receptors. Furthermore, the question remains how local memory T cells to one allergen in a mixture affect subsequent exposures to other allergens within the mixture.

Reference List

1. Diepgen, T. L., R. F. Ofenloch, M. Bruze, P. Bertuccio, S. Cazzaniga, P. J. Coenraads, P. Elsner, M. Goncalo, A. Svensson, and L. Naldi. 2016. Prevalence of contact allergy in the general population in different European regions. *Br. J Dermatol* 174: 319-329.

2. Liden, C., K. Yazar, J. D. Johansen, A. T. Karlberg, W. Uter, and I. R. White. 2016. Comparative sensitizing potencies of fragrances, preservatives, and hair dyes. *Contact Dermatitis* 75: 265-275.

3. Uter, W., K. Yazar, E. M. Kratz, G. Mildau, and C. Liden. 2013. Coupled exposure to ingredients of cosmetic products: I. Fragrances. *Contact Dermatitis* 69: 335-341.

4. Uter, W., K. Yazar, E. M. Kratz, G. Mildau, and C. Liden. 2014. Coupled exposure to ingredients of cosmetic products: II. Preservatives. *Contact Dermatitis* 70: 219-226.

5. DiIulio, N. A., H. Xu, and R. L. Fairchild. 1996. Diversion of CD4+ T cell development from regulatory T helper to effector T helper cells alters the contact hypersensitivity response. *Eur. J Immunol.* 26: 2606-2612.

6. Larsen, J. M., C. Geisler, M. W. Nielsen, L. Boding, E. M. Von, A. K. Hansen, L. Skov, and C. M. Bonefeld. 2007. Cellular dynamics in the draining lymph nodes during sensitization and elicitation phases of contact hypersensitivity. *Contact Dermatitis* 57: 300-308.

7. Vocanson, M., A. Hennino, M. Cluzel-Tailhardat, P. Saint-Mezard, J. Benetiere, C. Chavagnac, F. Berard, D. Kaiserlian, and J. F. Nicolas. 2006. CD8+ T cells are effector cells of contact dermatitis to common skin allergens in mice. *J Invest Dermatol* 126: 815-820.

8. Wang, B., H. Fujisawa, L. Zhuang, I. Freed, B. G. Howell, S. Shahid, G. M. Shivji, T. W. Mak, and D. N. Sauder. 2000. CD4+ Th1 and CD8+ type 1 cytotoxic T cells both play a crucial role in the full development of contact hypersensitivity. *J Immunol.* 165: 6783-6790.

9. Askenase, P. W., A. Itakura, M. C. Leite-de-Moraes, M. Lisbonne, S. Roongapinun, D. R. Goldstein, and M. Szczepanik. 2005. TLR-dependent IL-4 production by invariant Valpha14+Jalpha18+ NKT cells to initiate contact sensitivity in vivo. *J Immunol.* 175: 6390-6401.

10. Bennett, C. L., R. E. van, S. Jung, K. Inaba, R. M. Steinman, M. L. Kapsenberg, and B. E. Clausen. 2005. Inducible ablation of mouse Langerhans cells diminishes but fails to abrogate contact hypersensitivity. *J Cell Biol.* 169: 569-576.

11. Enk, A. H., and S. I. Katz. 1992. Early events in the induction phase of contact sensitivity. *J Invest Dermatol* 99: 39S-41S.

12. Enk, A. H., and S. I. Katz. 1992. Early molecular events in the induction phase of contact sensitivity. *Proc. Natl. Acad. Sci. U. S. A* 89: 1398-1402.

13. Enk, A. H., V. L. Angeloni, M. C. Udey, and S. I. Katz. 1993. An essential role for Langerhans cell-derived IL-1 beta in the initiation of primary immune responses in skin. *J Immunol.* 150: 3698-3704.

14. Honda, T., S. Nakajima, G. Egawa, K. Ogasawara, B. Malissen, Y. Miyachi, and K. Kabashima. 2010. Compensatory role of Langerhans cells and langerin-positive dermal dendritic cells in the sensitization phase of murine contact hypersensitivity. *J Allergy Clin. Immunol.* 125: 1154-1156.

15. Itakura, A., M. Szczepanik, R. A. Campos, V. Paliwal, M. Majewska, H. Matsuda, K. Takatsu, and P. W. Askenase. 2005. An hour after immunization peritoneal B-1 cells are activated to migrate to lymphoid organs where within 1 day they produce IgM antibodies that initiate elicitation of contact sensitivity. *J Immunol.* 175: 7170-7178.

16. Kaplan, D. H., M. C. Jenison, S. Saeland, W. D. Shlomchik, and M. J. Shlomchik. 2005. Epidermal langerhans cell-deficient mice develop enhanced contact hypersensitivity. *Immunity.* 23: 611-620.

17. Nielsen, M. M., P. Lovato, A. S. MacLeod, D. A. Witherden, L. Skov, B. Dyring-Andersen, S. Dabelsteen, A. Woetmann, N. Odum, W. L. Havran, C. Geisler, and C. M. Bonefeld. 2014. IL-1beta-dependent activation of dendritic epidermal T cells in contact hypersensitivity. *J Immunol.* 192: 2975-2983.

18. Nielsen, M. M., B. Dyring-Andersen, J. D. Schmidt, D. Witherden, P. Lovato, A. Woetmann, N. Odum, S. S. Poulsen, W. L. Havran, C. Geisler, and C. M. Bonefeld. 2015. NKG2D-dependent activation of dendritic epidermal T cells in contact hypersensitivity. *J Invest Dermatol* 135: 1311-1319.

19. O'Leary, J. G., M. Goodarzi, D. L. Drayton, and U. H. von Andrian. 2006. T cell- and B cell-independent adaptive immunity mediated by natural killer cells. *Nat. Immunol.* 7: 507-516.

20. Gaide, O., R. O. Emerson, X. Jiang, N. Gulati, S. Nizza, C. Desmarais, H. Robins, J. G. Krueger, R. A. Clark, and T. S. Kupper. 2015. Common clonal origin of central and resident memory T cells following skin immunization. *Nat. Med.* 21: 647-653.

21. Friedmann, P. S. 2007. The relationships between exposure dose and response in induction and elicitation of contact hypersensitivity in humans. *Br. J Dermatol* 157: 1093-1102.

22. Jin, H., L. Kumar, C. Mathias, D. Zurakowski, H. Oettgen, L. Gorelik, and R. Geha. 2009. Toll-like receptor 2 is important for the T(H)1 response to cutaneous sensitization. *J Allergy Clin. Immunol.* 123: 875-882.

23. Martin, S. F., J. C. Dudda, E. Bachtanian, A. Lembo, S. Liller, C. Durr, M. M. Heimesaat, S. Bereswill, G. Fejer, R. Vassileva, T. Jakob, N. Freudenberg, C. C. Termeer, C. Johner, C. Galanos, and M. A. Freudenberg. 2008. Toll-like receptor and IL-12 signaling control susceptibility to contact hypersensitivity. *J Exp. Med.* 205: 2151-2162.

24. Nakamura, N., R. Tamagawa-Mineoka, M. Ueta, S. Kinoshita, and N. Katoh. 2015. Toll-like receptor 3 increases allergic and irritant contact dermatitis. *J Invest Dermatol* 135: 411-417.

25. Schmidt, M., B. Raghavan, V. Muller, T. Vogl, G. Fejer, S. Tchaptchet, S. Keck, C. Kalis, P. J. Nielsen, C. Galanos, J. Roth, A. Skerra, S. F. Martin, M. A. Freudenberg, and M.

Goebeler. 2010. Crucial role for human Toll-like receptor 4 in the development of contact allergy to nickel. *Nat. Immunol.* 11: 814-819.

26. Esser, P. R., U. Wolfle, C. Durr, F. D. von Loewenich, C. M. Schempp, M. A. Freudenberg, T. Jakob, and S. F. Martin. 2012. Contact sensitizers induce skin inflammation via ROS production and hyaluronic acid degradation. *PLoS. One.* 7: e41340.

27. Galbiati, V., A. Papale, C. L. Galli, M. Marinovich, and E. Corsini. 2014. Role of ROS and HMGB1 in contact allergen-induced IL-18 production in human keratinocytes. *J Invest Dermatol* 134: 2719-2727.

28. Raghavan, B., S. F. Martin, P. R. Esser, M. Goebeler, and M. Schmidt. 2012. Metal allergens nickel and cobalt facilitate TLR4 homodimerization independently of MD2. *EMBO Rep.* 13: 1109-1115.

29. Suzuki, H., B. Wang, G. M. Shivji, P. Toto, P. Amerio, M. A. Tomai, R. L. Miller, and D. N. Sauder. 2000. Imiquimod, a topical immune response modifier, induces migration of Langerhans cells. *J Invest Dermatol* 114: 135-141.

30. Thatcher, T. H., I. Luzina, R. Fishelevich, M. A. Tomai, R. L. Miller, and A. A. Gaspari. 2006. Topical imiquimod treatment prevents UV-light induced loss of contact hypersensitivity and immune tolerance. *J Invest Dermatol* 126: 821-831.

31. Sutterwala, F. S., Y. Ogura, M. Szczepanik, M. Lara-Tejero, G. S. Lichtenberger, E. P. Grant, J. Bertin, A. J. Coyle, J. E. Galan, P. W. Askenase, and R. A. Flavell. 2006. Critical role for NALP3/CIAS1/Cryopyrin in innate and adaptive immunity through its regulation of caspase-1. *Immunity.* 24: 317-327.

32. Watanabe, H., O. Gaide, V. Petrilli, F. Martinon, E. Contassot, S. Roques, J. A. Kummer, J. Tschopp, and L. E. French. 2007. Activation of the IL-1beta-processing inflammasome is involved in contact hypersensitivity. *J Invest Dermatol* 127: 1956-1963.

33. Vennegaard, M. T., B. Dyring-Andersen, L. Skov, M. M. Nielsen, J. D. Schmidt, M. Bzorek, S. S. Poulsen, A. R. Thomsen, A. Woetmann, J. P. Thyssen, J. D. Johansen, N. Odum, T. Menne, C. Geisler, and C. M. Bonefeld. 2014. Epicutaneous exposure to nickel induces nickel allergy in mice via a MyD88-dependent and interleukin-1-dependent pathway. *Contact Dermatitis* 71: 224-232.

34. Watanabe, H., S. Gehrke, E. Contassot, S. Roques, J. Tschopp, P. S. Friedmann, L. E. French, and O. Gaide. 2008. Danger signaling through the inflammasome acts as a master switch between tolerance and sensitization. *J Immunol.* 180: 5826-5832.

35. Weber, F. C., P. R. Esser, T. Muller, J. Ganesan, P. Pellegatti, M. M. Simon, R. Zeiser, M. Idzko, T. Jakob, and S. F. Martin. 2010. Lack of the purinergic receptor P2X(7) results in resistance to contact hypersensitivity. *J Exp. Med.* 207: 2609-2619.

36. Onami, K., Y. Kimura, Y. Ito, T. Yamauchi, K. Yamasaki, and S. Aiba. 2014. Nonmetal haptens induce ATP release from keratinocytes through opening of pannexin hemichannels by reactive oxygen species. *J Invest Dermatol* 134: 1951-1960.

37. Gamerdinger, K., C. Moulon, D. R. Karp, B. J. Van, F. Koning, D. Wild, U. Pflugfelder, and H. U. Weltzien. 2003. A new type of metal recognition by human T cells: contact residues for peptide-independent bridging of T cell receptor and major histocompatibility complex by nickel. *J Exp. Med.* 197: 1345-1353.

38. Lu, L., J. Vollmer, C. Moulon, H. U. Weltzien, P. Marrack, and J. Kappler. 2003. Components of the ligand for a Ni++ reactive human T cell clone. *J Exp. Med.* 197: 567-574.

39. Moulon, C., J. Vollmer, and H. U. Weltzien. 1995. Characterization of processing requirements and metal cross-reactivities in T cell clones from patients with allergic contact dermatitis to nickel. *Eur. J Immunol.* 25: 3308-3315.

40. Moulon, C., D. Wild, A. Dormoy, and H. U. Weltzien. 1998. MHC-dependent and -independent activation of human nickel-specific CD8+ cytotoxic T cells from allergic donors. *J Invest Dermatol* 111: 360-366.

41. Sieben, S., Y. Kawakubo, M. T. Al, H. F. Merk, and B. Blomeke. 2002. Delayed-type hypersensitivity reaction to paraphenylenediamine is mediated by 2 different pathways of antigen recognition by specific alphabeta human T-cell clones. *J Allergy Clin. Immunol.* 109: 1005-1011.

42. Thierse, H. J., C. Moulon, Y. Allespach, B. Zimmermann, A. Doetze, S. Kuppig, D. Wild, F. Herberg, and H. U. Weltzien. 2004. Metal-protein complex-mediated transport and delivery of Ni2+ to TCR/MHC contact sites in nickel-specific human T cell activation. *J Immunol.* 172: 1926-1934.

43. Vollmer, J., H. U. Weltzien, and C. Moulon. 1999. TCR reactivity in human nickel allergy indicates contacts with complementarity-determining region 3 but excludes superantigen-like recognition. *J Immunol.* 163: 2723-2731.

44. Albanesi, C., A. Cavani, and G. Girolomoni. 1999. IL-17 is produced by nickel-specific T lymphocytes and regulates ICAM-1 expression and chemokine production in human keratinocytes: synergistic or antagonist effects with IFN-gamma and TNF-alpha. *J Immunol.* 162: 494-502.

45. Albanesi, C., C. Scarponi, A. Cavani, M. Federici, F. Nasorri, and G. Girolomoni. 2000. Interleukin-17 is produced by both Th1 and Th2 lymphocytes, and modulates interferon-gamma- and interleukin-4-induced activation of human keratinocytes. *J Invest Dermatol* 115: 81-87.

46. Dyring-Andersen, B., L. Skov, M. B. Lovendorf, M. Bzorek, K. Sondergaard, J. P. Lauritsen, S. Dabelsteen, C. Geisler, and C. M. Bonefeld. 2013. CD4(+) T cells producing interleukin (IL)-17, IL-22 and interferon-gamma are major effector T cells in nickel allergy. *Contact Dermatitis* 68: 339-347.

47. Larsen, J. M., C. M. Bonefeld, S. S. Poulsen, C. Geisler, and L. Skov. 2009. IL-23 and T(H)17-mediated inflammation in human allergic contact dermatitis. *J Allergy Clin. Immunol.* 123: 486-492.

48. Cavani, A., F. Nasorri, C. Ottaviani, S. Sebastiani, P. O. De, and G. Girolomoni. 2003. Human CD25+ regulatory T cells maintain immune tolerance to nickel in healthy, nonallergic individuals. *J Immunol.* 171: 5760-5768.

49. Rustemeyer, T., B. M. von Blomberg, I. M. van Hoogstraten, D. P. Bruynzeel, and R. J. Scheper. 2004. Analysis of effector and regulatory immune reactivity to nickel. *Clin. Exp. Allergy* 34: 1458-1466.

50. Berenbaum, M. C. 1989. What is synergy? *Pharmacol. Rev.* 41: 93-141.

51. Johansen, J. D., L. Skov, A. Volund, K. Andersen, and T. Menne. 1998. Allergens in combination have a synergistic effect on the elicitation response: a study of fragrance-sensitized individuals. *Br. J Dermatol* 139: 264-270.

52. Calandra, M. J., Y. Wang, J. Impellizzeri, S. Frank, J.-Y. de Saint Laumer, S. Leocata, and A. Chaintreau. 16 A.D. Terpene hydroperoxide chemistry in citrus oils;reaction with endogenous aldehydes to form peroxyhemiacetals. *Flavour and Fragrance Journal* 31: 241-249.

53. Karlberg, A. T., K. Magnusson, and U. Nilsson. 1994. Influence of an anti-oxidant on the formation of allergenic compounds during auto-oxidation of d-limonene. *Ann. Occup. Hyg.* 38: 199-207.

54. Pistoor, F. H., M. L. Kapsenberg, J. D. Bos, M. M. Meinardi, M. E. von Blomberg, and R. J. Scheper. 1995. Cross-reactivity of human nickel-reactive T-lymphocyte clones with copper and palladium. *J Invest Dermatol* 105: 92-95.

55. Viemann, D., M. Schmidt, K. Tenbrock, S. Schmid, V. Muller, K. Klimmek, S. Ludwig, J. Roth, and M. Goebeler. 2007. The contact allergen nickel triggers a unique inflammatory and proangiogenic gene expression pattern via activation of NF-kappaB and hypoxia-inducible factor-1alpha. *J Immunol.* 178: 3198-3207.

56. Li, X., and F. Zhong. 2014. Nickel induces interleukin-1beta secretion via the NLRP3-ASC-caspase-1 pathway. *Inflammation* 37: 457-466.

57. Lee, Y. H., S. B. Su, C. C. Huang, H. M. Sheu, J. C. Tsai, C. H. Lin, Y. J. Wang, and B. J. Wang. 2014. N-acetylcysteine attenuates hexavalent chromium-induced hypersensitivity through inhibition of cell death, ROS-related signaling and cytokine expression. *PLoS. One.* 9: e108317.

58. Wang, B. J., H. M. Sheu, Y. L. Guo, Y. H. Lee, C. S. Lai, M. H. Pan, and Y. J. Wang. 2010. Hexavalent chromium induced ROS formation, Akt, NF-kappaB, and MAPK activation, and TNF-alpha and IL-1alpha production in keratinocytes. *Toxicol. Lett.* 198: 216-224.

59. Bonefeld, C. M., M. M. Nielsen, M. T. Vennegaard, J. D. Johansen, C. Geisler, and J. P. Thyssen. 2015. Nickel acts as an adjuvant during cobalt sensitization. *Exp. Dermatol* 24: 229-231.

60. Brasch, J., and J. Geier. 1997. Patch test results in schoolchildren. Results from the Information Network of Departments of Dermatology (IVDK) and the German Contact Dermatitis Research Group (DKG). *Contact Dermatitis* 37: 286-293.

61. Curtis, A., J. Morton, C. Balafa, S. MacNeil, D. J. Gawkrodger, N. D. Warren, and G. S. Evans. 2007. The effects of nickel and chromium on human keratinocytes: differences in viability, cell associated metal and IL-1alpha release. *Toxicol. In Vitro* 21: 809-819.

62. Ermolli, M., C. Menne, G. Pozzi, M. A. Serra, and L. A. Clerici. 2001. Nickel, cobalt and chromium-induced cytotoxicity and intracellular accumulation in human hacat keratinocytes. *Toxicology* 159: 23-31.

63. Frosch, P. J., S. C. Rastogi, C. Pirker, T. Brinkmeier, K. E. Andersen, M. Bruze, C. Svedman, A. Goossens, I. R. White, W. Uter, E. G. Arnau, J. P. Lepoittevin, J. D. Johansen, and T. Menne. 2005. Patch testing with a new fragrance mix - reactivity to the individual constituents and chemical detection in relevant cosmetic products. *Contact Dermatitis* 52: 216-225.

64. Kienhuis, A. S., W. Slob, E. R. Gremmer, J. P. Vermeulen, and J. Ezendam. 2015. A Dose-Response Modeling Approach Shows That Effects From Mixture Exposure to the Skin Sensitizers Isoeugenol and Cinnamal Are in Line With Dose Addition and Not With Synergism. *Toxicol. Sci.* 147: 68-74.

65. Basketter D, K. I. 2011. Predictive tests for irritants and allergens and their use in quantitative risk assessment. In *Contact Dermatitis*, 5 ed. P. J. F. J. L. J.D.Johansen, ed. 229-240.

66. Mann, J., J. P. McFadden, J. M. White, I. R. White, and P. Banerjee. 2014. Baseline series fragrance markers fail to predict contact allergy. *Contact Dermatitis* 70: 276-281.

67. Bonefeld, C. M., M. M. Nielsen, I. M. Rubin, M. T. Vennegaard, S. Dabelsteen, E. Gimenez-Arnau, J. P. Lepoittevin, C. Geisler, and J. D. Johansen. 2011. Enhanced sensitization and elicitation responses caused by mixtures of common fragrance allergens. *Contact Dermatitis* 65: 336-342.

68. Bonefeld, C. M., M. M. Nielsen, E. Gimenez-Arnau, M. Lang, M. T. Vennegaard, C. Geisler, J. D. Johansen, and J. P. Lepoittevin. 2014. An immune response study of oakmoss absolute and its constituents atranol and chloroatranol. *Contact Dermatitis* 70: 282-290.

69. Opdyke, D. L. 1976. Inhibition of sensitization reactions induced by certain aldehydes. *Food Cosmet. Toxicol.* 14: 197-198.

70. Basketter, D. 2000. Quenching: fact or fiction? *Contact Dermatitis* 43: 253-258.

71. Kligman, A. M. 1966. The identification of contact allergens by human assay. 3. The maximization test: a procedure for screening and rating contact sensitizers. *J Invest Dermatol* 47: 393-409.

72. Aeby, P., T. Sieber, H. Beck, G. F. Gerberick, and C. Goebel. 2009. Skin sensitization to p-phenylenediamine: the diverging roles of oxidation and N-acetylation for dendritic cell activation and the immune response. *J Invest Dermatol* 129: 99-109.

73. Coulter, E. M., C. Jenkinson, Y. Wu, J. Farrell, B. Foster, A. Smith, C. McGuire, C. Pease, D. Basketter, C. King, P. S. Friedmann, M. Pirmohamed, B. K. Park, and D. J. Naisbitt. 2008. Activation of T-cells from allergic patients and volunteers by p-phenylenediamine and Bandrowski's base. *J Invest Dermatol* 128: 897-905.

74. Krasteva, M., J. F. Nicolas, G. Chabeau, J. L. Garrigue, H. Bour, J. Thivolet, and D. Schmitt. 1993. Dissociation of allergenic and immunogenic functions in contact sensitivity to para-phenylenediamine. *Int. Arch. Allergy Immunol.* 102: 200-204.

75. Jowsey, I. R., D. A. Basketter, and A. Irwin. 2008. Proliferative responses in the local lymph node assay associated with concomitant exposure to 1,4-phenylenediamine and methyldibromo glutaronitrile: evidence for synergy? *Contact Dermatitis* 59: 90-95.

76. Bonefeld, C. M., J. M. Larsen, S. Dabelsteen, C. Geisler, I. R. White, T. Menne, and J. D. Johansen. 2010. Consumer available permanent hair dye products cause major allergic immune activation in an animal model. *Br. J Dermatol* 162: 102-107.

77. Rubin, I. M., S. Dabelsteen, M. M. Nielsen, I. R. White, J. D. Johansen, C. Geisler, and C. M. Bonefeld. 2010. Repeated exposure to hair dye induces regulatory T cells in mice. *Br. J Dermatol* 163: 992-998.

78. Schmidt, J. D., J. D. Johansen, M. M. Nielsen, E. Zimersson, C. Svedman, M. Bruze, K. Engkilde, S. S. Poulsen, C. Geisler, and C. M. Bonefeld. 2014. Immune responses to hair dyes containing toluene-2,5-diamine. *Br. J Dermatol* 170: 352-359.

79. Kligman, A. M. 1966. The identification of contact allergens by human assay. II. Factors influencing the induction and measurement of allergic contact dermatitis. *J Invest Dermatol* 47: 375-392.

80. Cumberbatch, M., R. C. Scott, D. A. Basketter, E. W. Scholes, J. Hilton, R. J. Dearman, and I. Kimber. 1993. Influence of sodium lauryl sulphate on 2,4-dinitrochlorobenzene-induced lymph node activation. *Toxicology* 77: 181-191.

81. De Jong, W. H., M. Tentij, S. W. Spiekstra, R. J. Vandebriel, and L. H. Van. 2002. Determination of the sensitising activity of the rubber contact sensitisers TMTD, ZDMC, MBT and DEA in a modified local lymph node assay and the effect of sodium dodecyl sulfate pretreatment on local lymph node responses. *Toxicology* 176: 123-134.

82. van der Valk, P. G., M. H. Kruis-de Vries, J. P. Nater, E. Bleumink, and M. C. de Jong. 1985. Eczematous (irritant and allergic) reactions of the skin and barrier function as determined by water vapour loss. *Clin. Exp. Dermatol* 10: 185-193.

83. Palmer, C. N., A. D. Irvine, A. Terron-Kwiatkowski, Y. Zhao, H. Liao, S. P. Lee, D. R. Goudie, A. Sandilands, L. E. Campbell, F. J. Smith, G. M. O'Regan, R. M. Watson, J. E. Cecil, S. J. Bale, J. G. Compton, J. J. DiGiovanna, P. Fleckman, S. Lewis-Jones, G. Arseculeratne, A. Sergeant, C. S. Munro, H. B. El, K. McElreavey, L. B. Halkjaer, H.

Bisgaard, S. Mukhopadhyay, and W. H. McLean. 2006. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat. Genet.* 38: 441-446.

84. Carlsen, B. C., J. D. Johansen, T. Menne, M. Meldgaard, P. B. Szecsi, S. Stender, and J. P. Thyssen. 2010. Filaggrin null mutations and association with contact allergy and allergic contact dermatitis: results from a tertiary dermatology clinic. *Contact Dermatitis* 63: 89-95.

85. Novak, N., H. Baurecht, T. Schafer, E. Rodriguez, S. Wagenpfeil, N. Klopp, J. Heinrich, H. Behrendt, J. Ring, E. Wichmann, T. Illig, and S. Weidinger. 2008. Loss-of-function mutations in the filaggrin gene and allergic contact sensitization to nickel. *J Invest Dermatol* 128: 1430-1435.

86. Oyoshi, M. K., G. F. Murphy, and R. S. Geha. 2009. Filaggrin-deficient mice exhibit TH17-dominated skin inflammation and permissiveness to epicutaneous sensitization with protein antigen. *J Allergy Clin. Immunol.* 124: 485-93, 493.

87. Scharschmidt, T. C., M. Q. Man, Y. Hatano, D. Crumrine, R. Gunathilake, J. P. Sundberg, K. A. Silva, T. M. Mauro, M. Hupe, S. Cho, Y. Wu, A. Celli, M. Schmuth, K. R. Feingold, and P. M. Elias. 2009. Filaggrin deficiency confers a paracellular barrier abnormality that reduces inflammatory thresholds to irritants and haptens. *J Allergy Clin. Immunol.* 124: 496-506, 506.

88. Schmidt, J. D., M. G. Ahlstrom, J. D. Johansen, B. Dyring-Andersen, C. Agerbeck, M. M. Nielsen, S. S. Poulsen, A. Woetmann, N. Odum, A. R. Thomsen, C. Geisler, and C. M. Bonefeld. 2016. Rapid allergen-induced interleukin-17 and interferon-gamma secretion by skin-resident memory CD8+ T cells. *Contact Dermatitis*.

89. Fisher, A. A. 1986. Compound ("synergistic") allergy to contactants. *Cutis* 38: 101-102.

90. Johansen, J. D., K. Aalto-Korte, T. Agner, K. E. Andersen, A. Bircher, M. Bruze, A. Cannavo, A. Gimenez-Arnau, M. Goncalo, A. Goossens, S. M. John, C. Liden, M. Lindberg, V. Mahler, M. Matura, T. Rustemeyer, J. Serup, R. Spiewak, J. P. Thyssen, M. Vigan, I. R. White, M. Wilkinson, and W. Uter. 2015. European Society of Contact Dermatitis guideline for diagnostic patch testing - recommendations on best practice. *Contact Dermatitis* 73: 195-221.

91. Krautheim, A., W. Uter, P. Frosch, A. Schnuch, and J. Geier. 2010. Patch testing with fragrance mix II: results of the IVDK 2005-2008. *Contact Dermatitis* 63: 262-269.

92. McLelland, J., and S. Shuster. 1990. Contact dermatitis with negative patch tests: the additive effect of allergens in combination. *Br. J Dermatol* 122: 623-630.

93. Carlsen, B. C., T. Menne, and J. D. Johansen. 2007. 20 Years of standard patch testing in an eczema population with focus on patients with multiple contact allergies. *Contact Dermatitis* 57: 76-83.

94. Schnuch, A., J. Brasch, H. Lessmann, J. Geier, and W. Uter. 2007. A further characteristic of susceptibility to contact allergy: sensitization to a weak contact allergen is associated with polysensitization. Results of the IVDK. *Contact Dermatitis* 56: 331-337.

95. Hegewald, J., W. Uter, A. Pfahlberg, J. Geier, and A. Schnuch. 2005. A multifactorial analysis of concurrent patch-test reactions to nickel, cobalt, and chromate. *Allergy* 60: 372-378.

96. Dhingra, N., A. Shemer, R. J. Correa da, M. Rozenblit, J. Fuentes-Duculan, J. K. Gittler, R. Finney, T. Czarnowicki, X. Zheng, H. Xu, Y. D. Estrada, I. Cardinale, M. Suarez-Farinas, J. G. Krueger, and E. Guttman-Yassky. 2014. Molecular profiling of contact dermatitis skin identifies allergen-dependent differences in immune response. *J Allergy Clin. Immunol.* 134: 362-372.