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Stratum corneum levels of inflammatory mediators and natural moisturizing factor in patch test reactions to thiurams and fragrances and their possible role in discrimination between irritant and allergic reactions to hapten mixtures

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Abstract

Background: Patch test (PT) reactions to thiuram mix (TM) and fragrance mix (FM) I or II without concomitant reactions to their single constituents are potentially caused by the irritant properties of the mixes.

Objective: Comparing inflammatory profiles of PT reactions to TM, FM I, FM II, and their constituents and assessing their potential in discrimination of irritant and allergic reactions.

Patients and Methods: Levels of 14 cytokines and natural moisturizing factor (NMF) were determined in *stratum corneum* samples collected from PT reactions to TM, FM I or II, their constituents, and petrolatum (pet.) control sites in 36 individuals.

Results: Levels of interleukin (IL)-16, chemokine (CXC motif) ligand (CXCL) 8, CXCL10, chemokine (CC motif) ligand (CCL) 17, and CCL22 were significantly increased in reactions (+, ++) to thiurams and fragrances compared to their petrolatum controls, except for PT reactions to FM I/II with negative breakdown testing in which, however, decreased levels of NMF were observed. In doubtful reactions to FM I/II with negative breakdown testing, NMF was significantly lower than in petrolatum controls.

Conclusions: PT reactions to thiurams and fragrances indicate a Th2-skewed inflammation. The inflammatory profiles suggest that weak or doubtful FM I/II reactions without accompanying reaction to a constituent were irritant. IL-16 might be suitable to distinguish irritant from allergic reaction.

KEYWORDS

allergic, contact dermatitis, cytokines, fragrances, irritant, mix, natural moisturizing factor, patch test, rubber, thiurams

1 | INTRODUCTION

About 20% to 25% of the general population has contact allergy,[1] a T cell-mediated delayed-type hypersensitivity caused by haptens, which are recognized by the immune system after binding to epidermal proteins and may lead to allergic contact dermatitis (ACD).[2,3] However, the underlying mechanisms are only partially understood.

Patch testing is the gold standard for diagnosis of contact allergy. Due to an inherent irritant potential of many haptens, differentiation between true allergic and irritant patch test reactions could be challenging, in particular when hapten mixtures are tested. Fragrances and rubber accelerators such as thiurams are among the most common causes of contact allergy.[4-7] They are patch tested as screening mixtures in the European and most national baseline series. When a patch test reaction to thiuram mix (TM) or fragrance mix (FM) I or FM II occurs, testing their single ingredients is recommended. A patch test reaction to a mix without concomitant reaction to at least one of its single ingredients is fairly common when testing the TM (7.3%-53.6%),[8-10] FM I (42.7%-67.3%),[4,11,12] or FM II (17.0%-43.0%).[4,11,12] This phenomenon occurs more frequently when the reaction to the mix is weak, and it may indicate an irritant reaction to the hapten mixture.[13] However, also false-negative reactions to the single components have been discussed.

Haptens have different physicochemical properties eliciting different cutaneous inflammatory responses.[2] Inflammatory profiles may help to distinguish between haptens, and possibly even between allergic and irritant responses.[14] RNA microarrays have been used to investigate changes in gene expression in patch test reactions to various haptens showing a selective upregulation of several chemokines in nickel-induced ACD,[15] and different types of immune polarization with respect to Th1/Th17, Th22, and Th2 components for patch testing with haptens including fragrances and thiurams.[16] Previously it was demonstrated that the levels of various inflammatory mediators in the *stratum corneum* (SC), the uppermost layer of the epidermis, differed after patch testing with nickel, chromium, methylchloroisothiazolinone/methylisothiazolinone (MCI/MI), and the skin irritant sodium lauryl sulfate (SLS). Levels of interleukin (IL)-16 were increased in patch test reactions to all haptens but not to SLS rendering it a potential biomarker to differentiate between ACD and irritant contact dermatitis (ICD).[17] Moreover, SC levels of the natural moisturizing factor (NMF), which mainly consists of degradation products of the epidermal protein filaggrin, were decreased after patch testing with SLS, and similarly with MCI/MI, which was explained by its irritant properties.[18]

In the present study we investigated the inflammatory SC profiles of patch test reactions to TM, FM I, FM II, and their respective constituents. In addition, we assessed the suitability of theses profiles to potentially discriminate between irritant and allergic reactions to the mixes.

2 | MATERIALS AND METHODS

2.1 | Selection of patients and patch testing

The experimental protocol followed the Declaration of Helsinki principles and was approved by the ethics committee of the University of Osnabrück. As part of the routine diagnostic procedure, patients with predominantly work-related hand dermatitis were patch tested at the Institute for interdisciplinary Dermatologic Prevention and Rehabilitation (iDerm) at the University of Osnabrück, Germany, with the DKG (German Contact Dermatitis Research Group) baseline series and other relevant series, such as the DKG rubber chemical series. Patients with a patch test reaction to FM I and/or FM II in the baseline series underwent an additional patch test with their respective or remaining single ingredients 1 week later. Patch testing was performed with allergen preparations from SmartPractice Europe (Greven, Germany) which were filled in Allergeaze clear chambers (SmartPractice Europe) and placed on the upper back of the patients. All test preparations were loaded in the test chambers immediately before application. The chambers were removed after 24 hours in accordance with the DKG guidelines valid at the time of the study, which recommended an occlusion time of either 24 or 48 hours.[19] Grading of patch test reactions (?+, +, ++, +++) were performed on day (D)1, D2, D3, D4, and D7. As controls, petrolatum (pet.) and 0.25% SLS aq. were tested. The DKG baseline series contained TM 1% pet., FM I 8% pet., and FM II 14% pet. as well as hydroxyisohexyl 3-cyclohexene carboxyldehyde (HICC) 5% pet., a single ingredient of FM II. The TM components tetramethylthiuram monosulfide (TMTM), tetramethylthiuram disulfide (TMTD), tetraethylthiuram disulfide (TETD), and dipentamethylenethiuram disulfide (DPTD) were tested in the mix and as single substances in the DKG rubber chemical series at a concentration of 0.25% pet. each.[20] FM I consists of amyl cinnamal, cinnamal, cinnamyl alcohol, eugenol, geraniol, hydroxycitronellal, isoeugenol, and oakmoss absolute (*Evernia prunastri*).[21] They were tested in the mix at a concentration of 1% pet. each, resulting in a combined concentration of 8%. For breakdown testing they were tested also at 1% pet. The test preparations of FM I and several of its single ingredients contained the emulsifier sorbitan sesquioleate (SSO) at concentrations of 5% and 1%, respectively.[22] FM II consisted of citral (1%), citronellol (0.5%), coumarin (2.5%), farnesol (2.5%), α-hexyl cinnamal (5.0%), and hydroxyisohexyl 3-cyclohexene carboxyldehyde (HICC) (2.5%). They were tested in the mix at a concentration of 14% pet.[23] Its single components were tested in pet. at double the concentration that they have in the mix.[24] Patients with patch test reactions (?+, + or ++) to FM I, FM II, or TM at D3 were invited to participate in the study, and written informed consent was obtained from each participant.

2.2 | Sequential tape stripping of *stratum corneum*

Tape stripping was performed at D3 with round adhesive discs (3.8 cm^2, D-Squame, CuDerm, Dallas, Texas) from patch test reactions to the mixes, and if present, to single constituents of the respective mixes as well as skin sites patch tested with pet.[25] Eight consecutive discs from each skin site were collected. Each disc was pressed on for

10 seconds with standardized force using a disc pressure applicator (CuDerm).

2.3 | Natural moisturizing factor (NMF) analysis

NMF was defined as the sum of the concentrations of histidine, 2-pyrrolidone-5-carboxylic acid, and *trans*- and *cis*- isomers of urocanic acid. NMF was extracted from tape strips number six with 600 μL of millipore water and subsequently analyzed by high-performance liquid chromatography (HPLC-UV). NMF levels were corrected for the amount of protein on the tape, which was determined with the D-Squame Scan 850A instrument (Heiland electronic, Wetzlar, Germany).[25,26]

2.4 | Multiplex analysis

The samples were extracted from the tape strips number eight by 0.5 mL of phosphate-buffered saline containing 0.05% Tween 20 and sonicated for 15 minutes, as described previously.[17] After vortexing, the extract aliquots were distributed in vials and stored at −80°C until analysis. The analysis of inflammatory mediators from the extracts was performed using the V-Plex multiplex assays and a MESO QuickPlex SQ 120 reader (both MSD, Rockville, Maryland). Based on a previous study,[17] the following cytokines and chemokines (Th1, Th2, and innate markers) were included: eotaxin-1/CCL11, eotaxin-3/CCL26, IL-1α, IL-1β, IL-6, IL-8/CXCL8, IL-16, interferon (IFN)-γ-induced protein (IP)-10/CXCL10, monocyte chemoattractant protein (MCP)-1/CCL2, MCP-4/CCL13, macrophage-derived chemokine (MDC)/CCL22, macrophage inflammatory proteins (MIP)-1α/CCL3, MIP-1β/CCL4, and thymus and activation-regulated chemokine (TARC)/CCL17. As the amount of SC on the tape varies, the concentrations of the mediators were normalized for the total amount of protein on the tape, which was determined with a Pierce Micro BCA protein assay kit (Thermo Fischer Scientific, Rockford, Illinois).

2.5 | Data analysis

Calculations were performed by using Prism 8 software (GraphPad, San Diego, California). Distribution of data was tested using the Shapiro-Wilk normality test. Differences in the levels of all parameters between individual pet. controls and patch test reactions were determined by Wilcoxon matched-pairs signed-rank test. Differences in the cytokine and NMF levels between patch test reactions (+) to FM I/II with concomitant reaction to a single constituent and patch test reactions (+ or ?+) to FM I/II without concomitant reaction to a single constituent were tested by Kruskal-Wallis test followed by Dunn's multiple comparison test. The association between the strength of positive patch test reactions (+ and ++) and the levels of inflammatory mediators and NMF was assessed using Spearman's correlation test. The levels of the corresponding petrolatum controls were set as baseline. Doubtful reactions were excluded from the correlation analysis.

3 | RESULTS

3.1 | Patients and patch test reactions

We included 36 patients (25 women, average age: 47 years) with 75 patch test reactions in the study (Table S1). Two patients were excluded (#4 and #17) because they withdrew from the study before sample collection was completed. In 14 patients, a patch test reaction to TM was tape stripped (group T-mix). All of these patients had a concomitant patch test reaction to ≥1 single constituent of the TM (Table S2). Tape stripping was done from all 23 reactions to single thiurams (group T-single). Among them, patch test reactions to TETD were most common (n = 12). Twenty-three patients had 25 patch test reactions to FM I (n = 16) and/or FM II (n = 9). Tape stripping was done from 12 patch test reactions either to FM I or FM II, without subsequent patch test reaction to a single component of the respective mix (group F-mix$^{w/o}$), and from 13 patch test reactions to either FM I or FM II, with subsequent patch test reaction to a single component (group F-mix^{with}). Moreover, all 13 patch test reactions to the single fragrances were tape stripped (group F-single). Patch test reactions to isoeugenol (n = 4) were most common. The strengths of patch test reactions are presented in Tables 1 and S1. Most patch test reactions were + (73.3%). The highest share of doubtful reactions was in group F-mix$^{w/o}$ (50.0%).

3.2 | Inflammatory mediators

Only the results of + and ++ patch test reactions were included in the first analysis (Figure 1 and S1). Compared with the corresponding pet. controls, the levels of IL-16, IL-8/CXCL8, IP-10/CXCL10, TARC/CCL17, and MDC/CCL22 were significantly elevated in reactions to TM (T-mix, n = 13), to TM constituents (T-single, n = 23), to FM I or II with subsequent patch test reaction to a single constituent (F-mix^{with}, n = 12), and to FM I/II constituents (F-single, n = 13). Only in the latter group statistical significance was not reached for IP-10/CXCL10. The levels for IL-1β were significantly increased only in F-mix^{with}. Unlike patch test reactions to FM I/II with accompanying reaction to a single constituent (F-mix^{with}), no significant changes for any of the

TABLE 1 Strengths of patch test reactions

		Strengths of patch test reactions		
Group	n	?+ n (%)	+ n (%)	++ n (%)
T-mix	14	1 (7.1)	10 (71.4)	3 (21.4)
T-single	23	0	17 (73.9)	6 (26.1)
F-mix$^{w/o}$	12	6 (50.0)	6 (50.0)	0
F-mix^{with}	13	1 (7.7)	10 (76.9)	2 (15.4)
F-single	13	0	12 (92.3)	1 (7.7)

Abbreviations: F-mix$^{w/o}$, reaction to fragrance mix I or II without positive breakdown testing; F-mix^{with}, reaction to fragrance mix I or II with positive breakdown testing; F-single, reaction to single fragrance of fragrance mix I or II; T-mix, reaction to thiuram mix; T-single, reaction to single thiuram of thiuram mix.

inflammatory mediators compared with the corresponding pet. controls were observed in patch test reactions to FM I/II without subsequent patch test reaction to a single constituent (F-mix$^{w/o}$, n = 6).

To investigate whether the observed differences between patch test reactions (+, ++) to FM I/II with and to FM I/II without subsequent reaction to a single fragrance were related to the different strengths of reactions in both groups, a second analysis was done that included + reactions only of F-mix$^{w/o}$ (n = 6) and F-mix^{with} (n = 10) compared with the corresponding pet. controls. In addition, the doubtful (?+) patch test reactions of F-mix$^{w/o}$ (n = 6) were compared with their corresponding pet. controls. This was done for all inflammatory mediators that had shown significant differences for F-mix^{with} in the first analysis (IL-16, IL-8/CXCL8, IP-10/CXCL10, TARC/CCL17, MDC/CCL22, and IL-1β). In F-mix^{with} with subsequent reaction to a single fragrance, the levels of all mediators were still significantly higher than in the pet. controls (Figure 2). No significant differences compared to corresponding pet. controls were found for doubtful (?+) or + reactions of F-mix$^{w/o}$. In some + patch test reactions of F-mix$^{w/o}$, the levels of inflammatory mediators increased. However, these changes were overall not as consistent and strong as in F-mix^{with}. The median levels detected in + patch test reactions of F-mix^{with} were significantly higher than the ones in doubtful (?+) reactions of F-mix$^{w/o}$ for IP-10/CXCL10 ($P < .05$) and CCL17 ($P < .05$). Comparing the median levels in + patch test reactions between F-mix^{with} and F-mix$^{w/o}$, only IP-10/CXCL10 was significantly higher in F-mix^{with} ($P < .05$).

3.3 | Natural moisturizing factor (NMF)

In + and ++ patch test reactions of FM I/II with positive breakdown testing (F-mix^{with}), TM (T-mix), single thiurams (T-single), and single fragrances (F-single), the levels of NMF were not significantly

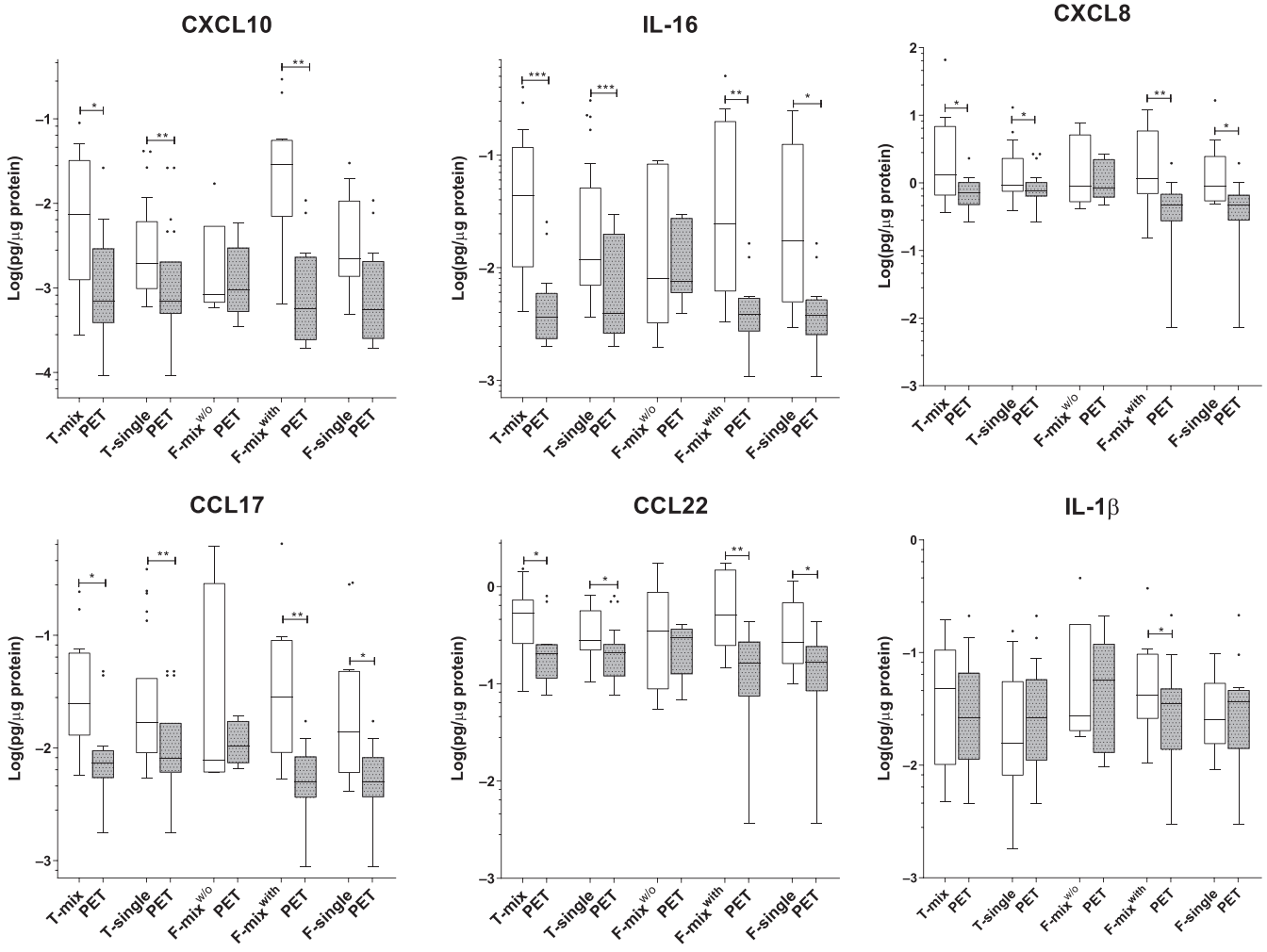

FIGURE 1 Levels of inflammatory mediators (with at least one significant result) in patch test reactions (+, ++) to thiuram mix (T-mix), single thiurams (T-single), fragrance mix I/II without subsequent reaction to a single constituent (F-mix$^{w/o}$), fragrance mix I//II with subsequent reaction to a single constituent (F-mix^{with}), and single fragrances (F-single) compared with their corresponding pet. controls. Data are given as median with interquartile ranges. A non-parametric Wilcoxon matched-pairs signed-rank test was used. *$P < .05$, **$P < .01$, ***$P < .001$

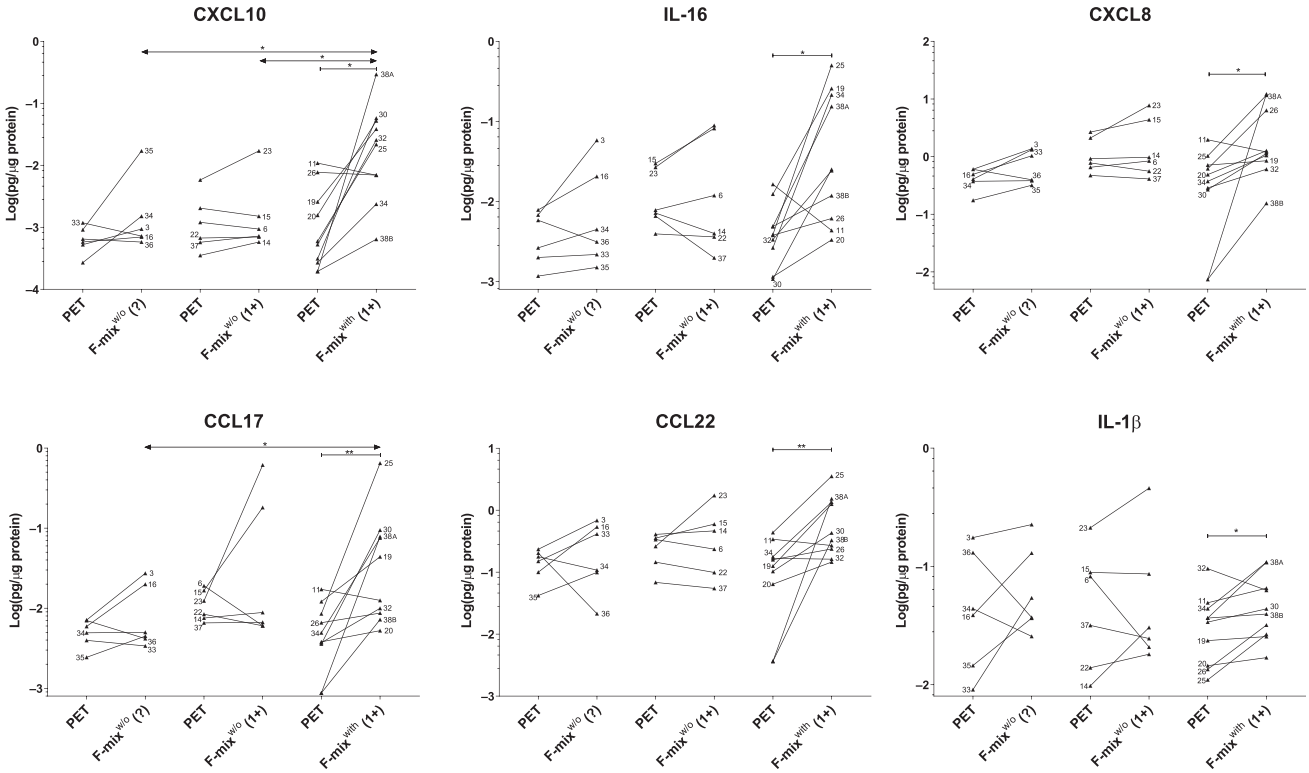

FIGURE 2 Levels of inflammatory mediators in patch test reactions (?+ or +) to fragrance mix I/II without subsequent reaction to a single constituent (F-mix$^{w/o}$) and patch test reactions (+) to fragrance mix I//II with subsequent reaction to a single constituent (F-mix^{with}) compared with their corresponding pet. controls. The labels indicate the individual number of each patient. Patient #38 reacted both to fragrance mix I (38A) and fragrance mix II (38B). A non-parametric Wilcoxon matched-pairs signed-rank test was used for comparison between patch test reactions and corresponding pet. controls. Kruskal-Wallis test followed by Dunn's multiple comparison test was used for comparison between reactions (?+ or +) to F-mix$^{w/o}$ and reactions (+) to F-mix^{with}. *P < .05, **P < .01

different from their corresponding pet. controls (Figure 3A). The levels of NMF were reduced in patch test reactions of FM I/II without subsequent patch test reaction to a single constituent (F-mix$^{w/o}$). However, compared to the corresponding petrolatum controls this difference was not statistically significant (P = .80). Next, we compared NMF levels in + patch test reactions only of F-mix^{with} (n = 10) as well as + and doubtful (?+) patch test reaction of F-mix$^{w/o}$ (n = 6 each) with their corresponding pet. controls (Figure 3B). The median levels of NMF were significantly lower in doubtful (?+) patch test reactions of F-mix$^{w/o}$ compared with their corresponding pet. controls. This was in contrast to the + reactions of F-mix$^{w/o}$ and F-mix^{with}. The NMF levels in 6 of ten patch test reaction of F-mix^{with} were decreased, with one outlier showing marked NMF increase (Figure 3B). When excluding this outlier, still no significant difference compared to pet. controls was found for the remaining levels of NMF (P = .07).

3.4 | Correlation analysis

Significant correlations were found between levels of several inflammatory mediators and strength of patch test reactions (Table S3). The strongest positive correlations were found for IL-16 (r = .55, P < .0001), TARC/CCL17 (r = .53, P < .0001), MDC/CCL22 (r = .45, P < .0001), IL-8/CXCL8 (r = .46, P < .0001), IP10/CXCL10 (r = 0.49, P < .0001), MCP-1/CCL2 (r = 0.40, P < .0001), and MIP-1β/CCL4 (r = .37, P = .0001). No significant correlation was found for NMF.

4 | DISCUSSION

Immune mechanisms of ACD are incompletely understood and involve a complex interplay between dendritic cells, keratinocytes, T cell activation, and regulatory T cell–mediated suppression.[2,3] Cytokines and chemokines are crucial for cell migration, cell adhesion, and cell activation during immune responses. Profiles of such inflammatory mediators may be hapten-specific and help to discriminate between ACD and ICD.[14,27] Previously, Koppes et al used similar methods to investigate patch test reactions to nickel, chromium, MCI/MI, and SLS and revealed that SC inflammation profiles were different between ACD and ICD as well as between the various haptens, although most of them showed similar patterns.[17] The only mediator that showed a significantly increased level in reactions to all haptens, but not to the irritant SLS, was IL-16. Similarly, in the present study, the levels for IL-16 were significantly elevated in patch test reactions to thiurams and fragrances, supporting the previous notion that IL-16 is a potential marker for

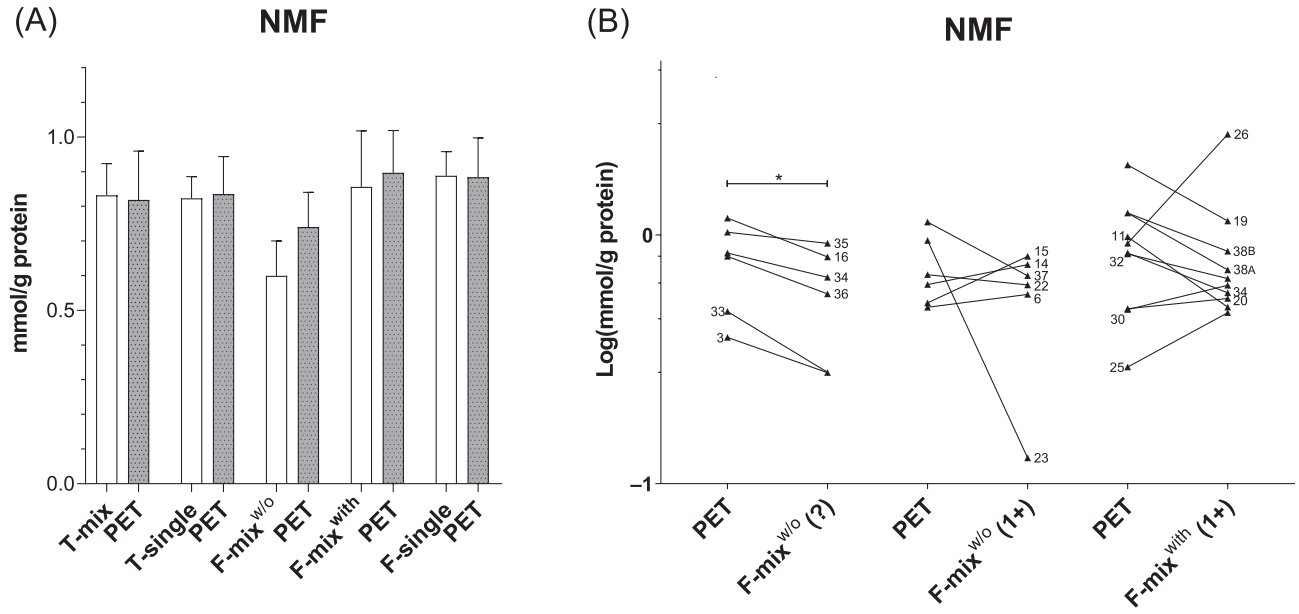

FIGURE 3 (**A**) Levels of natural moisturizing factor (NMF) in patch test reactions (+, ++) to thiuram mix (T-mix), single thiurams (T-single), fragrance mix I/II without subsequent reaction to a single constituent (F-mix$^{w/o}$), fragrance mix I//II with subsequent reaction to a single constituent (F-mix^{with}), and single fragrances (F-single) compared with their corresponding pet. controls. Data are given as median with interquartile ranges. A non-parametric Wilcoxon matched-pairs signed-rank test was used. (**B**) Levels of NMF in patch test reactions (?+ or +) to fragrance mix I/II without subsequent reaction to a single constituent (F-mix$^{w/o}$) and patch test reactions (+) to fragrance mix I//II with subsequent reaction to a single constituent (F-mix^{with}) compared with their corresponding pet. controls. The labels indicate the individual number of each patient. Patient #38 reacted both to fragrance mix I (38A) and fragrance mix II (38B). A non-parametric Wilcoxon matched-pairs signed-rank test was used for comparison between patch test reaction and corresponding pet. controls. Kruskal-Wallis test followed by Dunn's multiple comparison test was used for comparison between reactions (?+ or +) to F-mix$^{w/o}$ and reactions (+) to F-mix^{with}. *P < .05

ACD. IL-16 is produced by epidermal cells, in particular keratinocytes, during sensitization and elicitation phases of hapten-induced contact hypersensitivity and attracts CD4+ T cells and dendritic cells.[28] It was demonstrated in a study by Masuda et al that its production is induced by haptens, but not by primary irritants.[28] Of interest, a case-control study revealed that a promoter polymorphism in the gene encoding IL-16 is associated with contact allergy.[29]

Apart from IL-16, another 4 of the 14 mediators analyzed in the present study (TARC/CCL17, MDC/CCL22, IL-8/CXCL-8, and IP-10/CXCL-10) showed significant differences for TM, FM I/II, and their single constituents compared to the corresponding pet. controls. Only in patch test reactions to single fragrances, the difference for IP-10/CXCL-10 did not reach statistical significance. Most of these mediators belong to the keratinocyte-derived cytokines. This was very similar to the previously reported results for patch test reactions to MCI/MI,[17] suggesting common inflammatory pathways for MCI/MI, thiurams, and fragrances. In a study by Dhingra et al, RNA microarrays were used to analyze skin biopsies from patch test reactions to different haptens, including nickel (n = 10), fragrances (*Myroxylon pereirae*; balsam of Peru: n = 2, FM I: n = 1), and rubbers (carba mix: n = 5, thiurams: n = 2).[16] Although nickel induced a strong Th1/Th17 immune response, test reaction to fragrances and rubbers demonstrated a greater messenger RNA (mRNA) expression of Th2-related chemokines, including TARC/CCL17 and MDC/CCL22, and only a low Th1/Th17 contribution. Both TARC/CCL17 and MDC/CCL22 bind to the skin-homing receptor CCR4 on T cells, which subsequently promotes their migration into inflamed skin.[30,31] CCR4 is expressed to a greater degree on Th2 cells relative to Th1 cells.[32] An amplification loop has been suggested as TARC/CCL17 and MDC/CCL22 attract IL-4 releasing Th2 cells, which in turn enhance Th2-related chemokine production.[33] In addition to mediating T cell migration, TARC/CCL17 plays an important role in cutaneous dendritic cell migration into draining lymph nodes.[34] In mouse models, TARC/CCL17 was shown to promote contact hypersensitivity.[35,36] In humans, a pronounced increase of TARC/CCL17 expression was detected in patch test reactions to nickel, and it was suggested that it plays a major role during the late elicitation phase of ACD.[37] Similarly, Meller et al found an upregulation of TARC/CCL17 and IP-10/CXCL-10 in skin biopsies from patch test reactions to nickel, but not from ICD.[33] In a study by Kamsteeg et al, TARC/CCL17, IP-10/CXCL10, and IL-8/CXCL-8 showed high gene expression levels in punch biopsies from patch test reactions to nickel and FM I, but not from ICD.[38]

IP-10/CXCL10 is produced by keratinocytes upon stimulation with the Th1-related cytokine IFN-γ. It attracts T cells by binding to the CXCR3 receptor and mediates the inflammatory response in ACD.[27,39] Together with the other two CXCR3 ligands CXCL9 and CXCL11, IP-10/CXCL-10 is selectively upregulated in nickel-induced ACD as compared to atopic dermatitis.[15] CXCL8 is a marker of innate immunity and plays a role in the initial hapten-induced chemokine response.[27] Activation by haptens upregulates the production of

CXCL-8/IL-8 in monocyte-derived dendritic cells, whereas irritant exposures leads to decreased CXCL-8/IL-8 production.[40] Similarly, in the study by Dinghra et al, IL-8/CXCL-8 was upregulated significantly in patch test reactions to most haptens tested.[16] Another marker for innate immunity is IL-1β, which is important for mobilization and migration of epidermal Langerhans cells.[41] Its expression was increased in punch biopsies from patch test reactions to nickel and FM I, but not from ICD.[38] In the present study, levels for L-1β were only significantly increased in patch test reactions to FM I/II with positive breakdown testing (F-mix^{with}). In some of the other patch test reactions, its levels were even decreased compared to the corresponding pet. controls. Even though level of significance was not reached, this was similar to the study by Koppes et al, in which decreased SC levels for IL-1β were found in patch test reactions to all haptens and SLS.[17] This was explained by early release of this cytokine from corneocytes and subsequent depletion. Notably, the levels of most inflammatory mediators with significant differences compared with their pet. controls (IL-16, TARC/CCL17, MDC/CCL22, IL-8/CXCL8, IP10/CXCL10) showed strong correlations with the strength of positive patch test reactions.

A large proportion of individuals with patch test reactions to TM, FM I, or FM II do not react to their constituents when tested separately.[4,8-12] In the present study, this was at least true for the FMs. Negative patch test reactions to the single fragrances despite a reaction to FM I or FM II are considered to be an indicator for an irritant reaction to the mix. However, it is still a matter of debate whether negative breakdown testing indicates rather an irritant reaction to the FM, or a false-negative reaction to its single constituents. A likely reason for an irritant reaction to the mix could be the high total test concentration in the mix and the inherent irritant potential of fragrances.[13] In contrast, several potential reasons for a false-negative reaction to the single fragrances have been suggested. Possibly, some test preparations for single fragrances are less stable than when tested in the mix,[42] or co-exposure of fragrances in the mix may have additive or synergistic effects, thereby lowering the elicitation threshold, for example, by increased skin penetration, irritant reactions, or alteration of inflammatory responses.[43-45] Furthermore, it was argued that the emulsifier SSO, which is included in FM I, but not in all of the test preparations for its single ingredients, may enhance skin penetration of fragrances in the mix. Moreover, it might be possible that 1% is too low of a test concentration for the single fragrances of FM I, in particular in individuals with a low-grade sensitization.[46,47] Notably, the single fragrances of FM II are already tested double the concentration that they have in the mix, which may suggest that at least weak reactions to FM II with a negative breakdown test are irritant.[11] Another suggested reason for negative breakdown testing is that co-exposure of fragrances in the mix may cause formation of new allergens and thus a compound allergy that is not discovered by testing of the single constituents.[48] Hence, it remains difficult to judge if only FM reactions with positive breakdown testing are truly allergic.

To the best of our knowledge, the present study is the first to investigate the potential of inflammatory profiles and NMF as biomarkers for differentiating between irritant and allergic patch test reactions to FMs. When analyzing + and ++ patch test reactions, only those of FM I/II with subsequent reaction to a single constituent (F-mix^{with}) showed inflammatory profiles similar to test reactions to TM (T-mix) as well as to the single thiurams (T-single) and single fragrances (F-single), with significantly elevated SC levels of IL-16, TARC/CCL17, MDC/CCL22, IL-8/CXCL-8, and IP-10/CXCL-10 compared to pet. controls. As discussed before, these inflammatory mediators are potential indicators for hypersensitivity reactions. Instead, no significant changes for any of the inflammatory mediators compared with the corresponding pet. controls were found in patch test reactions to FM I/II without subsequent patch test reaction to a single constituent (F-mix$^{w/o}$). This difference to the other groups suggests that the reactions to FM I/II with negative breakdown testing were rather not allergic. It could be argued that the difference was related to missing ++ reactions in this subgroup. However, when comparing only + reactions of F-mix^{with} and F-mix$^{w/o}$, the results were similar, indicating a substantial difference between these two groups. Moreover, the results of + reactions in group F-mix$^{w/o}$ resembled those of doubtful (?+) reactions, which are usually considered to be irritant. The changes of inflammatory mediators in both groups were overall not as consistent and strong as in the reactions to FM I/II with positive breakdown testing. Compared to the results of + reactions in group F-mix^{with}, some of the levels of inflammatory mediators were significantly lower in doubtful (?+) reactions of F-mix$^{w/o}$, which could be related to both a missing allergic reaction and the weaker inflammatory response in the latter.

Experimental studies show that various skin irritants, including SLS, NaOH, fruit acids, and aliphatic alcohols, significantly decrease the SC levels of NMF.[49-52] Recently, Koppes et al demonstrated that NMF levels were decreased in patch test reactions to MCI/MI and SLS, but not to nickel, chromate, or *p*-phenylenediamine.[18] It was speculated that the reduction in NMF levels after patch testing with MCI/MI was at least partly caused by the irritant properties of this sensitizer. In the present study, the levels of NMF were significantly decreased in doubtful (?+) reactions of F-mix$^{w/o}$ compared with their corresponding pet. controls. This was in contrast to all other groups and fits with the assumption that the doubtful reactions were irritant. Although not significantly different from pet. controls, 6 of 10 patch test reactions (+) to FM I/II with positive breakdown testing (F-mix^{with}) showed decreased NMF levels. Overall this suggests that NMF levels may decrease in patch test reactions to fragrances as seen for MCI/MI due to their inherent irritant potential. This certainly hampers the potential of NMF for differentiation between irritant and allergic patch test reactions to these haptens.

Some limitations of the present study should be considered. The sample size is limited and only a selection of inflammatory mediators was analyzed. Because the levels of inflammatory mediators are probably time- and concentration-dependent, temporal changes could have been missed. Moreover, the measured SC concentrations may differ from concentrations in the viable epidermis where many inflammatory mediators are formed and where they primarily exert their activity; however, the SC sampling method offers a non-invasive approach to measuring a wide range of inflammatory mediators in vivo in the skin. The occlusion time of 24 hours instead of 48 hours

may have led to a higher number of false-negative or weak patch test reactions. However, the occlusion time was the same for all haptens and by choosing the shorter occlusion time we have instead avoided potentially irritant, false-positive reactions.

In conclusion, the present study shows that the inflammatory profiles of patch test reactions to thiurams and fragrances are similar and indicate a Th2-skewed inflammation. The potential of IL-16 as biomarker for ACD was confirmed, whereas a decrease of NMF may indicate skin irritation. Our results support the hypothesis that doubtful or weak positive patch test reactions to FM I or FM II without concomitant reaction to a single ingredient are rather caused by an irritant than an allergic reaction.

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AUTHOR CONTRIBUTIONS

Richard Brans: Conceptualization; data curation; formal analysis; investigation; project administration; supervision; writing-original draft; writing-review and editing. **Ivone Jakasa:** Formal analysis; investigation; writing-review and editing. **Sanja Goc:** Formal analysis; investigation; writing-review and editing. **Swen Malte John:** Funding acquisition; resources; writing-review and editing. **Sanja Kezic:** Conceptualization; data curation; formal analysis; investigation; resources; supervision; writing-original draft; writing-review and editing.

CONFLICT OF INTEREST

There are no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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