Stratum corneum biomarkers for inflammatory skin diseases
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CHAPTER 5

General Discussion
GENERAL DISCUSSION

This chapter discusses the main findings, methodological considerations, and the relevance of the investigated biomarkers for clinical practice. Conclusions and recommendations for future research and clinical practice are then presented.

The aim of the present research was to explore skin barrier and immune response related biomarkers for allergic contact dermatitis (ACD), irritant contact dermatitis (ICD), and atopic dermatitis (AD) that can be derived from the stratum corneum (SC). The corresponding research objectives and research questions were formulated as follows:

Objective I: To gain insight into the SC biomarkers related to skin barrier and immune response in ACD and ICD
Research question I: Which biomarkers are known to be related to ACD and ICD, and what is their potential for use in research and clinical settings? (Chapter 2)

Research question II: Which skin barrier and immune response related parameters obtained from the SC can serve as biomarkers to distinguish ACD from ICD? (Chapter 3)

Objective II: To evaluate the suitability of various SC biomarkers for clinical practice related to AD
Research question III: Which skin barrier and immune response related biomarkers obtained from the SC are suitable for the monitoring of therapy in atopic dermatitis? (Chapter 4)
MAIN FINDINGS

Research question I: Which biomarkers are known to be related to ACD and ICD, and what is their potential for use in research and clinical settings? (Chapter 2)

Two non-systematic reviews of current literature identified several skin barrier and inflammatory mediators as key molecules in the sensitization or elicitation phase of ACD. However, no biomarkers are currently used in the clinical practice to, for example, differentiate ACD from ICD, predict individual response, or serve as a surrogate endpoint in patch testing. For ICD, literature data consistently show that skin exposure to various irritants leads to changes in IL-1α, IL-RA, and NMF. However, as most of the studies were conducted in an experimental setting, their potential as biomarkers for ICD to, for instance, differentiate ACD from ICD or identify the skin irritating properties of a compound, should be further confirmed in clinical practice.

Research question II: Which skin barrier and immune response related parameters obtained from the SC can serve as biomarkers to distinguish ACD from ICD? (Chapter 3)

In a clinical study (Chapter 3), a large number of inflammatory mediators and skin barrier biomarkers were investigated after patch testing to common contact allergens (Ni, MCI/MI, Cr and PPD) and an irritant, SLS (which is frequently used in research on ICD). The results show that DTI, a quantitative measure of corneocyte surface texture, significantly increased in SLS-induced ICD, whereas none of the investigated allergens affected DTI. This indicates DTI as a promising biomarker to distinguish ICD from ACD. NMF was also affected by SLS, showing significantly lower values compared to the control skin. However, also one of the investigated allergens, namely MCI/MI, caused a significant NMF decrease, suggesting that NMF cannot discriminate ICD from ACD. MCI/MI, as the only allergen, also caused remarkable morphological changes of the corneocytes on a microscopic scale (without affecting DTI). Furthermore, MCI/MI caused comparable alterations in the protease activity and IL-1α, which was also observed in SLS, but not in other allergens. When focusing on inflammatory mediators, MCI/MI induced a larger
and broader difference in the immune response than the other tested allergens, suggesting that skin barrier damage caused by MCI/MI enhances its allergenic response.

In total, 32 inflammatory mediators representing innate, Th1, and Th2 immune responses were detected in the SC samples of ACD and ICD. Overall, the profiles of most mediators in the patch test reactions were similar among the investigated allergens and SLS, reflecting common inflammatory pathways. However, several cytokines showed distinct differences between ICD and ACD, of which IL-16 seems to be the most promising to differentiate between the two dermatoses, as this cytokine is increased by Ni, MCI/MI, and Cr, but not by the skin irritant SLS.

**Research question III: Which skin barrier and immune response related biomarkers obtained from the SC are suitable for the monitoring of therapy in atopic dermatitis? (Chapter 4)**

To investigate the suitability of SC-derived biomarkers for the monitoring of therapy in AD, three clinical studies were conducted. In the first, the relationship between NMF and corneocyte surface texture (expressed as DTI) in therapy-naïve AD patients with and without a filaggrin loss-of-function (FLG LOF) mutation was investigated (Chapter 4.1). The results showed that DTI strongly correlates with NMF levels, both before and after therapy. Furthermore, a high DTI and a low NMF persist in patients with FLG LOF mutations despite clinical improvement due to therapy. In addition to changes in corneocyte texture, immunolabeling with corneodesmosine (Cdsn), a protein important for desquamation, showed aberrant Cdsn expression in patients with FLG LOF mutations, suggesting impaired cell maturation. These results indicate that AD patients with FLG LOF mutations have a unique skin barrier subtype, and that the monitoring of skin barrier function in these patients is of particular importance. To monitor skin barrier, DTI and NMF seem to be suitable biomarkers.

In the second study, which was performed in patients with mild to moderate AD (Chapters 4.2 and 4.3), skin barrier biomarkers (NMF, TEWL, skin hydration), a large array of inflammatory mediators, and clinical symptoms (SCORAD)
were used to monitor three topical therapies: a cream containing ceramides and magnesium, hydrocortisone acetate, and unguentum leniens (cold cream). The results showed that despite a significant improvement in clinical symptoms after all three therapies, hydrocortisone and unguentum leniens caused a decrease in NMF concentration, an effect that was not observed for ceramide–magnesium cream. This was consistent with data on skin hydration, which showed the greatest improvement following the application of ceramide–magnesium cream. As NMF is an important factor in the hydration of the skin, a temporary decrease might affect the long-term course of disease, and thus its levels should be monitored when evaluating therapy. In addition to skin barrier biomarkers, a wide range of Th1 and Th2 of cytokines and chemokines were measured in the SC of AD lesions treated with ceramide–magnesium cream, collected before and after therapy. Several inflammatory mediators decreased significantly in concentration after topical therapy. In patients with moderate AD, the decrease in TARC/CCL17 and IL-8 concentration was correlated with the decrease in disease severity. Furthermore, the levels of these two chemokines at baseline were correlated with disease severity, indicating that these chemokines might be useful biomarkers for investigating the course of the disease and the effect of local therapy.

The third study (Chapter 4.4) compared two noninvasive techniques for the determination of NMF in the SC: Raman confocal microscopy and SC tape stripping followed by high-pressure liquid chromatography (HPLC). The study revealed that the NMF concentrations determined by both techniques, show a good correlation ($r^2=0.61$). Good agreement between measurements ($r^2=0.90$) obtained on the left and the right arm indicate the robustness and good reproducibility of both methods. The choice of technique therefore largely depends on practical considerations, such as price, accessibility of the technique, available expertise, and time constraints.

**METHODOLOGICAL CONSIDERATIONS**

Measuring inflammatory mediators for ACD, ICD, and AD in the SC has some limitations (Chapters 3.2 and 4.3). In contrast to IL-1α and IL-1RA, which are present constitutively in substantial amounts in the SC, most of measured inflammatory
mediators are induced in the epidermis upon skin irritants of contact allergens, from where they diffuse into the SC. The diffusion kinetics of these mediators from the lower epidermis might not be the same for all inflammatory mediators, thus their SC concentrations might not reflect their concentrations in the epidermis or dermis where they are produced and have their targets. However, this is of less importance when using biomarkers as a tool to distinguish ACD from ICD or for the monitoring of disease severity. Next, in the study described in Chapter 3.2, inflammatory mediators in ACD were measured at 72 hours after patch testing. Although this is a common readout time-point in clinical practice, the kinetics of immune response are allergen and individual specific(1). Similarly, the timing of sampling is also crucial in ICD. Levels of SC IL-1α show consistent decreases after SLS(2-4). This decrease probably represents a depletion of a preformed IL-1α pool in the SC. However, as a consequence of inflammation de novo synthesis of IL-1α will occur lower in the epidermis, which will result in an increase in IL-1α levels at a later stage. Another limitation of this study is the group size, especially in the experimental study with contact allergens. As only four subjects were included in the PPD group, the results for PPD should be interpreted as an indication of a trend.

Some of studies presented in this thesis included large sets of biomarkers, for example the inflammatory mediators in the studies described in Chapters 3.2 and 4.3. In such a case, correction for multiple testing is recommended to reduce type I errors (false-positive results). However, the explorative nature of our studies demanded high sensitivity and thus no correction for multiple testing was performed. Results are therefore “exploratory results” and the further confirmation of findings is needed(5).

INTERPRETATION OF RESULTS AND RELEVANCE FOR CLINICAL PRACTICE

Biomarkers in diagnostics
Contact dermatitis (CD) is one of the most frequently seen occupational diseases(6). A major challenge for clinicians is to distinguish between the two subtypes, ACD and ICD, which have similar clinical features but different pathophysiology, treatment
options, and prevention strategies(7). Patch testing with allergens is the current diagnostic tool for discriminating between ACD and ICD. However, a positive patch test does not prove that clinical symptoms are caused by that particular allergen(8). Moreover, the absence of a positive patch test does not automatically imply ICD. In the present studies, we investigated several skin barrier biomarkers, as the primary cause of ICD is damage to the skin barrier. This was also confirmed in the present studies: Skin barrier related biomarkers, including NMF, protease activity, DTI, and IL-1α, were affected by SLS. NMF reduction has been reported for several other skin irritants, including NaOH, n-propanol, and acetic acid, suggesting that NMF might be regarded as a biomarker of skin irritation(9). For other biomarkers such as DTI and proteases, data for skin irritants other than SLS are largely lacking and it has still to be investigated whether DTI and investigated protease activity can be used as general biomarkers for ICD. Although skin barrier biomarkers such as NMF seem to be promising to identify ICD, their use to distinguish ICD from ACD is hampered by the skin damaging properties of some contact allergens. This was also found in the present study for MCI/MI, which induces a similar effect to that of SLS for protease activity, NMF, and IL-1α. The skin damaging effect might contribute to the high allergenic potential of MCI/MI. It has previously been suggested that an irritant reaction or skin barrier damage might act as a “danger signal,” initiating the sensitization or elicitation of ACD(10). Some allergens can induce such irritant reactions and can thus produce the danger signals necessary for sensitization(11). This has also been shown for the potent allergen 2,4-dinitrochlorobenzene (DNCB), which upregulates TNF-α, a proinflammatory cytokine that plays a role in skin irritation and the sensitization process(10). It is thus of importance to identify the irritant potency of allergens by, for example, using skin barrier related biomarkers.

Although MCI/MI showed a similar pattern to SLS regarding changes of NMF, proteases, and IL-1α, one of the investigated biomarkers – DTI, which expresses surface texture – showed a clear difference between ACD and ICD. DTI is a novel biomarker of skin barrier damage, which showed its value also in AD. As shown in Chapter 4.1 (a study in which DTI was introduced as a skin barrier biomarker), DTI is closely related to FLG LOF mutations and NMF. The group of AD patients with FLG mutations is shown to have a divergent characteristics compared to AD patients without FLG mutations. The skin barrier defect in AD patients with FLG
mutations, in this case represented as a high DTI, is persistent over time, even when clinical symptoms improve. Although the exact role of DTI in the pathogenesis of AD is still unclear, knowledge on the diversity within the AD patient population might aid in the stratification of AD patients and for a personalized therapeutic approach.

In the study on the inflammatory mediators of ACD and SLS-induced ICD presented in Chapter 3.2, similar profiles were found, suggesting that ICD and ACD share a common inflammatory pathway. However, a different pattern was shown by several cytokines, of which IL-16 is the most promising. IL-16 is produced by keratinocytes during the sensitization and elicitation phases of ACD. Masuda et al. showed that haptns, but not primary irritants, induce IL-16 in the skin(12), which is in agreement with the findings presented in this thesis. Interestingly, polymorphisms in the gene encoding IL-16 modify susceptibility to contact allergens(12, 13), further supporting IL-16 as an important cytokine in contact allergy. These findings need to be confirmed for other allergens and irritants before the results can be generalized, but the results do show that allergens of very diverse chemical groups can produce similar patterns of inflammatory mediators among allergens. This is somewhat surprising, as allergens have different mechanisms by which innate pathway signaling is triggered. For example nickel, cobalt, and palladium directly bind to Toll-like receptor 4, inducing a proinflammatory cascade(14-16). Furthermore, chromium (VI) – but not chromium (III) or Ni – induces innate immune response through the generation of oxidative species (ROS), which activate NLRP3 inflammasome(17). Also Dhingra et al. found differences between various allergens in m-RNA levels for several cytokines; however, the m-RNA values might not be directly comparable to the protein expressions that were measured in the present studies(18). A biomarker differentiating ACD from ICD would be most valuable for diagnostics, as the clinical presentation is very similar. Therefore, IL-16 as an objective biomarker deserves to be studied further.

Therapy monitoring

Therapy monitoring is of importance in clinical practice, as well as in evaluating the efficacy of new interventions. Biomarkers might offer an advantage over clinical outcome scoring tools such as SCORAD or EASI, as the latter include subjective
parameters like sleep deprivation or itching, and are subject to intra- and inter-
observer variability(19, 20). When biomarkers are used to evaluate therapy or assess
disease severity, they are usually derived from blood samples. The invasiveness of
this method makes it less suited for large cohorts of patients and poses a barrier
to the implementation of biomarkers in pediatric medicine where AD is a major
topic(21). Moreover, AD usually presents on certain body areas and thus the
monitoring of biomarkers in the treated skin in the case of topical therapy might be
more relevant than their blood levels. Biomarkers that are suggested as candidate
biomarkers for monitoring or of disease severity mainly concern inflammatory
mediators(22, 23). Elevated total and/or allergen-specific serum immunoglobulin
IgE levels are commonly associated with AD, but only moderately correlate with
disease severity and are not specific; some AD patients have normal IgE levels(24).
In a recent meta-analysis by Thijs et al. on AD biomarkers, serum TARC/CCL17
levels showed the best correlation with disease severity in cross-sectional and
longitudinal studies(25). TARC (thymus- and activation-regulated chemokine, also
known as CCL17) is a chemokine produced by the keratinocytes in AD lesions and
is important in mediation of the acute Th2 inflammatory reaction of the disease(26).
Thijs et al.’s results are consistent with those from the study described in Chapter
3.2, which shows that the SC concentration of TARC/CCL17 is correlated with
disease severity in patients with moderate AD. Furthermore, the changes in the
TARC/CCL17 levels were associated with the changes in SCORAD, indicating that
TARC/CCL17 is a suitable biomarker to monitor therapy efficacy as well as disease
severity. The fact that TARC/CCL17 can be measured in the SC might contribute
to a more feasible monitoring of disease course in research and clinical practice.
SC-derived TARC/CCL17 can be used as a noninvasive tool to objectively monitor
therapy success in patients who receive local therapy, including children (in which
AD primarily arises). It can also be used as a surrogate endpoint in clinical trials,
which would improve the comparison of study results, an option suggested earlier
by Thijs et al.(27) In addition to TARC/CCL17, in the present studies several other
inflammatory mediators showed a significant decrease in the SC after therapy,
including Th2 and Th1 cytokines/chemokines, regulatory cytokines, and vascular
adhesion molecules. For most of these cytokines, data on their levels in the SC are
lacking and their suitability for therapy monitoring should be further investigated
in larger studies.
In contrast to inflammatory mediators, skin barrier biomarkers are largely neglected as biomarkers for AD with the exception of TEWL, which is mainly used in research to assess skin barrier function. This is surprising, as AD is currently regarded as disease that is at least partially caused by a skin barrier dysfunction resulting from, for example, filaggrin deficiency(28). NMF, which contains degradation products of filaggrin, has been shown to be a feasible biomarker of the FLG genotype, but is also affected by disease severity(29, 30). Furthermore, NMF concentrations have been shown to decrease in ICD(9, 31, 32). In the RCT presented in Chapter 3.2, NMF was used to monitor the effect of local therapy on the skin barrier. In this trial, despite a clinical improvement, AD patients showed a decrease in NMF when treated with hydrocortisone, which is a low-potency corticosteroid. Such an effect was also found by Danby et al. for a more potent corticosteroid, namely betamethasone valerate(33). The mechanisms that underlie this decrease are unclear. However, the implications are significant, as these creams are widely used in the treatment of inflammatory skin diseases, and in some countries hydrocortisone acetate is available as an over-the-counter product. If these treatments cause a (temporal) decrease in NMF concentration, it could be expected that this has a negative effect on the skin barrier function, making the skin more vulnerable to the effect of such external factors as skin irritants(34). NMF can be used to detect such negative effects and might also serve as an early effect biomarker. Protective measures in the form of moisturizing creams that compensate for the loss of NMF could be implemented. In a 2017 Cochrane Review on the use of moisturizers in AD, it was concluded that moisturizers are effective in prolonging time to new flares and reducing the amount of topical corticosteroids needed(35). Furthermore, the authors did not find reliable evidence that one moisturizer is more effective than another. Skin barrier biomarkers and inflammatory mediators are rarely used in the evaluation of therapy efficacy. Biomarkers could have an important role to play in such evaluations, helping practitioners to decide which moisturizer to choose.
CONCLUSION

Stratum corneum (SC) derived biomarkers might play a large role in research and clinical practice related to inflammatory skin diseases, as they provide information on both skin barrier and inflammatory status. The SC samples can be collected in a noninvasive manner and the levels of a large number of key molecules of skin barrier and immune response can be measured. In diagnostics, the number of circular-nano objects on the corneocyte surface (DTI) and SC derived IL-16 seem to be promising biomarkers to distinguish ACD from ICD. Studies on more contact allergens and skin irritants and their validation in clinical practice should provide evidence whether DTI and IL-16 are suitable for diagnostic purposes. In AD therapy monitoring, of the investigated SC derived biomarkers, TARC/CCL17, IL-8, and NMF are the most promising. SC-derived TARC/CCL17 and IL-8 are correlated with both disease severity and decrease in clinical symptoms during therapy. NMF is helpful in the assessment of skin barrier damage resulting from, for example, exposure to skin irritants, some contact allergens, and corticosteroid therapy. Finally, the carriers of filaggrin loss-of-function mutations alter the corneocyte surface texture. This persists even after clinical improvement, which might aid the stratification of AD patients and thus contribute to a more personalized therapeutic approach.

Recommendations for future research

1. More insight into the nature of circular-nano objects and their role in the pathophysiology of AD and ICD is needed. This could be achieved by studying carriers and non-carriers of FLG LOF with and without atopic dermatitis or murine models deficient for structural SC proteins.

2. The adverse effect of local therapy on the NMF and skin barrier in general should be investigated for anti-inflammatory drugs and emollients of different compositions.

3. The predictive value of NMF as an early effect parameter of occupational irritant contact dermatitis should be evaluated preferably in a prospective cohort study in the occupational setting.
4. Evaluation of the DTI and IL-16 as biomarkers for the differentiation between irritant and allergic contact dermatitis should be performed with more allergens and skin irritants, and validation should be performed in patients with contact dermatitis.

**Recommendations for future practice**

SC-derived biomarkers of skin barrier function and inflammatory mediators have potential in both diagnostic practice and therapy evaluation of ACD, ICD, and AD. However, a thorough validation is needed before SC biomarkers can be implemented in clinical practice.
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