Stratum corneum biomarkers for inflammatory skin diseases
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General Introduction
GENERAL INTRODUCTION

Our skin acts as a barrier to the outside world. This barrier function mainly resides in the stratum corneum (SC), the uppermost layer of the skin. This layer is the body’s first line of defense, and it is vulnerable to external treats such as physical insults, and irritating or allergenic substances. Furthermore, some individuals have an intrinsically compromised skin barrier due to their genetic makeup. An impaired skin barrier is known to play a major role in the pathophysiology of contact dermatitis, a dermatosis that is frequently seen in the occupational setting. There are two types of contact dermatitis: allergic contact dermatitis (ACD) and irritant contact dermatitis (ICD) (1, 2). In the USA, 15.2% of all cases of nonfatal occupational illnesses reported in 2014 were occupational skin disorders (OSDs) (3). Most of these cases concerned occupational contact dermatitis (OCD), a term that refers to ACD and ICD induced by work activities (4). Recent data from the Netherlands show that 71% of all reported OSD cases were a form of OCD (mainly ICD)(5). These disorders have a major impact on the quality of life of patients in general and on their work ability in particular (6). Almost half of workers with OCD have a history of atopic dermatitis (AD), an inflammatory skin disease that is characterized by an impaired skin barrier (7-9). Strong attenuation of the risk for OCD by AD and its high prevalence (7.2% of the general population in US adults in 2015) underlines its high relevancy when studying OCD10. In the workplace, ACD, ICD, and AD often coincide. As they share the same clinical features, the diagnostics is challenging (11). Accurate diagnosis of OCD and identifying the causative agent(s) are both of major importance: When diagnostics are not adequate and timely, OCD can become a chronic disease that causes extensive suffering for patients and has a large socioeconomic impact, such as sustained and prolonged sick leave, changing of work tasks or job, job loss and long-term unemployment (12, 13). Despite similar clinical and histological characteristics, the underlying pathophysiological mechanism of ACD, ICD, and AD is different. In contrast to direct skin barrier damage induced by an irritant activating innate immunity, ACD and AD are characterized by the induction of antigen-specific effector and memory T cells. A disturbed barrier also plays an important role in ACD and AD and its immune response (Fig. 1). Immune responses in ACD, ICD, and AD are mediated by a large number of immune mediators (Fig. 1). The identification of biomarkers
of the key molecular or cellular events that are specific to ACD, ICD, and AD might assist in diagnostics and therefore the prevention of OSD. A biomarker is defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (14). In addition to diagnostics, biomarkers might also be useful to monitor the efficacy of therapy or other interventions, as currently there is a lack of valid and reliable outcome measures (11, 15). Most of the currently studied biomarkers in these diseases are determined from blood samples or in skin biopsies (16). However, blood samples provide information at a systemic level and skin biopsies are invasive (17, 18). SC biomarkers may overcome these drawbacks, as SC can be collected from the skin site of interest in a noninvasive manner.

SC harbors a large number of molecules that are essential for the skin barrier function and immune response, such as lipids, proteases, inflammatory mediators, and natural moisturizing factors (NMF) (19-22). Changes in SC morphology might provide valuable information on the structural damage to the skin barrier. However, up to now, an SC tape stripping technique has been used for a limited number of immunological biomarkers mainly due to the poor sensitivity of the assays. Furthermore, morphological characteristics of the SC have only been assessed qualitatively, and at present there are no quantitative morphological biomarkers for ICD, ACD, or AD (22). The development of highly sensitive multiplex assays offers new possibilities for the analysis of a large set of inflammatory mediators from a single sample (23). Advances have also been made in the standardization of the tape stripping procedures, in particular in the assessment of the amount of SC harvested by a tape (18, 24). Furthermore, the recent development of automated imaging analysis of corneocyte surface texture obtained by atomic force microscopy, provides for the first time a quantitative measure that has potential as a suitable biomarker of skin barrier damage.

Methodological development and advances in our understanding of the mechanistic pathways that underlie ICD, ACD, and AD have paved the way for the potential use of SC biomarkers in research and clinics. In the present research, advances in techniques will be utilized to assess a large sets of biomarkers from the SC. The aim was to gain new insights into local inflammatory milieu and skin barrier function.
in ACD, ICD, and AD, and potentially identify biomarkers that might be useful for diagnostics and therapy monitoring.

In the following sections, background information is provided on the SC, addressing structural and molecular components important for its barrier function and thus relevant as a potential biomarker of the skin barrier. Furthermore, a brief overview of the etiology and challenges in current diagnostics of ACD, ICD, and AD is presented.

**Stratum corneum**

The stratum corneum (SC) is the uppermost layer of the epidermis (Fig. 2) and it is the major barrier to chemical transfer through the skin. It harbors a large number of molecules that are crucial for its homeostasis including lipids, proteases, antimicrobial peptides, cytokines, and natural moisturizing factors (NMF) (22). At most body locations, the SC is a relatively small part of the epidermis, consisting of approximately 10 \( \mu \text{m} \) of terminally differentiated and interconnected keratinocytes called corneocytes embedded in a lipid matrix (see Fig. 2 and 3) (25). Keratinocytes are produced in the lower regions of the epidermis, the stratum basale (see Fig. 2). During the maturation process to corneocytes, keratinocytes gradually move up toward the stratum corneum, losing their nucleus and reorganizing their inner structure, a process that takes about four weeks. Keratin fibers make up the greatest part of the cytoskeleton within the corneocytes and are aligned by a protein called filaggrin (contraction of “filament-aggregating protein”) (25). Filaggrin also plays a role in SC moisturization, as some of its degradation products, which are constituents of NMF, are hygroscopic. The outer layer of the keratinocyte is replaced by the cornified envelope, which is formed beneath the plasma membrane in terminally differentiating squamous cells (26). The cornified envelope has a complex structure consisting of crosslinked proteins, including filaggrin, loricrin, keratins, and involucrin, that are surrounded by a lipid envelope (27). The corneocytes are linked by corneodesmosomes, which provide mechanical strength and preserve the horizontal alignment (28). When the corneodesmosomes are degraded by kallikrein-related peptidases (KLKs) in the outer SC layers, the corneocytes are shed from the skin (29). On average, one layer of corneocytes is shed every day, rejuvenating the skin barrier day by day. This ensures a steady state of a “fresh”
corneocyte-based barrier. In addition to the corneocytes, the intercellular lipid bilayers also have a vital role in the skin barrier, especially in water homeostasis. The highly organized lipid bilayers are important in making the skin waterproof. They are comprised of approximately 45–50% ceramides, 25% cholesterol, 10–15% free fatty acids, and 5% other lipids (30).

Fig. 1: Inflammatory mediators in ICD, ACD, and AD. Source: Julia K. Gittler, JACI 2013

Fig. 2. A cross-section of the epidermis
**Allergic contact dermatitis (ACD)**

**Etiology**

ACD is an overreaction of the adaptive immune system to a low molecular chemical called allergen or hapten. ACD is characterized by a sensitization phase that is followed by an elicitation phase. In the sensitization phase, after penetrating the stratum corneum, haptens form a complex with endogenous proteins. Keratinocytes are essential in this step, as they provide the enzymes required for the conversion of pro-haptens into biologically active haptens. Keratinocytes also provide alarmins and cytokines, which are needed for the activation of dendritic cells (DCs). Activated DCs present these hapten–protein complexes to T cells. As a consequence, T cells get activated if the hapten–carrier complex can bind to the T cell receptor, under the condition that sufficient cell membrane-bound and soluble mediators are present. The T cells then start to proliferate and become hapten-primed effector T cells (32-34). In addition to this T cell activation, the presence of innate proinflammatory signals can lower the activation threshold of naïve T cells (35). How this proinflammatory signal is induced is not fully understood, but some allergens, such as nickel, cobalt, and palladium, are known to directly bind to Toll-like receptor 4 and by doing so, induce a proinflammatory cascade (35-37). Recent research also indicates that skin barrier damage plays an important role in the sensitization phase of ACD, as an impaired skin barrier facilitates the penetration of contact sensitizers and also induces so-called danger signals (38, 39). The allergen-
induced proinflammatory cascade and the other mediating proinflammatory processes and the role of the skin barrier are shown in Fig. 1.

After the sensitization phase, re-exposure to the allergen can induce an inflammatory reaction, namely the elicitation phase. In this phase, the hapten-specific T cells formed in the sensitization phase, recognize the haptens and together with other inflammatory cells release cytokines, resulting in an inflammatory cascade in which the keratinocytes again play an important role (40, 41).

Diagnosis
Diagnosing ACD is mainly done by patch testing. Based on the patient history, clinical examination, and information about possible exposure to allergens and/or irritants, suspected allergens are tested epicutaneously (42). Deciding which allergens should be included in the patch testing is difficult, especially for less common allergens and unclear exposure patterns (43). To overcome this problem, in clinical practice series with multiple allergens and mixes of allergens are tested. This broad range of testing can, however, result in false positive or clinically irrelevant reactions. Another problem is the occurrence of irritant reactions to allergens. Although training helps, it can be hard for the practitioner to determine whether the observed patch test reaction is an allergic or an irritant reaction, which is problematic as many allergens can induce both (44, 45). In this light, objective biomarkers that can distinguish ACD from ICD can have a valuable place in the diagnostic toolbox and improve clinical practice.

Irritant contact dermatitis

Etiology
Irritant contact dermatitis (ICD) occurs as a result of a single or cumulative exposure to physical or chemical irritants that react with the proteins of the stratum corneum leading to skin barrier damage. Genetic predisposition and existing barrier dysfunction in the form of AD can increase this risk; workers with AD and a filaggrin loss-of-function (FLG-LOF) mutation have a four- to fivefold increased risk for the development of ICD (7, 46). The irritant potency of an agent is dependent on the physio-chemical properties of the irritant, which governs its
interaction with SC components. For example, solvents, soaps, and detergents can affect the SC lipids, structural proteins, and NMF, making the skin dry, less flexible, and prone to damage. Unsurprisingly, workers who frequently use these products, for example nurses, cleaners, and hairdressers, often suffer from OCD. The innate immune response in ICD is rapid and largely localized on the skin site that is in contact with the irritant (47, 48). One of the first responses to skin damage is the release of a preformed pool of IL-1\(\alpha\) stored in the corneocytes, initiating a cascade of inflammatory mediators (see Fig. 1) (49). Studies on immune response in ICD focuses mainly on IL-1 cytokines, and data on other immune mediators in the SC are scarce (50, 51). Skin barrier damage has largely been investigated by measuring transepidermal water loss (TEWL) and skin hydration, whereas very few studies have addressed changes in the molecular composition of the SC (52).

### Diagnosis

The diagnosis of ICD can be challenging. The main focus is on ruling out ACD (43). Clinicians therefore mainly relay on the patient’s history. As the pathophysiology of ICD largely concerns the damage to the skin barrier, and ICD pathogenesis differs from ACD, biomarkers that reflect these two mechanistic pathways may potentially be used to discriminate between the two diseases.

### Atopic dermatitis

Atopic dermatitis (AD) has a multifactorial pathogenesis, and the focus in AD research has long been on the immune component of the disease, with T cell activation as the main player in the pathophysiology and skin barrier changes being an epiphenomenon (53). Th2 cells are predominant in the acute phase, whereas chronic lesions are driven by a Th1 response. Other T cell populations (e.g. Th17 and Th22) are also detectable in the skin, leading to the increased local production of cytokines (Fig. 1). After Palmer et al. showed in 2006 that loss-of-function mutations in the gene encoding for the epidermal protein filaggrin (FLG) predisposes for AD, the skin barrier became a new field of interest in AD research (54). Filaggrin is a protein formed from a large precursor protein (pro-filaggrin) by post-translational hydrolysis. During the maturation of keratinocytes, filaggrin monomers aggregate the keratin filaments that make up the cytoskeleton of the keratinocytes and corneocytes. This matured keratin cytoskeleton structure is of great importance.
Filaggrin also contributes indirectly to the SC structure by regulating skin hydration. In the SC, filaggrin is degraded into hygroscopic amino acids and their derivatives, which include trans-urocanic acid (UCA) and pyrrolidine carboxylic acid (PCA). These degradation products are important constituents of collectively called “natural moisturizing factor” (NMF). As the name implies, NMF retains water in the SC and thus contributes to its structural integrity. Furthermore, the acidic components of the NMF regulate the pH of the SC and maintain its slight acidity, which is important for the antibacterial properties of the SC and the activity of various stratum corneum proteases (56). The important role of NMF in the pathophysiology of AD and its easy accessibility for sampling (it resides in the SC) makes NMF a good candidate for the evaluation of skin barrier in general and therapy efficacy in AD in particular.

**Diagnosis**
The diagnosis of AD is primarily based on clinical and anamnestic features, which are defined in the Hanifin and Rajka criteria, later updated by the UK working party (57, 58). To assess disease severity, several clinical scoring systems are used (such as SCORAD and EASI) (1, 7, 11). However, these scoring systems often include subjective information from the patient and the interpretation of the practitioner. Furthermore, they cannot reveal subclinical adverse effects of a therapy, such as skin barrier impairment. Objective biomarkers might overcome this obstacle. Most studies on biomarkers focus on the immune component of AD. Thijs et al. recently evaluated immune mediators and found that the chemokine “serum thymus and activation-regulated chemokine” (TARC/CCL17) correlated strongest to disease severity and is thus also a candidate for the assessment of therapy efficacy (59).

In general, in most of these studies blood or skin biopsies were used to determine biomarkers, although it has been recognized that they are less suitable for use in the field and there is a strong need for noninvasive alternatives (60).

**Objectives**
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 obtained from the stratum corneum (SC). To address this, the following objectives and their corresponding research questions were formulated:
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)

Thesis Outline

To provide an insight into existing biomarkers for ACD and ICD, Chapters 2.1 and 2.2 present two comprehensive non-systematic literature reviews. A variety of molecules and morphological parameters involved in skin barrier and immune response function are addressed. Chapters 3.1 and 3.2 present the results of a study in which various SC biomarkers for distinguishing ACD from ICD were evaluated. In Chapter 3.1, the skin barrier biomarkers, including NMF, stratum corneum proteases, and morphological changes of the corneocyte surface, are explored. Chapter 3.2 focuses on immune mediators.

To investigate which skin barrier and immune related biomarkers can be used for the monitoring of therapy in atopic dermatitis, three clinical studies were conducted. Chapter 4.1 addresses a novel biomarker of skin barrier based on the measurement of corneocyte surface morphology in relation to filaggrin deficiency. Chapter 4.2 reports on a randomized clinical trial that used NMF, skin barrier function parameters, and disease severity to assess therapy efficacy. The efficacy of a ceramide–magnesium cream was compared to that of a low-potency corticosteroid cream and a lipid-rich emollient in patients with mild to moderate AD. In Chapter 4.3, the changes in the profiles of a large number of immune mediators and their
relation to disease severity are described for one of the treatments. Chapter 4.4 describes a comparison of two techniques that determine the concentration of NMF in patients with AD. Chapter 5 presents the general discussion of this work, as well as the conclusions and guidance for future research and practice in this field.
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