Stratum corneum biomarkers for inflammatory skin diseases

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Link to publication

Citation for published version (APA):

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Current knowledge on biomarkers of allergic contact dermatitis

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(Contact Dermatitis. 2017 May 12: (Epub ahead of print))
ABSTRACT

Contact sensitization is common and affects up to 20% of the general population. The clinical manifestation of contact sensitization is allergic contact dermatitis. This is a clinical expression which is sometimes difficult to distinguish from other types of dermatitis, e.g. irritant and atopic dermatitis. Several studies have examined the pathogenesis and severity of allergic contact dermatitis by measuring the absence or presence of various biomarkers. In this review article, we provide a non-systematic overview of biomarkers which have been studied in allergic contact dermatitis. These include genetic variations and mutations, inflammatory mediators, alarmins, proteases, immunoproteomics, lipids, natural moisturizing factors, tight junctions, and antimicrobial peptides. We conclude that despite the enormous amount of data, convincing specific biomarkers for allergic contact dermatitis are yet to be described.
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACD</td>
<td>allergic contact dermatitis</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin-converting enzyme</td>
</tr>
<tr>
<td>AD</td>
<td>atopic dermatitis</td>
</tr>
<tr>
<td>AMP</td>
<td>antimicrobial peptide</td>
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<tr>
<td>CLA</td>
<td>cutaneous leukocyte antigen</td>
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<tr>
<td>CS</td>
<td>contact sensitization</td>
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<tr>
<td>DAMP</td>
<td>damage-associated molecular pattern</td>
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<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DNCB</td>
<td>2,4-dinitrochlorobenzene</td>
</tr>
<tr>
<td>FLG</td>
<td>Flaggrin gene</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione-S-transferase</td>
</tr>
<tr>
<td>HMGB1</td>
<td>high-mobility group box-1 protein</td>
</tr>
<tr>
<td>HAS</td>
<td>human serum albumin</td>
</tr>
<tr>
<td>ICD</td>
<td>irritant contact dermatitis</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>LC</td>
<td>Langerhans cell</td>
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<tr>
<td>LCE3</td>
<td>late cornified envelope-3</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MCI</td>
<td>methylchloroisothiazolinone</td>
</tr>
<tr>
<td>MI</td>
<td>methylisothiazolinone</td>
</tr>
<tr>
<td>MMP-12</td>
<td>matrix metalloproteinase-12</td>
</tr>
<tr>
<td>NA T</td>
<td>N-acetyltransferase</td>
</tr>
<tr>
<td>NMF</td>
<td>natural moisturizing factor</td>
</tr>
<tr>
<td>PPD</td>
<td>p-phenylenediamine</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SC</td>
<td>stratum corneum</td>
</tr>
<tr>
<td>SNP</td>
<td>single-nucleotide polymorphism</td>
</tr>
<tr>
<td>SERPIN</td>
<td>serine protease inhibitor</td>
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<tr>
<td>TEWL</td>
<td>transepidermal water loss</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>Th T</td>
<td>helper</td>
</tr>
<tr>
<td>TJ</td>
<td>tight junction</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
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<tr>
<td>Treg</td>
<td>regulatory T cell</td>
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<tr>
<td>ZO</td>
<td>zonula occludens</td>
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INTRODUCTION

Contact sensitization (CS), the underlying pathomechanism of allergic contact dermatitis (ACD), is highly prevalent, affecting up to 20% of the general population in European countries (1). When a sensitized individual is re-exposed to the culprit contact sensitizer in sufficient concentrations, ACD occurs at the site of skin exposure. Although numerous contact sensitizers exist, they have different physicochemical properties, resulting in different abilities to penetrate the epidermal barrier, bind to proteins, and elicit an inflammatory response (2).

Little is currently known about individual factors that can affect the clinical response to contact sensitizers (3, 4). However, exposure to some allergens, for example preservatives and fragrances, is very common, but causes CS in only a minority of exposed persons, whereas exposure to other contact sensitizers, such as poison ivy, causes CS in most individuals (5, 6). Obviously, to increase our understanding, the mechanisms underlying CS need to be elucidated for a range of contact sensitizers with different physicochemical properties and allergenic potencies. Ideally, such insights will result in the development of biomarker profiles, which can be used to differentiate between the various contact sensitizers, and possibly even between the response to a contact sensitizer and that to an irritant substance. Traditionally, biomarker research in CS has been focused on immune mediators such as cytokines and chemokines, and only recently have studies on proteins involved in skin barrier homeostasis, xenobiotic metabolism and cellular stress responses been conducted.

This non-systematic review on biomarkers was initiated by a working group of international experts who met on several occasions to discuss the aetiology of, and susceptibility to, occupational skin diseases, including ACD. The framework was based on a grant donated by the European Cooperation in Science and Technology (COST) Action StanDerm (TD-1206) to increase research in occupational skin disease (www.standerm.eu). In this article, we provide an extensive overview of the pathogenesis of ACD by summarizing the main findings on the phenotypic and genotypic biomarkers in ACD, which, in the future, may be used for diagnostic purposes, the identification of susceptible individuals, and the development of more tailored prevention and therapy. A biomarker was defined by the World Health
Organization international programme on chemical safety biomarkers in risk assessment ‘as any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease’ (7).

**Inflammatory Mediators**

Although the induction and elicitation of ACD normally represent two distinct and separate phases of the disease, they may sometimes occur during the same exposure. For clarity, the phases are here described separately.

**Sensitization phase**

An essential step in the sensitization process is the activation of the innate immune system by contact sensitizers. Because of their low molecular weight and polarity, and sometimes facilitated by pre-existing skin barrier dysfunction, contact sensitizers can penetrate the stratum corneum (SC) of the epidermis and either covalently bind to or, in the case of metal ions, form complexes with endogenous proteins. The formation of such sensitizer–protein complexes (see ‘Immunoproteomics’ for further details) is crucial for the activation of the innate immune system, and for the efficient priming of T cells (8, 9). Another signal for efficient sensitization is the generation of alarmins, which are danger signals that induce immune responses. These include damage-associated molecular patterns (DAMPs), which are sensed by so-called pattern recognition receptors (PRRs) such as the Toll-like receptors (TLRs) (see ‘Alarmins’ for further details). Interestingly, recruited T helper (Th) 1 cells have been found to release significant quantities of the DAMP molecule extradomain A+ fibronectin, which is an endogenous ligand of TLR4. This triggers a positive-feedback mechanism that further reinforces immune activation in ACD (10, 11).

Keratinocytes are key players in the sensitization phase, as they contain enzymes that are required for the conversion of prohapten into biologically active hapten, thereby facilitating their binding to endogenous proteins and making them immunogenic (12). Keratinocytes also provide sets of alarmins and cytokines that generate a proinflammatory microenvironment in the skin, which is necessary for innate immune system activation. Some alarmins activate TLR2, TLR4, and the NLRP3 inflammasome of skin dendritic cells (DCs) such as Langerhans cells (LCs) and dermal DCs, leading to their activation (2).
TLR2 and TLR4 activation induces the production of nuclear factor-κB-dependent proinflammatory cytokines and chemokines such as interleukin (IL)-6, IL-12, and tumour necrosis factor (TNF)-α, and of pro-IL-1β and pro-IL-18. The activated NLRP3 inflammasome complex activates caspase-1, which cleaves pro-IL-1β and pro-IL-18 into their mature and secreted forms, IL-1β and IL-18 (2, 13). Mice that lack components of the inflammasome complex, or the ATP-triggered P2X7 receptor, which can activate the inflammasome, fail to develop ACD. In the same context, the IL-1 receptor antagonist anakinra has been shown to prevent CS (14, 15). Notably, P2X7R-deficient mice became susceptible again following injection of recombinant IL-1β (15), implying that IL-1β and the inflammasome are crucial in priming adaptive immunity.

Secreted IL-1β and IL-18 induce keratinocytes to release IL-1α, TNF-α and granulocyte–macrophage colony-stimulating factor, and promote LC migration from the epidermis (16). IL-1α has been shown to have a marked effect on skin sensitization, as ear swelling in response to 2,4,6-trinitrobenzenesulfonic acid is impaired in IL-1α-deficient mice, but not in IL-1β-deficient mice (17). Whereas IL-1β is mainly produced by LCs, keratinocytes are the main source of IL-1α. This implies that IL-1α is required for the induction of skin sensitization, whereas IL-1β plays an important role in LC migration.

Activated DCs upregulate costimulatory molecules. Exposure to sensitizers (nickel, chromium, copper and 2,4-dinitrochlorobenzene (DNCB)) upregulates CD83, CD86 and the chemokine CXCL8 (IL-8) in monocyte-derived DCs, whereas irritant exposure leads to decreased CXCL8 production (18). DC activation as measured by induction of CD86, CXCL8 or CD54 is used in in vitro assays for CS identification, such as the human cell line activation test (THP-1 cells) (OECD guideline test 442E) and the peripheral blood monocyte-derived DC assay (19, 20).

Activated DCs migrate to the skin-draining lymph nodes and present contact sensitizers in the context of major histocompatibility complex (MHC) molecules to naïve T cells. In the dermis, endothelial and lymphatic cells produce CCL19 and CCL21. These chemokines are recognized by the upregulated CCR7 chemokine receptor of sensitizer-activated DCs, which migrate to afferent lymphatic vessels (21,
DC migration has been measured in MUTZ3-LCs in vitro. Whereas migration of irritant-treated MUTZ-LCs was dependent on CCR5, contact sensitizer treatment induced CXCR4 upregulation and CXCL12-dependent dermal migration. CXCL12 can be secreted by, for example, keratinocytes (23, 24).

The activation of sensitizer-specific naive T cells by activated DCs in the skin-draining lymph nodes is the crucial step and concludes the sensitization phase (21, 22). Upon activation, T cells produce IL-2, which is a T cell growth factor, resulting in abundant T cell expansion (22). Moreover, they receive instructive signals from the skin DCs, resulting in the expression of a combination of homing receptors, that is, chemokine receptors and adhesion molecules, that directs them to the skin.

The immunological microenvironment (comprising the amount of sensitizer, danger signals, and other soluble mediators) determines the final phenotype of effector T cells. In the skin-draining lymph nodes, sensitizer-activated DCs produce IL-12 and interferon (IFN)-γ, promoting the differentiation of Th1 and Tc1 cells, which release IFN-γ and TNF (22, 25). The microenvironment containing IL-6, transforming growth factor (TGF)-β, IL-21, IL-23 and IL-1β leads to Th17/22 polarization and the production of IL-17 and IL-22. The presence of IL-4 leads to Th2 polarization and subsequent IL-4, IL-5 and IL-13 production. IL-2 and TGF-β in the microenvironment promote the differentiation of regulatory T cells (Tregs), which secrete immunosuppressive IL-10, an important cytokine that limits the extent and duration of ACD and promotes tolerance (22, 26, 27). Moreover, in addition to driving the cytokine polarization of T cells, DCs from skin induce the expression of a skin-specific T cell-homing receptor profile (e.g. cutaneous leukocyte antigen (CLA), CCR4, and CCR10) in skin-draining lymph nodes (25, 28). CLA binds to E-selectin on dermal endothelial cells, whereas CCR4 and CCR10 receptors promote T cell migration to the epidermis, where keratinocytes produce the corresponding chemokines CCL17 and CCL27, as well as CXCL8, CXCL9, CXCL10, CXCL11, and adhesion molecules (intercellular adhesion molecule-1) (22). As a result, primed T cells will home into the tissue of origin of the corresponding DCs, that is, the skin. In addition, these chemokines attract more immune cells to the exposed skin area, thereby strengthening the immune responses (29). It has been speculated that the strength of the innate inflammation caused by the contact
sensitizer is responsible for the immunogenic or tolerogenic state of DCs and the subsequent effector/memory T cell/Treg ratio (13). Both sensitizing and tolerizing pathways are induced during sensitization, and the balance of these pathways determines the final outcome (27).

**Elicitation phase**

Effector T cells specific for a contact sensitizer are recruited into the skin upon contact with the same sensitizer. Upon re-exposure to the contact sensitizer, the innate inflammatory response triggers the release of cytokines (IL-1β, TNF-α, and IL-18) from keratinocytes and LCs (21, 22). In fact, keratinocyte activation can be measured by IL-18 production in the human keratinocyte cell line activation test (NCTC2544) (30). IL-18 causes activated DCs to mature and migrate. Endothelial cells are activated (expressing, for example, E-selectin), and the contact sensitizer-specific T cells (expressing, for example, CLA) infiltrate the skin (13, 22). T cell-attracting chemokines (CXCL9/10, CCL17, CCL20, and CCL27) are produced by keratinocytes.

Keratinocytes are also important in the elicitation phase of ACD, because, upon re-exposure, they upregulate costimulatory molecules such as CD80, and are able to function as antigen-presenting cells, facilitating activation of hapten-specific effector T cells (22). On the other hand, keratinocytes also suppress the immune response by secreting LL-37 (cathelicidin), which inhibits hyaluronan-induced cytokine release, and the immunosuppressive cytokine IL-10 (21, 25).

Skin-infiltrating T cells release IFN-γ, IL-4, IL-17, and TNF-α (21, 25, 31). IFN-γ-activated keratinocytes upregulate their adhesion molecules and cytokines/chemokines, increasing the recruitment of T cells, natural killer cells, macrophages, mast cells and/or eosinophils to the site of sensitizer exposure, promoting the killing of sensitizer-bearing cells (31). With time and repeated contact sensitizer exposure, a Th2 response begins to dominate the ACD reaction (22).

The identification of specific combinations of cytokines and chemokines as biomarkers that are unique to ACD is challenging. These mediators are commonly also found in other inflammatory conditions. However, it is tempting to hypothesize
that the distinction between irritant contact dermatitis (ICD) and ACD could be made on the basis of T cell-related factors, as ICD does not involve antigen-specific T cells (32, 33). Interestingly, CXCL9, CXCL10 and CXCL11 were recently found to be selectively upregulated in human skin in nickel-induced ACD as compared with atopic dermatitis (AD) (34).

Alarmins

In addition to secretion of cytokines, skin keratinocytes and other skin cells have the capacity to regulate immune responses through the production of alarmins, which are molecules that activate the immune system and represent danger signals. These include DAMPs (35, 36). Alarmins include structurally diverse and evolutionarily unrelated multifunctional endogenous molecules, including DNA, RNA, uric acid, ATP, reactive oxygen species (ROS), mitochondrion-derived molecules, haem, and several intracellular proteins (high-mobility group box-1 protein (HMGB1), IL-33, IL-1α, heat shock proteins, S100 proteins, and antimicrobial peptides (AMPs)) (37). Many of the alarmins are passively released upon cellular stress, damage, or necrotic cell death. Once released extracellularly, some alarmins promote activation of both innate immune cells, including antigen-presenting cells through PRRs, such as TLRs, and other receptors. Interestingly, alarmins are able to initiate, amplify and sustain the inflammatory responses even in absence of external pathogens, causing sterile inflammation (38).

Alarmins play a key role in the pathogenesis of different inflammatory skin diseases, including ACD (35, 39). One route in the sensitization phase is the generation of low molecular weight alarmins (ROS and uric acid) in keratinocytes upon exposure to contact sensitizers (40). The stressed keratinocytes start to express a set of alarmins such as HMGB1, calgranulins (S100A8/S100A9), and LL-37 (41-43). Upon continuous exposure to cellular stress, these primary intracellular proteins are released and continue to amplify the innate immune responses via activation of TLR2, TLR4, TLR9 and receptor of advanced glycation end products, leading to the generation of IL-1 family cytokines (IL-1α, IL-1β, IL-18, IL-33, and IL-36) (44). The balance between proinflammatory and anti-inflammatory cytokines of the IL-1 family is crucial in human ACD pathogenesis (44). Interestingly, for more efficient stimulation of cells, some of the alarmins can undergo post-translational
modifications, and can form immunostimulatory complexes with cytokines and other endogenous and exogenous factors, including self-DNA (45).

Despite the well-established role of alarmins in the pathogenesis of ACD, their use as biomarkers to distinguish different types of skin inflammatory conditions is questionable, as most of these markers are common inflammatory mediators and cannot be used as specific disease-associated markers. However, the level of alarmins correlates with disease activity, and they can be used as reliable markers to detect local inflammatory activities and to predict the disease outcome (46, 47).

**Proteases**

Proteases are currently classified into six broad groups based on their catalytic domain: serine proteases, cysteine proteases, aspartate proteases, threonine proteases, glutamic acid proteases, and metalloproteases (48). In the skin, various proteases contribute to a protease/protease inhibitor balance. Exogenous proteases are derived from bacteria, fungi, or viruses. Local endogenous proteases comprise, for example, kallikreins, caspase-14, and prostasin, and are tightly controlled by local serine protease inhibitors such as lympho-epithelial Kazal-type-related inhibitor, (SERPINs, or cystatins) (49). Identified protease targets include structural proteins such as filaggrin, and cytokines and receptors that are involved in epidermal barrier function, the immune response, and/or antimicrobial defence mechanisms. More specifically, serine proteases are critical for epidermal barrier homeostasis, and aberrant expression and/or activity have been associated with AD in human studies (50). Airborne proteins such as the cysteine peptidase Der p1 produced by house dust mites and cockroaches have the ability to penetrate into the epidermis and exacerbate AD (51, 52). Those show innate proteolytic activity on the skin, and can thus directly contribute to barrier impairment and increased local inflammation (53). A role for mannose receptor-positive M2 macrophages, by producing matrix metalloproteinase12 (MMP-12), in the development of contact hypersensitivity has been shown (54). The authors suggest that MMP-12 activity is required to trigger skin inflammation, presumably through the induction of chemokine expression. Expression of the cysteinyl-aspartate-specific proteinase caspase-14 is reduced in skin biopsies from patients with ACD, further supporting its role in inflammatory skin conditions (55). Mouse models of experimentally induced ACD have shown a
regulatory role of protease-activated receptor-2 during skin inflammation and the immune response (56, 57). Disruption of tight junction (TJ) morphology associated with cleavage of zonula occludens (ZO)-1 and occludin has been reported, although a second study showed, rather, initiation of apoptosis independently of TJ proteolysis (58, 59). Overall, although the involvement of proteases in ACD is becoming increasingly evident, their role as promising biomarkers for ACD still remains to be confirmed.

**Genetic Markers**

Despite similar exposures to contact sensitizers, some individuals develop CS resulting in ACD, whereas others are spared. Genetic factors may modify this individual susceptibility. Polymorphisms in several candidate genes have been studied (3, 60, 61), as they may influence the individual immune responses, skin barrier function, or metabolizing capacities (online table S1). The TNFA-308A allele causes increased production of the proinflammatory cytokine TNF-α, and was found more frequently in patients with CS to a para-substituted aryl compound and at least one more unrelated contact sensitizer (62). This single-nucleotide polymorphism (SNP) was additionally associated with an increased risk of ICD (63-65), and thus could have an impact on the development of CS via unspecific trigger factors, as suggested by the ‘danger model’ (66). It was also significantly linked to the risk of severe generalized dermatitis caused by trichloroethylene, and CS to p-phenylenediamine (PPD) (67, 68) and chromium (69). However, no effect on susceptibility to CS to a para-substituted aryl compound and at least one more unrelated contact sensitizer was found for polymorphisms in the genes encoding IL-1β, IL-1 receptor antagonist, and IL-6 (62). In contrast, the IL16-295*C/C genotype was significantly overrepresented among individuals with CS to a para-substituted aryl compound and at least one more unrelated contact sensitizer (70), whereas the CXCL11*A/A genotype (rs6817952) was associated with polysensitization, defined as reaction to three or more unrelated sensitizers (71). A link was found between SNPs in the gene encoding the immunosuppressive cytokine IL-10 (IL10-1082G→A and IL10-819C→T) and CS to parthenium (72). No association was found between IL4-590 polymorphism and CS to chromium (69). Angiotensin-converting enzyme (ACE) cleaves substance P, β-endorphins and other peptides with immunomodulatory functions, and thus modulates the inflammatory response.
to allergens, but not to irritants. Insertion polymorphisms in ACE were associated with an increased risk of CS to PPD (73).

An impaired skin barrier function may facilitate the penetration of contact sensitizers, and thus the development of CS (3, 60, 61). Molin et al. reported an association between ACD on the hands and combined deletions in genes encoding late cornified envelope-3 (LCE3B and LCE3C) (74). Moreover, SNPs in the gene encoding the TJ claudin-1 were associated with CS to fragrances and nickel in individuals without ear piercings (75). The effect of filaggrin gene (FLG) loss-of-function mutations on the development of CS is controversial. Mutations in FLG have been associated with combined ICD and ACD of the hands in dermatitis patients (76). However, no associations between FLG mutations and CS were found in a small twin sample or in patients with multiple allergies (77-79). In contrast, in a cohort of individuals with AD and recurrent hand eczema, FLG mutations conferred a strongly increased risk of CS to sensitizers other than nickel, probably indicating that chronic and/or severe dermatitis is associated with barrier deficiency and increased topical exposures (80). An association between FLG mutations and CS to nickel has been reported in individuals with a history of intolerance to fashion jewellery and in individuals without piercing (81, 82). In a cohort of patients with occupational contact dermatitis of the hands, FLG mutations were associated with CS to lanolin alcohol (83). Individuals with AD and FLG mutations have a higher prevalence of CS to ethylenediamine and neomycin than wild-type carriers without AD (84). However, the high prevalence of CS to substances commonly found in topical preparations could be related to the increased use of such products by FLG mutation carriers, because of dry or inflamed skin.

Individuals may differ in their ability to activate or detoxify contact sensitizers upon skin exposure, possibly because of polymorphisms in genes encoding xenobiotic-metabolizing enzymes (3, 60, 61). Several studies have investigated SNPs in the gene encoding the enzyme glutathione-S-transferase (GST). A higher risk of CS to chromium was found in individuals with the GST-T1 null genotype (69). The prevalence of combined GSTT1 and GST-M1 deletions was more frequent in individuals with CS to thiomersal than in healthy controls (85). However, others could not confirm associations between SNPs in GST and CS (75, 86). Some
studies have focused on SNPs in genes encoding the metabolizing enzymes N-acetyltransferase (NAT)1 and NAT2, which have been linked with ‘rapid’ and ‘slow’ acetylator phenotypes (3, 60, 61). Carriers of the rapid NAT2*4 allele showed increased susceptibility to CS to para-substituted aryl compounds, including PPD (87). The slow acetylator phenotype associated with NAT2*5b/2*6a was significantly less common in the disease group. Smaller studies on the effects of SNPs in NAT2 supported the notion that a rapid acetylator phenotype may increase the risk of CS to PPD (88, 89). Even though N-acetylation is generally regarded as a detoxifying reaction, it may also result in transformation of para-substituted aryl compounds or their intermediates into stronger haptens, which may explain the reported increased risk of sensitization in ‘rapid’ acetylators. However, others reported that the rapid acetylator NAT1*10 allele was less frequent in patients with CS to PPD (90). No association was found between two polymorphisms (ALA-9Val and Ile58Thr) in the gene encoding manganese superoxide dismutase and the risk of CS to PPD (91).

Even though the results of the reviewed studies indicate the influence of genetic factors on susceptibility to CS, several limitations should be addressed (3, 60, 61). The pathogenesis of CS is complex and not completely understood. Most likely, a combination of environmental and genetic factors is involved, which may differ according to the contact sensitizer. Thus, the results can probably not be generalized. Many studies are further compromised by their small sample sizes. Moreover, inadequate definition and selection of cases and controls may limit the value of the results. The candidate gene approach is based on a pathogenic hypothesis, which may be misleading. The functional role of the selected polymorphisms is not always proven. Moreover, it is possible that the investigated genetic variation may not be directly involved in CS, but is rather genetically linked to an unknown susceptibility factor or to a concomitant disease such as AD. Therefore, further studies in much larger cohorts are warranted, in which stratification by other linked disorders is better accounted for. An overview of all genetic biomarkers is shown in online Table S1.

**Gene expression in contact sensitizer identification**

Contact sensitizers are being tested by the use of cell lines and reconstructed human epidermis models to develop in vitro assays for contact sensitizer identification (92,
93). For example, DCs derived from CD34+ cord blood progenitors MUTZ-3DC cells, HaCaT keratinocytes and the Episkin model are being used in gene expression profiling studies (94-97). These studies provide insights into the early events in the sensitization process. Metabolic processes, oxidative stress and cell cycling are triggered by contact sensitizers. One of the most prominent pathways that has been identified in this and other studies is the Keap1/Nrf2-dependent antioxidant phase 2 response, which is present in all cell types (98). Contact sensitizers can covalently bind to critical cysteine residues in the cytosolic protein Keap1, which is a sensor for oxidative and electrophilic stress. Keap1 normally ubiquitinylates the transcription factor Keap1, and thereby marks it for degradation by the proteasome. Upon modification by contact sensitizers, Nrf2 is no longer degraded, and it translocates into the nucleus, where it drives the expression of antioxidant response element-containing genes after its association with cofactors. These include genes that regulate glutathione-mediated redox homeostasis. Knockout mice lacking Nrf2 can be sensitized with lower concentrations of contact sensitizers, and ACD can even be induced with weak contact sensitizers that do not induce sensitization in wild-type mice (99).

Biomarkers related to this contact sensitizer-triggered response can be identified in, for example, DCs, and may be very useful (100). An in vitro test for contact sensitizer identification, the KeratinoSens™ assay, has been developed and was recently validated (OECD guideline test 442D) (101).

One important piece of information that is still missing is the extent of the overlap of the contact sensitizer-induced gene expression profiles with irritants, some of which may also engage pathways triggered by contact sensitizers. The extent of specificity of these profiles for contact sensitizers will only become evident when a large panel of sensitizers and irritants has been tested. It may well be that it is difficult to identify a general gene profile that unequivocally identifies all contact sensitizers. Owing to the different physicochemical properties and reaction mechanisms of the few thousands of chemicals that can cause ACD, there may be a need to identify ‘class-specific’ profiles.
Recent studies have addressed the changes in gene expression by using RNA microarrays in human skin treated with contact sensitizers or affected by inflammatory skin diseases such as atopy or psoriasis. Dhingra et al. analysed skin biopsies from pet.-reactive and sensitizer-reactive patches of 24 individuals 72h after the application of different contact sensitizers in a patch test (102). They identified a common ACD transcriptome that comprised 149 genes for all tested sensitizers as compared with pet. Even more genes relating to innate immunity, T cell trafficking and T cell subset polarization were differentially expressed when different contact sensitizers were compared. The authors emphasized different types of immune polarization with respect to Th1/Th17, Th22 and Th2 components for nickel, fragrance, and rubber. Quaranta et al. performed gene expression profiling with human skin samples from 24 individual patients simultaneously affected by psoriasis and non-atopic or AD lesions, thus avoiding problems of interindividual variability (34). In addition, eczematous skin from patients with nickel-induced ACD was included. Lesional skin was compared with autologous unaffected skin. Differentially expressed genes were associated with the immune response, AMPs, skin barrier and epidermal differentiation, and metabolism. There were single genes and signalling pathways that were common to the different skin diseases, as well as disease-specific ones. A set of 15 selected genes was then tested as a disease classifier for diagnosis in an independent patient cohort. Reverse transcription polymerase chain reaction analysis was performed with biopsies from the lesional skin. The classifier was able to correctly diagnose the relevant skin disease. When naturally occurring AD and nickel-induced ACD were compared, 172 genes were regulated only in ACD, 28 only in naturally occurring eczema, and 33 in both types of eczema. Whereas epithelial antimicrobial response genes (S100 family, some keratins) were regulated similarly, genes regulating epithelial differentiation, such as genes of the small proline-rich and LCE families, were regulated differently, and genes associated with an acute immune response were significantly regulated only in ACD. These were, for example, inflammasome-related genes such as those encoding IL-1β and AIM2, as well as those encoding neutrophil-attracting and Th1-associated chemokines. Most interestingly, NOS2 and CCL27 were identified as molecular classifiers that allow differentiation between psoriasis and eczema (103).
These interesting studies show that there are disease-specific gene signatures and gene signatures common to different inflammatory skin diseases. For ACD, common and contact sensitizer-specific gene signatures have been found. However, a larger panel of chemicals must be tested before general conclusions can be drawn. Nevertheless, these studies can be used to identify disease-specific classifiers for improved molecular diagnosis.

**Immunoproteomics**

As stated previously, the current concept of ACD implies direct sensitizer–protein interactions followed by antigen processing and immune recognition. This process is known as haptenation or hapten binding to self-proteins, or immunotoxico logically as a molecular initiating event (2, 104-107). This emphasizes that human self-proteins are essential sensitizer targets and important co-regulators in the disease’s pathogenesis. Even though self-proteins/peptides may significantly trigger sensitizer-specific T cell epitope generation, little is known so far about sensitizer-specific T cell epitopes. Thus, it is still unclear which role cryptic self-epitopes, cross-reactions or the p-I concept may have in this process (108, 109). Specifically for metal sensitizers such as nickel and beryllium, several clonal T cell epitopes have been described (110-113). However, because the reactions are of a polyclonal nature, a higher number of molecular epitopes for each single sensitizer has to be taken into account.

One potential physiological sensitizer target protein is human serum albumin (HSA), a multifunctional high molecular weight blood protein (∼69,000 Da) that is also present in human sweat and skin (114). Many important skin sensitizers have been shown to interact specifically with HSA, such as nickel, DNCB, PPD, and methylisothiazolinone (MI), whereas fragrances such as cinnamal, citronellol and eugenol have been shown to interfere with the related xenogeneic bovine serum albumin (115-122). Furthermore, some of these sensitizer–carrier-albumin molecules may become immunologically active and affect sensitizer-specific human T cell clone activation by, for example, by transferring nickel to the T cell receptor–MHC interface or by generating still unknown MI-specific T cell epitopes (120, 122, 123). It is remarkable that similar results were obtained with nickel bound to human transferrin, usually known as iron carrier, indicating several distinct parallel mechanisms in nickel-specific polyclonal T cell activation (124).
Fig. 1. Potential protein targets of human contact allergen nickel in human skin possibly co-triggering the immune response e.g. by affecting epitope generation and/or metabolic processes. Functional annotation cluster of nickel-binding proteins from human keratinocytes (y-axis) displays relationship to functionally similar terms (x-axis; enrichment score 9.93, all with significant p-values) like stress responses, chaperone and unfolded protein binding or ATP binding and nucleotide binding (green - corresponding protein/gene-term association positively reported; black - corresponding protein/ gene-term association not reported yet) CTHBP, cytosolic thyroid, hormone-binding protein; IFN, interferon; TCP1, T-complex protein 1; THBP1, thyroid hormone-binding 1; TNF, tumour necrosis factor.
To further determine sensitizer–protein interactions in human skin and elucidate potential early mechanisms of haptenation and/or direct or indirect sensitizer-dependent metabolic disproportion, we have investigated nickel–protein interactions in human antigen-presenting cells and human keratinocytes by using proteomic technologies (S. Ohnesorge pers. comm. 2017) (125). On application of the database for annotation, visualization and integrated discovery (DAVID) 6.7 to nickel-interacting proteins detectable in human keratinocytes, functional annotation clustering showed 24 annotation clusters, with cluster 1 showing similar terms, such as stress response, chaperone and unfolded protein binding or ATP and nucleotide binding, as those associated with one subgroup of nickel-binding skin molecules (Fig. 1) (S. Ohnesorge pers. comm. 2017) (126). Thus, combining immunoproteomic interaction analyses with nickel-specific human T cell clone reactions and nickel-specific activated keratinocytes will provide new molecular insights into the basic mechanisms of ACD, including hapten epitope generation, and innate inflammatory responses or metabolic pathways affected by reactive small molecules or sensitizing metals such as nickel.

**Structural Elements of the Epidermis**

**Lipids**

The sensitization and elicitation phases of ACD are concentration-dependent phenomena, and the skin barrier possibly influences the threshold concentration of a contact sensitizer for provoking an immune response (127). The permeability function of the skin largely depends on the spatial organization and composition of the three major SC lipid classes: ceramides, free fatty acids, and cholesterol (128). The depletion of these or alteration of their relative compositions results in reduced skin barrier function (129). In addition to their barrier function, SC lipids and their precursors and metabolites also play an important role in epidermal signalling and modulation of innate immunity (130). It is likely that aberrant lipid composition will facilitate the skin penetration of sensitizers, particularly if these are water-soluble. However, studies addressing a relationship between SC lipids and ACD are scarce. Jungersted et al. found no difference in the ceramide profile in non-lesional skin between patients with ICD and ACD on the hands and patients with hyperkeratotic hand eczema (131). So far, there are no other studies that have investigated the SC
lipids as a susceptibility parameter for ACD. Therefore, future studies are needed to shed more light on the role of the skin lipids in ACD, including their contribution to barrier function and epidermal signalling.

**Natural moisturizing factors (NMFs)**

Filaggrin and its degradation products, which contribute to a pool of hygroscopic compounds collectively called NMFs, affect the structure and composition of the SC, which is the principal barrier of the skin (132). The levels of NMFs in the SC can be affected by both genetic and environmental factors, with the loss-of-function mutations in FLG being as a major determinant (133). Theoretically, NMF deficiency could influence the development of ACD in different ways. The enhanced skin permeability will increase the likelihood that the threshold concentration of the contact sensitizer for inducing sensitization or elicitation will be reached. Percutaneous absorption can also be affected by the binding of a sensitizer to the SC. It has recently been shown that filaggrin chelates nickel, which might lower the amount of nickel that penetrates across the SC into viable epidermis (134). Increased SC penetration of trivalent chromium was shown in filaggrin-deficient mouse skin (135), and carriers of FLG mutations have an increased risk of nickel-induced sensitization as compared with wild-type carriers (81, 82, 136). However, Ross-Hansen et al. did not find a difference in the dose–response relationship for nickel elicitation between FLG mutation carriers and non-carriers in a small pilot study (134). Another limitation of that study was that the sensitization dose, which is known to substantially influence the dermatitis reaction, was not taken into account (137).

In contrast to ICD and AD, little is known about the effect of the cytokine and chemokine milieu in ACD on the epidermal filaggrin and NMF levels. Howell et al. showed that the expression of filaggrin is downregulated in AD, owing to Th2-mediated inflammation (138-142). Kezic et al. (142) also showed that NMF levels are lower in AD patients than in healthy controls, and that the decrease in NMF levels was associated with disease severity. As many contact sensitizers also show Th2 inflammatory responses, it might be expected that the NMF levels in ACD would be reduced (102). In a study by Koppes et al. the NMF levels were decreased after patch testing with methylisothiazolinone/methylchloroisothiazolinone
(MCI), but not after patch testing with nickel, PPD, and chromium, although all investigated contact sensitizers induced similar clinical responses (143). As skin irritants markedly decrease NMF levels, it might be speculated that the reduction in NMF levels after patch testing with MI/MCI is caused by the irritant properties of this sensitizer (139-141, 143).

To summarize, there are very few studies that have addressed the role of NMFs in ACD. As the effect of sensitizers on the NMF levels proved to be sensitizer-specific, it might be interesting to further investigate this phenomenon with more contact sensitizers.

**Tight junctions**

TJs are cell–cell junctions that are composed of transmembrane proteins (e.g. claudins 1–24, occludin, tricellulin, and junctional adhesion molecules A–C) and cytoplasmic plaque proteins (e.g. ZO proteins 1–3 and cingulin). The definite composition depends on the cell type, the differentiation state, and physiological and non-physiological stimuli (144). TJs have been shown to form a functional paracellular barrier to hydrophilic molecules with molecular weights of ≥557 and lanthanum in the granular cell layer of the epidermis (145). For molecules with molecular weights of <557 and other ions, experimental data are still missing; however, because of the barrier to lanthanum, a barrier for these molecules/ions could also be hypothesized. In addition, TJ proteins are also found in other epidermal layers, which means outside of TJ structures, with a characteristic distribution pattern for each protein (145). Besides their barrier function, TJs and/or TJ proteins have been shown to be involved in proliferation, differentiation, apoptosis, and cell–cell adhesion (146).

Little is known about TJs in ACD. A general population study showed a genetic correlation of CS and Cldn-1 SNPs (75). In a mouse model of allergic dermatitis, Cldn-1 is downregulated. In this study, an increase in TJ permeability could be shown for molecules with molecular weights of 557–5000, whereas there was no change for molecules with molecular weights of ~30000 (147). Again, smaller molecules have not been tested. In conclusion, more research on TJ proteins in ACD, including the proof of barrier function for molecules with molecular weights of <557 and ions other than lanthanum, is needed to elucidate their role in this cutaneous disease. In
general, TJ-dependent and TJ-independent functions of TJ proteins are of interest. In addition, it will be of special interest whether different patterns of TJ protein alterations can be seen in the different kinds of dermatitis, and thus whether these proteins may help to distinguish between the different entities AD, ACD, and ICD.

**Antimicrobial Peptides**

AMPs are small cationic peptides that are produced predominantly in the epidermis, and transported to the SC, where they play a vital role in the skin barrier. They act as multifunctional effector molecules, with broad antimicrobial activity (148, 149) as well as immune-modulating properties, linking the innate and adaptive immune responses (150-152). In healthy skin, a low constitutive level of AMPs provides a defence against microbial pathogens. During infection or injury to the skin, upregulation will take place to create a stronger antibacterial shield and to modulate the immunological response.

Increased levels of AMPs are found after tape-stripping of both healthy skin and non-lesional AD (153-155). Furthermore, the expression of LL-37 is important for barrier recovery in murine studies, in which knockout mice lacking murine LL-37 show significant delays in barrier recovery (156).

Not much is known about the role of AMPs in relation to CS. In vivo expression studies of AMPs from skin biopsies have shown increased protein levels of LL-37 in ACD patients as compared with healthy controls and AD patients (157), and decreased protein levels of elafin and human β-defensin-2, but increased mRNA levels, in ACD patients as compared with AD patients (158). Interestingly, murine studies have shown LL-37 to have ‘anti-inflammatory’ properties that down-modulate ACD in vivo. In knockout mice, the ACD response was enhanced in the absence of LL-37 (159, 160).

Despite there being few studies on the expression of AMPs in ACD, roles for AMPs in modulating skin inflammation and in the recovery of barrier function seem plausible. The importance of AMPs in skin conditions such as AD and psoriasis is well reported (154, 161-163), and their use as biomarkers for local inflammation and disease severity is credible. To fully understand their role in inflammatory skin
conditions such as ACD and their role in maintaining an optimal skin barrier and modulating the immune response, more research is needed in this field.

**Bioengineering Parameters**

ACD is characterized by cellular infiltration and reactivity in the skin. The responsiveness and degree of sensitization in the individual to whose skin a contact sensitizers is applied are also important factors determining the magnitude of the response. Contact sensitizers penetrate the epidermis, most often without harming the barrier significantly, and then induce an inflammatory response that leads to secondary skin barrier impairment (164). The barrier defect measured as increased transepidermal water loss (TEWL) in ACD is primarily explained by the inflammatory response. As stated before, an impaired skin barrier function will necessarily increase the risk of sensitization and elicitation of ACD. In line with this, combined exposure to irritants and sensitizers is known to significantly augment the response as assessed by measurement of TEWL and erythema (165).

Bioengineering methods are useful for quantification of allergic skin reactions, and may be used to follow up reactions over time in experimental studies, and to quantitatively study the kinetics of the pathophysiology of ACD reactions in vivo (166). Although the response to some irritants with direct barrier-harming effects, such as detergents, may easily be differentiated from allergic reactions by measurement of TEWL, bioengineering methods cannot generally differentiate between allergic and irritant skin reactions (167). ACD mainly causes inflammation, and bioengineering methods directed at assessment of blood flow or oedema may be even more suitable than TEWL for assessment.

Measurement of both TEWL and erythema may be useful for quantification of ACD, in particular patch test responses, and both methods have been widely used for this purpose (165, 168, 169).
CONCLUDING REMARKS

The prevalence of ACD in the general population is high. In the framework of COST Action StanDerm, we have reviewed several potential biomarkers, such as inflammation mediators, skin barrier function, and genetic susceptibility markers, and methods to be used in the quantification of ACD (for an overview, see Table S2). Even though the biomarkers presented can be used in certain ways in the diagnosis of ACD, to assess the severity of ACD, or to identify ACD-susceptible individuals, the last of these being very challenging, our review also highlights the need for future research. For several promising biomarkers for ACD, there are few, and in some cases no, studies. The vast majority of the potential biomarkers mentioned here will most likely characterize inflammatory conditions in general. Specificity for eczematous reactions may be associated with skin-related biomarkers, and specificity for T cell-mediated eczema with biomarkers related to T cell immunity. The most challenging question is whether there are biomarkers that are specific for ACD. These should relate to the unique triggering mechanisms based on the protein reactivity of contact sensitizers. Here, technologies such as genomics and proteomics should be most useful, and promising research in this field is ongoing. With increasing knowledge, we will potentially be able to provide a mapping of biomarkers to enhance ACD diagnosis and identify susceptible individuals, and this may also be applicable in everyday clinical practice. Our review addresses topics to be investigated further with the goal of preventing the development of ACD.

Acknowledgements
This work was supported by COST Action TD1206 StanDerm and, in part, by a grant from the European Commission as part of the project ‘Novel Testing Strategies for In Vitro Assessment of Allergens (Sens-it-iv’) (LSHB-CT-2005-018681 to H.-J. Thierse and S. Ohnesorge).

Supporting Information
Additional Supporting Information may be found in the online version of this article: Table S1. Genetic susceptibility markers for contact sensitization. Table S2. Summary of studied molecular biomarkers relevant for allergic contact dermatitis.
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