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Current knowledge on biomarkers for contact sensitization and allergic contact dermatitis

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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.

Keywords: Allergic contact dermatitis, Biomarkers, contact allergy, contact sensitization

List of abbreviations.

ACD	Allergic contact dermatitis
ACE	Angiotensin-converting enzyme
AD	atopic dermatitis
AMP	antimicrobial peptide
ARE	antioxidant response element
CLA	cutaneous leukocyte antigen,
CLDN1	claudin-1
COST	Cooperation in Science and Technology
CS	contact sensitization
DAMP	damage associated molecular pattern
DCs	dendritic cells
DNCB	2,4-dinitrochlorobenzene
FLG	filaggrin gene
GST	glutathione-S-transferase
HMGB1	high-mobility group box-1 protein
HSA	human serum albumin
ICD	irritant contact dermatitis
IFN	interferon
IL	interleukin
LC	Langerhans cell
LCE3	late cornified envelope
LEKT-I	Lympho-epithelial Kazal-type-related inhibitor
MCI	methylchloroisothiazolinone
MI	methylisothiazolinone
MMP-12	metalloproteinase-12
MR	mannose receptor
NAT	N-acetyltransferase
NMF	natural moisturizing factors
PAR-2	protease-activated receptor

PELI-1	pellino homolog
PPD	*p*-phenylenediamine
RaGE	receptor of advanced glycation end products
SC	stratum corneum
SNP	single nucleotide polymorphism
SERPIN	serine protease inhibitors
TEWL	transepidermal water loss
TJ	tight junction
TLRs	Toll like receptors
TNBS	2,4,6-trinitrobenzenesulfonic acid
TNF	tumor necrosis factor
Tregs	regulatory T cells
ZO	zonula occludens

1. 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. While numerous contact sensitizers exist, they have different physico-chemical properties, resulting in a different ability to penetrate the epidermal barrier, bind to proteins, and elicit an inflammatory response (2).

Little is currently known about individual factors, which can affect the clinical response to contact sensitizers (3, 4). However, exposure to some allergens is very common, e.g. to preservatives and fragrances, but only causes CS in 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 understanding, the mechanisms underlying CS needs to be elucidated for a range of contact sensitizers with different physico-chemical properties and allergenic potencies. Ideally, such insight may result in 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 an irritant substance. Traditionally, biomarker research in CS has been focused on immune mediators such as cytokines and chemokines, and only recently studies on proteins involved in skin barrier homeostasis, xenobiotic metabolism and cellular stress responses have been conducted.

This non-systematic review article on biomarkers was initiated by a working group of international experts who met over on several occasions to discuss the aetiology and susceptibility to occupational skin disease, 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, identification of susceptible individuals, and

development of more tailored prevention and therapy. A biomarker was defined by the WHO international program 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).

2. Inflammatory mediators

While 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.

2.1 Sensitization phase

An essential step in the sensitization process is the activation of the innate immune system by contact sensitizers. Due to 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 as well as for the efficient priming of T cells (8, 9). Another signal for efficient sensitization is the generation of alarmins, 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 alarmin section for further details). Interestingly, recruited Th1 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 required for the conversion of pro-haptens into biologically active haptens, thereby facilitating their binding to endogenous proteins and making them immunogenic (12). Keratinocytes also provide sets of alarmins and cytokines that generate a pro-inflammatory microenvironment

in the skin, which is necessary for innate immune system activation. Some alarmins activate TLRs 2 and 4 and the NLRP3 inflammasome of skin dendritic cells (DC) such as Langerhans cells (LC) and dermal DCs, leading to their activation (2).

TLR2 and TLR4 activation induces the production of NF-κB dependent pro-inflammatory cytokines and chemokines such as interleukin(IL)-6, IL-12, tumor necrosis factor (TNF)-α and of pro-IL-1β and pro-IL-18. The activated NLRP3 inflammasome complex activates caspase-1 that 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 contact sensitization (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 GM-CSF 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 (TNBS) is impaired in IL-1α deficient mice, but not in IL-1β deficient mice (17). Whereas IL-1β is mainly produced by Langerhans cells, 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 co-stimulatory 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 human Cell Line Activation Test (hCLAT, THP-1 cells) (OECD guideline test 442E) and peripheral blood monocyte-derived DC (PBMDC) assay (19, 20).

Activated DCs migrate to the skin-draining lymph nodes and present contact sensitizers in the context of 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, 22). DC migration has been measured in MUTZ3-LCs *in vitro*. While migration of irritant treated MUTZ-LC was dependent on CCR5, contact sensitizer treatment induced CXCR4 upregulation and CXCL12 dependent dermal migration. CXCL12 can be secreted by e.g. keratinocytes (23, 24).

The activation of sensitizer-specific naïve 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, i.e. 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 (25) (22). The microenvironment containing IL-6, tumor growth factor (TGF)-β, IL-21, IL-23, IL-1β leads to Th17/22 polarization and production of IL-17 and IL-22. 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 differentiation of Tregs, which secrete immunosuppressive IL-10, an important cytokine limiting extent and duration of ACD and promoting 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 while CCR4 and CCR10 receptors promote T cell migration to the epidermis where keratinocytes produce the corresponding chemokines CCL17, CCL27 as well as CXCL8, CXCL9, CXCL10 and CXCL11 and adhesion molecules (ICAM-1) (22). As a result primed T cells will home into the tissue of origin of the corresponding DCs, i.e. 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 tolerising pathways are induced during sensitization and the balance of these pathways determines the final outcome(27).

2.2 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, 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 e.g. E-selectin), and the contact sensitizer-specific T cells (expressing e.g. CLA) infiltrate the skin (13, 22). T cell-attracting chemokines (CXCL9/10, CCL17, CCL20, CCL27) are produced by keratinocytes.

Keratinocytes are important in the elicitation phase of ACD as well, since 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, NK 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 found also in other inflammatory conditions. However, it is tempting to hypothesize that the distinction between irritant contact dermatitis (ICD) and ACD could be made based on T cell related factors since ICD does not involve antigen specific T cells (32, 33). Interestingly, CXCL9, 10

and 11 were recently found to be selectively upregulated in human skin in nickel-induced ACD as compared to atopic dermatitis (AD) (34).

3. 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; molecules which activate the immune system and represent danger signals. These include DAMPs (35, 36). Alarmins include structurally diverse and evolutionary unrelated multifunctional endogenous molecules, including DNA, RNA, uric acid, ATP, ROS, mitochondria derived molecules, heme and several intracellular proteins [high-mobility group box-1 protein (HMGB1), interleukins IL-33 and IL-1α, heat shock proteins, S100 proteins and antimicrobial peptides (AMPs)] (37). Many of the alarmins are passively released upon cellular stress, damage, or by 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, 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 (RAGE), leading to generation of IL-1 family cytokines (IL-1α, IL-1β, IL-18, IL-33 and IL-36) (44). The balance between pro- and anti-inflammatory cytokines of IL-1 family members 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 usage as biomarkers to distinguish different types of skin inflammatory conditions is questionable, since 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).

4. 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 derive from bacteria, fungi or viruses. Local endogenous proteases comprise e.g. kallikreins, caspase-14 and prostasin, and are tightly controlled by local serine protease inhibitors like e.g. LEKT-I, SERPINs, or cystatins (49). Identified protease targets include structural proteins such as filaggrin, cytokines and receptors that are involved in the epidermal barrier function, 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 exhibit innate proteolytic activity on the skin and can thus directly contribute to barrier impairment and increased local inflammation (53). A role for mannose receptor (MR)-positive M2 macrophages are demonstrated in the development of contact hypersensitivity by producing the metallo-proteinase MMP12(54). The authors suggest that MMP12 activity is required to trigger skin inflammation presumably through the induction of chemokine expression. The cysteinyl-aspartate-specific proteinase caspase 14 expression is reduced in skin biopsies from patients with ACD further supporting its role in inflammatory skin conditions (55). Mouse models of experimentally induced ACD demonstrate a regulatory role of the protease-activated receptor PAR-2 during skin inflammation and 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 rather revealed initiation of apoptosis independently of TJ

proteolysis (58, 59). Overall, although the implication of proteases in ACD becomes increasingly evident, their role as promising biomarkers for ACD still remains to be confirmed.

5. Genetic markers

Despite similar exposures to contact sensitizers, some individuals develop CS resulting in ACD, while 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 (Table 1). The *TNFA-308A* allele confers an increased production of the pro-inflammatory cytokine TNF-α and was found more frequently in patients with CS against a *para*-substituted aryl compound and at least one more unrelated contact sensitizer (62). This single nucleotide polymorphism (SNP) was additionally associated with increased risk of irritant contact dermatitis (63-65) and thus could have an impact on development of CS via unspecific trigger factors as suggested by the 'danger model' (66). It was also significantly linked to the risk for severe generalized dermatitis to trichloroethylene as well as 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 (RA) 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), while 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 function and thus, modulates the inflammatory response to allergens, but not to irritants. Insertion polymorphisms in the *ACE* gene 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 the late cornified envelope (LCE3B and LCE3C) (74). Moreover, SNPs in the gene encoding the tight junction claudin-1 (CLDN1) 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 association between *FLG* mutations and CS was found in a small twin sample and 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 for CS to sensitizers other than nickel, likely 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). Similarly, compared to wild-type carriers without AD, individuals with AD and *FLG* mutations have a higher prevalence of CS to ethylenediamine and neomycin (84). However, the high prevalence of CS to substances commonly found in topical preparations could be related to an increased use of such products by *FLG* mutation carriers due to dry or inflamed skin.

Individuals may differ in their ability to activate or detoxify contact sensitizers upon skin exposure, which may be due to polymorphisms in genes encoding xenobiotic metabolizing enzymes (3, 60, 61). Several studies 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 *GST-T1* 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 the *GST* gene and CS (75, 86). Some studies have focused on SNPs in genes encoding the metabolizing enzymes N-acetyltransferase (*NAT*) 1 and 2, which have been linked with 'rapid' and 'slow' acetylator phenotypes (3, 60, 61). Carriers of the rapid *NAT2*4* allele showed an 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 effect of SNPs in the *NAT2* gene supported the notion that a rapid acetylator phenotype may increase the risk for 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 CS to PPD (90). No association was found between two polymorphisms (*ALA-9Val* and *Ile58Thr*) in the gene encoding manganese superoxide dismutase (MnSOD) and the risk for CS to PPD (91).

Even though the results of the reviewed studies indicate the influence of genetic factors on the susceptibility for 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 depending on the contact sensitizer. Thus, the results can likely not be generalized. Many studies are further compromised by their small sample size. Moreover, an 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 bigger cohorts are warranted where stratification by other linked disorders is better accounted for. An overview of all genetic biomarkers can be found in our online supplementary table 1.

5.2 Gene expression in contact sensitizer identification

Contact sensitizers are being tested using 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-3 DC progenitor cells, HaCaT keratinocytes and the Episkin model are being used in gene expression profiling studies (94-97). These studies give insight 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; a sensor for oxidative and electrophilic stress. Keap1 normally ubiqitinylates the transcription factor Keap1 and thereby marks it for degradation by the proteasome. Upon modification by contact sensitizers, Nrf2 is no longer degraded and translocates into the nucleus where it drives the expression of antioxidant response element (ARE) containing genes after its association with cofactors. These include genes, which 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 which do not induce sensitization in wildtype mice (99).

Biomarkers related to this contact sensitizer-triggered response can be identified e.g. in 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 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. Due to the different physicochemical properties and reaction mechanisms of the few thousands of chemicals that can cause ACD, there may be the need to identify “class-specific” profiles.

Recent studies have addressed the changes in gene expression by 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 petrolatum- and sensitizer-reactive patches of 24 individuals 72 h after application of different contact sensitizers in a patch test (102). They identified a common ACD transcriptome that comprised 149 genes for all tested sensitizers compared to petrolatum. Even more genes relating to innate immunity, T cell trafficking and T cell subset polarisation 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 avoiding problems of inter-individual variability (34). In addition, eczematous skin from nickel-induced ACD was included. Lesional skin was compared to autologous unaffected skin. Differentially expressed genes were associated with the immune response, AMP, skin barrier and epidermal differentiation, and metabolism. Identified single genes and signalling pathways 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. RT-PCR analysis was performed using biopsies from the lesional skin. The classifier was able to correctly diagnose the relevant skin disease. When comparing naturally occurring AD and nickel-induced ACD, 172 genes were only regulated in ACD, 28 only in naturally occurring eczema and 33 genes were regulated in both types of eczema. While epithelial antimicrobial response genes (S100 family, some keratin) were regulated similarly, genes regulating epithelial differentiation such as genes of the SPRR (small proline-rich) and late cornified envelope (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 IL-1β, AIM2 as well as 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 reveal 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.

6. 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 immunotoxicologically as 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,

only 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 like nickel and beryllium, several clonal T cell epitopes have been described (110-113). Yet, since reactions are of 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 represented by human serum albumin (HSA), a multifunctional high molecular weight blood protein (69 kD) also occurring in human sweat and skin (114). Many important skin sensitizers have been demonstrated to interact specifically with HSA, such as nickel, DNCB, PPD and methylisothiazolinone (MI) while fragrances like 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 e.g. by transferring nickel to the TCR/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).

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 proteomic technologies (Ohnesorge et al., in preparation) (125). By applying the database for annotation, visualization and integrated discovery (DAVID) 6.7 to nickel-interacting proteins detectable in human keratinocytes, functional annotation clustering revealed 24 annotation clusters, and with cluster 1 showing similar terms like stress response, chaperone and unfolded protein binding or ATP and nucleotide binding associated with one sub-group of nickel-binding skin molecules (see figure 1) (Ohnesorge et al. in preparation) (126). Thus, combining immunoproteomic interaction analyses with nickel-specific human T cell clone reactions and nickel-specific activated keratinocytes will give new molecular insights into basic mechanisms of ACD, including

hapten epitope generation, and innate inflammatory responses or metabolic pathways affected by reactive small molecules or sensitizing metals like nickel.

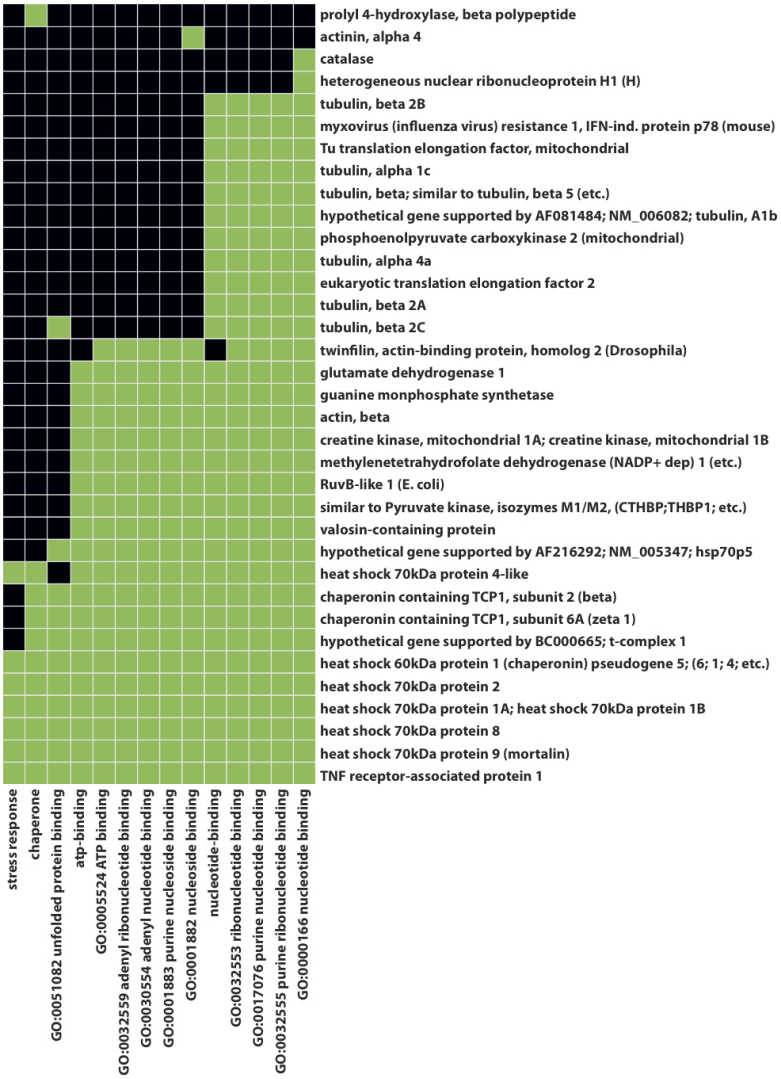

Figure. 1: *Potential protein targets of human contact allergen nickel in human skin possibly co-triggering the immune response e.g. by affecting epitope generation and/or metabolic processes. Functional annotation cluster of nickel-binding proteins from human keratinocytes (y-axis) displays relationship 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).

7. Structural elements of the epidermis

7.1 Lipids

The sensitization and elicitation phase of ACD are concentration-dependent phenomena, and the skin barrier possibly influences the threshold concentration of a contact sensitizer to provoke 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 or alteration of the relative composition of these lipid classes results in a 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 may facilitate skin penetration of sensitizers, in particular if these are water soluble. However, studies addressing a relationship between SC lipids and ACD are scarce. Jungersted et al. found no difference in 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 which 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 for ACD, including their contribution to the barrier function as well as epidermal signalling.

7.2 Natural moisturizing factors

Filaggrin and its degradation products which are contributing to a pool of hygroscopic compounds collectively called natural moisturizing factors (NMF) affect the structure and composition of the SC, the principal barrier of the skin (132). The levels of NMF in the SC can be affected by both genetic and environmental factors with the loss-of-function mutations in *FLG* as a major determinant (133). Theoretically, NMF deficiency can influence development of ACD in different ways. The enhanced skin permeability will increase the likelihood that the threshold concentration of the contact sensitizer to induce sensitization or elicitation will be reached. The 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 mice skin (135), and carriers of *FLG* mutations have an increased risk of nickel-induced sensitization compared to wild-type carriers (81, 136, 137). However, Ross-Hansen et al. did not find a difference in the dose-response relationship in Ni-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 largely influence dermatitis reaction has not been taken into account (138).

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, due to Th2 mediated inflammation (139-143). Kezic et al. (143) showed also that NMF levels are lower in AD patients as compared to healthy controls and that the decrease in NMF was associated with disease severity. As many contact sensitizers show (also) Th2 inflammatory responses, it might be expected that the NMF levels in ACD are reduced (144). In a study by Koppes et al., the NMF levels were decreased after patch testing with methylisothizolinone/methylchloroisothiazolinone (MI/MCI), but not after nickel, PPD and chromium although all investigated contact sensitizers induced similar clinical responses (145). As skin irritants markedly decrease NMF levels, it might be speculated that the NMF reduction after MI/MCI is caused by irritant properties of this sensitizer (140-142, 145).

To summarize, there are very few studies which have addressed the role of NMF 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.

7.3 Tight Junctions

TJs are cell-cell junctions that are composed of transmembrane proteins (e.g. claudins 1-24, occludin, tricellulin, junctional adhesion molecules A-C) and cytoplasmic plaque proteins (e.g. ZO proteins 1-3, cingulin). The definite composition depends on the cell type, differentiation state and physiologic and non-physiologic stimuli (146). TJs have been shown to form a functional paracellular barrier to hydrophilic molecules ≥557 Da and lanthanum in the granular cell layer of the epidermis (147). For molecules smaller than 557 Da and other ions experimental data are still missing, however, because of the barrier function to

lanthanum a barrier also for these molecules / ions could 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 (147). Besides barrier function, TJs and/or TJ proteins have been shown to be involved in proliferation, differentiation, apoptosis and cell-cell adhesion (148).

Little is known about TJs in ACD. A general population study demonstrated 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 of TJ permeability could be shown for molecules from 557-5000 Da while there was no change for molecules around 30 kDa (149). Again, smaller molecules have not been tested. In conclusion, more research on TJ proteins in ACD, including the proof of barrier function for molecules smaller than 557 Da and other ions 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.

8. Antimicrobial peptides

AMPs are small cationic peptides, 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 a broad antimicrobial activity (150, 151) as well as immune modulating properties, linking the innate and adaptive immune response (152-154). In healthy skin, a low constitutive level of AMPs provides a defence against microbial pathogens. During infection or injury to the skin, up-regulation will take place to create a stronger antibacterial shield as well as modulate the immunological response.

Increased levels of AMPs are found after tape-stripping of healthy skin as well as of non-lesional skin of AD (155-157). Furthermore, the expression of LL-37 is important for barrier recovery in murine studies, where knockout mice missing murine LL-37 display significant delay in barrier recovery (158).

Not much is known about the role of AMPs in relation to CS. In vivo expression of AMPs from skin biopsies have shown increased protein levels of LL-37 in ACD compared to healthy

controls and AD (159), and decreased protein levels of elafin and human beta defensin-2, but higher mRNA levels, in ACD compared to AD (160). Interestingly, murine studies have found LL-37 to have 'anti-inflammatory' properties that down-modulate ACD in vivo. Using knockout mice, ACD response was enhanced in the absence of LL-37 (161, 162).

Despite few studies on the expression of AMPs in ACD, a role for AMPs in modulating skin inflammation as well as in recovery of barrier function seems plausible. The importance of AMPs in skin conditions like AD and psoriasis is well reported (156, 163-165), and their use as biomarkers for local inflammation and disease severity is credible. To fully understand their role in inflammatory skin conditions like ACD and their role in maintaining an optimal skin barrier and modulating the immune response, more research is needed in this field.

9. Bioengineering parameters

ACD is characterized by cellular infiltration and reactivity in the skin. The responsiveness and degree of sensitization in the individual to whom a contact sensitizer is applied on the skin is also an important factor determining the magnitude of the response. Contact sensitizers penetrate the epidermis, most often without harming the barrier significantly, and then induce an inflammatory response which leads to secondary skin barrier impairment (166). The barrier defect measured as increased transepidermal water loss (TEWL) in ACD is primarily explained by the inflammatory response. As stated before, 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 (167).

Bioengineering methods are useful for quantification of allergic skin reactions, and may be used to follow-up on reactions over time in experimental studies, and to quantitatively study the kinetics of the pathophysiology of ACD reactions in vivo (168). Although the response to some irritants with direct barrier-harming effects like detergents may easily be differentiated from allergic reactions by measurement of TEWL, bioengineering methods cannot generally differentiate between allergic and irritant skin reactions (169). 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 (167, 170, 171).

10. 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 overview see online supplement Table 2). Even though the biomarkers presented can can be used in certain ways in the diagnosis of ACD, to assess the severity of ACD or to identify ACD susceptible individuals, the latter 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 for T cell–mediated eczema with biomarkers related to T cell immunity. The most challenging question is if 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, maybe applicable also for everyday clinical practice. Our review addresses topics to be investigated further in the goal of preventing development of ACD.

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