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S.A. KOPPES STRATUM CORNEUM BIOMARKERS FOR INFLAMMATORY SKIN DISEASES

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STRATUM CORNEUM BIOMARKERS FOR INFLAMMATORY SKIN DISEASES

S.A. KOPPES

AMSTERDAM
MMXVII

Stratum corneum biomarkers for inflammatory skin diseases

The studies described in this thesis were carried out at the Academic Medical Center, University of Amsterdam, Department: Coronel Institute of Occupational Health, Amsterdam, The Netherlands and the Department of Dermatology, VU University Medical Center, Amsterdam, the Netherlands.

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STRATUM CORNEUM BIOMARKERS FOR INFLAMMATORY SKIN DISEASES

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Contents

Chapter 1	**General introduction**	**9**
Chapter 2	**Biomarkers for contact dermatitis, literature reviews**	
2.1	Current knowledge on biomarkers of allergic contact dermatitis *(Contact Dermatitis. 2017 May 12: (Epub ahead of print))*	25
2.2	Current knowledge on biomarkers of irritant contact dermatitis (*under review*)	59
Chapter 3	**Biomarkers for contact dermatitis, experimental studies**	
3.1	Effect of allergens and irritants on levels of NMF and corneocyte morphology *(Contact Dermatitis. 2017 May;76(5):287-295)*	91
3.2	Stratum corneum profiles of inflammatory mediators in patch test reactions to common contact allergens and sodium lauryl sulfate *(British Journal of Dermatol. 2016 Nov 4: (Epub ahead of print))*	111
Chapter 4	**Biomarkers for atopic dermatitis, clinical studies**	
4.1	Filaggrin breakdown products determine corneocyte conformation in patients with atopic dermatitis *(J Allergy Clin Immunol. 2015 Dec;136(6):1573-80)*	129

4.2	Efficacy of a cream containing ceramides and magnesium in the treatment of mild to moderate atopic dermatitis: a randomized, double-blind, emollient- and hydrocortisone-controlled trial (*Acta Derm Venereol. 2016 Nov 2;96(7):948-953)*	145
4.3	Stratum corneum tape stripping: monitoring of inflammatory mediators in atopic dermatitis patients using topical therapy *(International Archives of Allergy Immunology 2016;170(3):187-93)*	163
4.4	Determination of natural moisturizing factors in the skin: Raman microspectroscopy versus HPLC (*Biomarkers. 2017 Jan 19:1-6)*	179
Chapter 5	**General discussion and conclusion**	197
Summary		213
Nederlandse samenvatting		221
About the author	Curriculum vitae	231
	Portfolio	233
	List of publications	235
Dankwoord		237

CHAPTER 1

General Introduction

GENERAL INTRODUCTION

Our skin acts as a barrier to the outside world. This barrier function mainly resides in the stratum corneum (SC), the uppermost layer of the skin. This layer is the body's first line of defense, and it is vulnerable to external treats such as physical insults, and irritating or allergenic substances. Furthermore, some individuals have an intrinsically compromised skin barrier due to their genetic makeup. An impaired skin barrier is known to play a major role in the pathophysiology of contact dermatitis, a dermatosis that is frequently seen in the occupational setting. There are two types of contact dermatitis: allergic contact dermatitis (ACD) and irritant contact dermatitis (ICD) (1, 2). In the USA, 15.2% of all cases of nonfatal occupational illnesses reported in 2014 were occupational skin disorders (OSDs) (3). Most of these cases concerned occupational contact dermatitis (OCD), a term that refers to ACD and ICD induced by work activities (4). Recent data from the Netherlands show that 71% of all reported OSD cases were a form of OCD (mainly ICD)(5). These disorders have a major impact on the quality of life of patients in general and on their work ability in particular (6). Almost half of workers with OCD have a history of atopic dermatitis (AD), an inflammatory skin disease that is characterized by an impaired skin barrier (7-9). Strong attenuation of the risk for OCD by AD and its high prevalence (7.2% of the general population in US adults in 2015) underlines its high relevancy when studying OCD10. In the workplace, ACD, ICD, and AD often coincide. As they share the same clinical features, the diagnostics is challenging (11). Accurate diagnosis of OCD and identifying the causative agent(s) are both of major importance: When diagnostics are not adequate and timely, OCD can become a chronic disease that causes extensive suffering for patients and has a large socioeconomic impact, such as sustained and prolonged sick leave, changing of work tasks or job, job loss and long-term unemployment (12, 13). Despite similar clinical and histological characteristics, the underlying pathophysiological mechanism of ACD, ICD, and AD is different. In contrast to direct skin barrier damage induced by an irritant activating innate immunity, ACD and AD are characterized by the induction of antigen-specific effector and memory T cells. A disturbed barrier also plays an important role in ACD and AD and its immune response (Fig. 1). Immune responses in ACD, ICD, and AD are mediated by a large number of immune mediators (Fig. 1). The identification of biomarkers

of the key molecular or cellular events that are specific to ACD, ICD, and AD might assist in diagnostics and therefore the prevention of OSD. A biomarker is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"(14). In addition to diagnostics, biomarkers might also be useful to monitor the efficacy of therapy or other interventions, as currently there is a lack of valid and reliable outcome measures (11, 15). Most of the currently studied biomarkers in these diseases are determined from blood samples or in skin biopsies16. However, blood samples provide information at a systemic level and skin biopsies are invasive (17, 18). SC biomarkers may overcome these drawbacks, as SC can be collected from the skin site of interest in a noninvasive manner.

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

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

in ACD, ICD, and AD, and potentially identify biomarkers that might be useful for diagnostics and therapy monitoring.

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

Stratum corneum

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

corneocyte-based barrier. In addition to the corneocytes, the intercellular lipid bilayers also have a vital role in the skin barrier, especially in water homeostasis. The highly organized lipid bilayers are important in making the skin waterproof. They are comprised of approximately 45–50% ceramides, 25% cholesterol, 10–15% free fatty acids, and 5% other lipids (30).

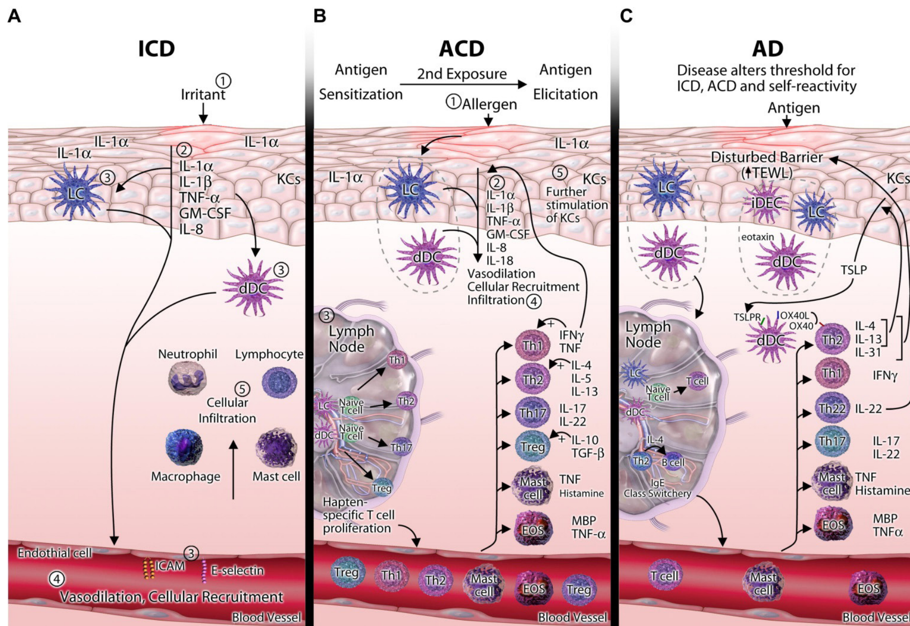

Fig. 1: Inflammatory mediators in ICD, ACD, and AD. Source: Julia K. Gittler, JACI 2013[31]

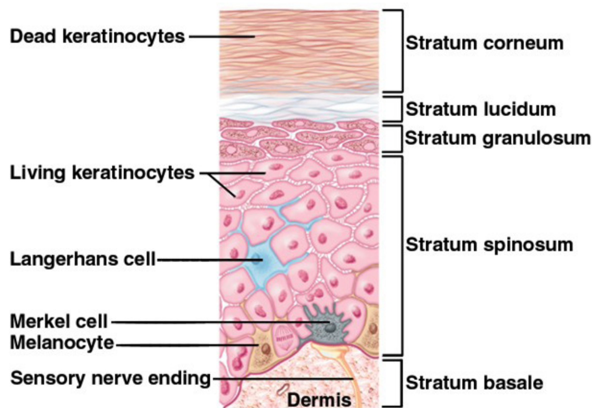

Fig. 2. A cross-section of the epidermis

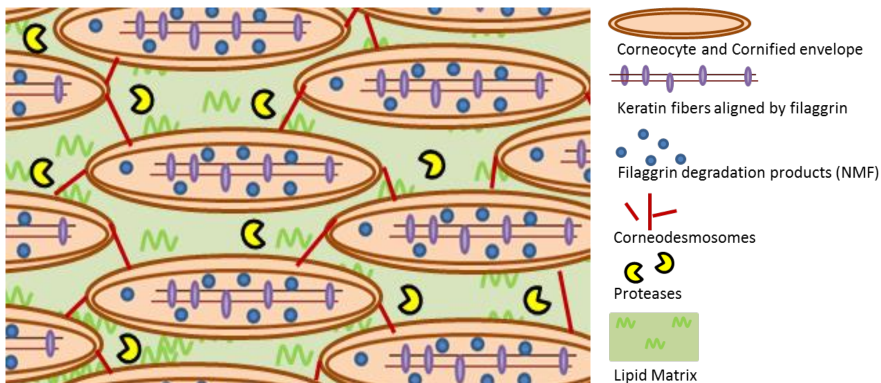

Fig. 3. Representation of the stratum corneum structure

Allergic contact dermatitis (ACD)

Etiology

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

induced proinflammatory cascade and the other mediating proinflammatory processes and the role of the skin barrier are shown in Fig. 1.

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

Diagnosis

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

Irritant contact dermatitis

Etiology

Irritant contact dermatitis (ICD) occurs as a result of a single or cumulative exposure to physical or chemical irritants that react with the proteins of the stratum corneum leading to skin barrier damage. Genetic predisposition and existing barrier dysfunction in the form of AD can increase this risk; workers with AD and a filaggrin loss-of-function (*FLG*- LOF) mutation have a four- to fivefold increased risk for the development of ICD (7, 46). The irritant potency of an agent is dependent on the physio-chemical properties of the irritant, which governs its

interaction with SC components. For example, solvents, soaps, and detergents can affect the SC lipids, structural proteins, and NMF, making the skin dry, less flexible, and prone to damage. Unsurprisingly, workers who frequently use these products, for example nurses, cleaners, and hairdressers, often suffer from OCD. The innate immune response in ICD is rapid and largely localized on the skin site that is in contact with the irritant (47, 48). One of the first responses to skin damage is the release of a preformed pool of IL-1α stored in the corneocytes, initiating a cascade of inflammatory mediators (see Fig. 1) (49). Studies on immune response in ICD focuses mainly on IL-1 cytokines, and data on other immune mediators in the SC are scarce (50, 51). Skin barrier damage has largely been investigated by measuring transepidermal water loss (TEWL) and skin hydration, whereas very few studies have addressed changes in the molecular composition of the SC (52).

Diagnosis

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

Atopic dermatitis

Atopic dermatitis (AD) has a multifactorial pathogenesis, and the focus in AD research has long been on the immune component of the disease, with T cell activation as the main player in the pathophysiology and skin barrier changes being an epiphenomenon (53). Th2 cells are predominant in the acute phase, whereas chronic lesions are driven by a Th1 response. Other T cell populations (e.g. Th17 and Th22) are also detectable in the skin, leading to the increased local production of cytokines (Fig. 1). After Palmer et al. showed in 2006 that loss-of-function mutations in the gene encoding for the epidermal protein filaggrin (FLG) predisposes for AD, the skin barrier became a new field of interest in AD research (54). Filaggrin is a protein formed from a large precursor protein (pro-filaggrin) by post-translational hydrolysis. During the maturation of keratinocytes, filaggrin monomers aggregate the keratin filaments that make up the cytoskeleton of the keratinocytes and corneocytes. This matured keratin cytoskeleton structure is of great importance

for the mechanical strength of the SC (55). Filaggrin also contributes indirectly to the SC structure by regulating skin hydration. In the SC, filaggrin is degraded into hygroscopic amino acids and their derivatives, which include trans-urocanic acid (UCA) and pyrrolidine carboxylic acid (PCA). These degradation products are important constituents of collectively called “natural moisturizing factor” (NMF). As the name implies, NMF retains water in the SC and thus contributes to its structural integrity. Furthermore, the acidic components of the NMF regulate the pH of the SC and maintain its slight acidity, which is important for the antibacterial properties of the SC and the activity of various stratum corneum proteases (56). The important role of NMF in the pathophysiology of AD and its easy accessibility for sampling (it resides in the SC) makes NMF a good candidate for the evaluation of skin barrier in general and therapy efficacy in AD in particular.

Diagnosis

The diagnosis of AD is primarily based on clinical and anamnestic features, which are defined in the Hanifin and Rajka criteria, later updated by the UK working party (57, 58). To assess disease severity, several clinical scoring systems are used (such as SCORAD and EASI) (1, 7, 11). However, these scoring systems often include subjective information from the patient and the interpretation of the practitioner. Furthermore, they cannot reveal subclinical adverse effects of a therapy, such as skin barrier impairment. Objective biomarkers might overcome this obstacle. Most studies on biomarkers focus on the immune component of AD. Thijs et al. recently evaluated immune mediators and found that the chemokine “serum thymus and activation-regulated chemokine” (TARC/CCL17) correlated strongest to disease severity and is thus also a candidate for the assessment of therapy efficacy (59). In general, in most of these studies blood or skin biopsies were used to determine biomarkers, although it has been recognized that they are less suitable for use in the field and there is a strong need for noninvasive alternatives (60).

Objectives

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

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

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

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

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

To investigate which skin barrier and immune related biomarkers can be used for the monitoring of therapy in atopic dermatitis, three clinical studies were conducted. **Chapter 4.1** addresses a novel biomarker of skin barrier based on the measurement of corneocyte surface morphology in relation to filaggrin deficiency. **Chapter 4.2** reports on a randomized clinical trial that used NMF, skin barrier function parameters, and disease severity to assess therapy efficacy. The efficacy of a ceramide–magnesium cream was compared to that of a low-potency corticosteroid cream and a lipid-rich emollient in patients with mild to moderate AD. In **Chapter 4.3,** the changes in the profiles of a large number of immune mediators and their

relation to disease severity are described for one of the treatments. **Chapter 4.4** describes a comparison of two techniques that determine the concentration of NMF in patients with AD. **Chapter 5** presents the general discussion of this work, as well as the conclusions and guidance for future research and practice in this field.

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CHAPTER 2

Biomarkers for contact dermatitis, literature reviews

2.1

Current knowledge on biomarkers of 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.

LIST OF ABBREVIATIONS

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

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 prohaptens 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 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 naive 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. 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 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 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 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 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 72 h 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 immunotoxicologically 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).

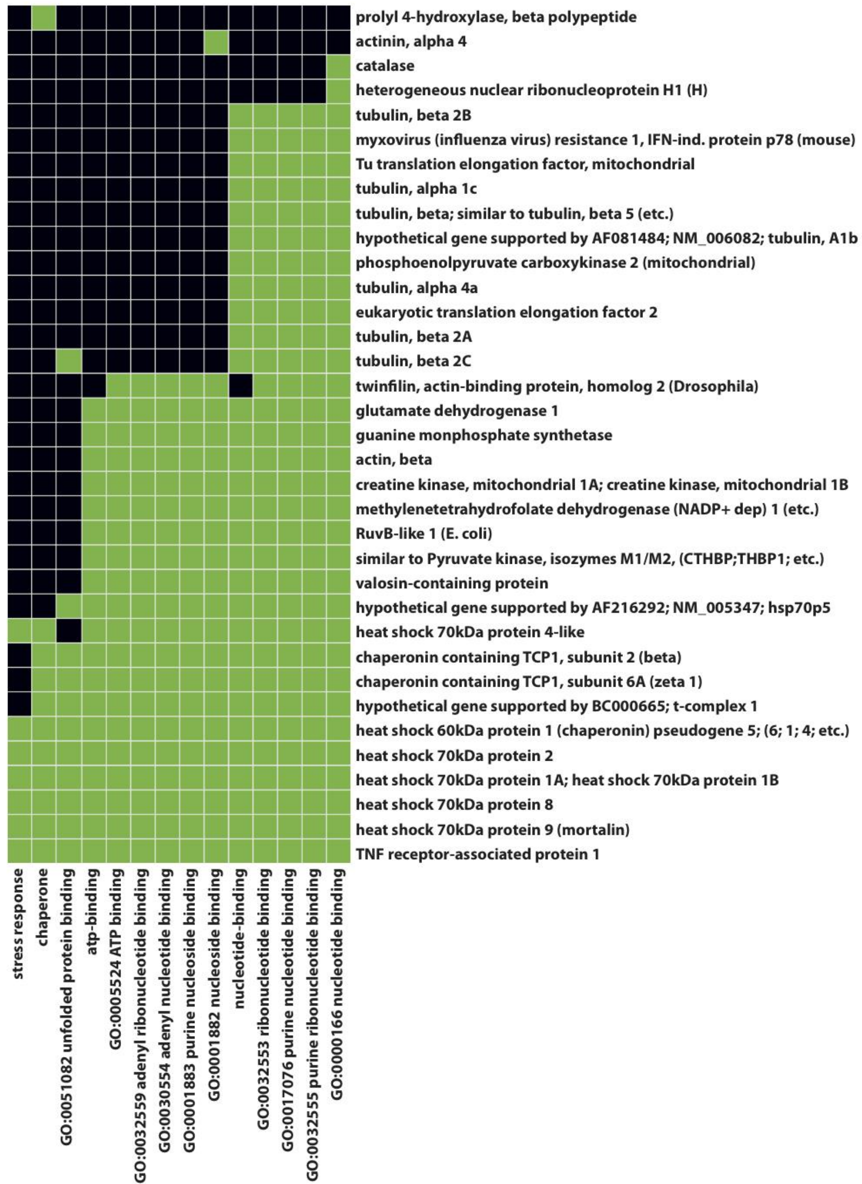

Fig. 1. Potential protein targets of human contact allergen nickel in human skin possibly co-triggering the immune response e.g. by affecting epitope generation and/or metabolic processes. Functional annotation cluster of nickel-binding proteins from human keratinocytes (y-axis) displays relationship 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 methylisothiozolinone/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 ~30 000 (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-37show 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 sensitizer 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.

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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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2.2

Current knowledge on biomarkers of irritant contact dermatitis

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ABSTRACT

The use of biomarkers is a valuable tool in both research and clinical practice to study disease mechanisms, identify susceptible individuals, assess disease severity, prognosis and treatment outcomes. Irritant contact dermatitis (ICD), and its most common clinical manifestation, hand eczema, is a leading cause of occupational skin disease worldwide. ICD has a considerable impact on life quality and may lead to occupational disability as well as the need for occupational retraining. Nevertheless, its pathogenesis remains incompletely understood, and there have been no established biomarkers for improved disease characterization so far. In the present review, we explore different parameters as potential biomarkers for ICD including genetic markers, inflammatory mediators, structural barrier proteins, proteases, lipids and biophysiological measures. The reviewed data point to a need for additional laboratory and patient-oriented clinical research to identify reliable and valid disease biomarkers for ICD.

INTRODUCTION

Irritant contact dermatitis (ICD) is a common inflammatory skin disease as a result of a single or cumulative exposure to physical or chemical irritants. Irritants interact with different components of the skin and elicit a diverse spectrum of reactions, extending from subclinical inflammation or mild sensory responses to severe, widespread disease with systemic involvement (1). ICD is the most frequent form of contact dermatitis and its most common clinical manifestation, hand eczema, is a leading cause of occupational skin disease worldwide. Although robust epidemiological data regarding ICD are scarce, literature-based analysis has shown an estimated point prevalence of hand eczema in the general population of 4.2%, 1-year prevalence of 9.7% and lifetime prevalence of 15% (2). In addition, based on the citation source, up to 80% of the ccluding cases of occupational contact dermatitis have been attributed to ICD (3, 4).

In contrast to allergic contact dermatitis (ACD), the pathogenesis of ICD has been less well studied and understood so far. Following a recent article focusing on ACD (5), in the present publication we aim to review potential biomarkers related to ICD. As for ACD, the present review was initiated by the working group "Etiology and susceptibility to occupational skin disease" of the European COST Action "StanDerm".

Genetic markers

Despite similar exposures some individuals are more prone to develop ICD than others, suggesting the existence of genetic variations affecting the individual response to skin irritants (6, 7). In agreement with this concept, a population-based twin cohort study has shown a higher risk of self-reported hand eczema in monozygotic as compared with dizygotic twin pairs (8). History of atopic skin disease is a known significant risk factor for ICD, however, large scale studies provide evidence that the genetic predisposition to ICD could not be explained by atopy alone (9, 10). So far, studies on genetic factors contributing to ICD have been scarce and focus primarily on polymorphisms and mutations in genes related to skin inflammation and barrier function.

Cytokines play a key role in the initiation and modulation of irritant-induced skin inflammation and genetically determined variations in the cytokine levels in the skin have been shown to influence the individual susceptibility to ICD (11). De Jongh et al. conducted a case-control study including 197 patients with chronic occupational ICD of the hands and 217 healthy apprentices in vocational training for at risk professions to investigate associations between ICD and 9 single nucleotide polymorphisms (SNPs) in genes encoding the pro-inflammatory cytokines interleukin (IL)-1α, IL-1β, IL-8 and tumor necrosis factor (TNF)-α as well as the anti-inflammatory cytokine IL-10 (12). The authors found a higher frequency of the *TNFA-308A* allele in the patients than in the healthy control group. The allele was most prevalent in patients with a low level of irritant exposure suggesting that higher levels of irritant exposure may mitigate the effect of genetic susceptibility factors. The *TNFA-308A* allele has been associated with increased TNF-α production and may consequently confer enhanced inflammatory responses to skin irritation. These observations have been supported by studies showing a lower irritation threshold to the model irritants sodium lauryl sulfate (SLS) and benzalkonium chloride in carriers of this allele variant (13, 14). Increasing the size of the study cohorts, Landeck et al. confirmed the impact of the *TNFA-308A* allele on developing occupational ICD of the hands (15) and showed that in contrast to *TNFA-308A, the TNFA-238A* allele conferred a protective effect (12). In addition, De Jongh et al. as well as Landeck et al. reported a protective effect of a C→T transition at position -889 within the IL-1 gene (*IL1*), associated with decreased levels of IL-1α in the stratum corneum of carriers of the IL-1A-889T allele (11, 12, 16).

Several studies provide evidence that carrying a loss-of-function mutation in the filaggrin gene (*FLG*) modifies the risk of developing occupational ICD. Filaggrin plays a key role in terminal differentiation and in the maintenance of the skin barrier function (17). A pilot study by De Jongh et al. found a higher combined carrier frequency of the *FLG* loss-of-function alleles R501X and 2282del4 in patients with chronic occupational ICD of the hands, nearly doubling the risk of its occurrence (OR 1.91, 95% CI: 1.02-3.59) (18). Increasing the numbers of patients and controls, the same research group demonstrated that *FLG* mutations and atopic dermatitis (AD) were independently of each other associated with occupational ICD of the hands (19). The adjusted OR for having ICD in those with *FLG* mutations,

corrected for AD, was 1.62 (95% CI: 1.01-2.58). A history of AD increased the risk of developing ICD of the hands approximately 3-fold (OR 2.89, 95% CI: 2.08-4.03), whereas individuals with a combination of a history of AD and *FLG* mutations had the highest risk (4.7-fold). Similarly, Timmerman et al. reported an association between *FLG* mutations and contact dermatitis in Dutch construction workers however, no differentiation between ICD and ACD was made (20). A prospective study from the Netherlands showed that apprentice nurses with *FLG* mutations and AD had the highest risk of developing ICD during their training (OR 3.6, 90% CI: 1.7-7.5) (21). Furthermore, the results of another study in patients with occupational ICD showed that *FLG* mutation carriers had an unfavorable prognosis with regard to disease course and remaining in the workforce (22). Taken together the findings of these investigations provide evidence that genetic variations modify the risk of developing ICD and its course. Nevertheless, there is a need for further large scale studies to support these observations.

Inflammatory mediators

In contrast to earlier assumptions that irritants induce non-immunologic skin responses, the role of the innate immunity in the pathogenesis of ICD is nowadays widely acknowledged (23, 24). The innate immune response is rapid, does not require previous sensitization and involves neither specific T cells nor immunological memory.

Keratinocytes (KCs) are integral to the innate immune response. They are a dominant cellular source of immune mediators in ICD. In the earliest stage, irritant exposure leads to cytotoxic cell damage and release of pre-stored IL-1α from the epidermal cells (25-27). Activation of IL-1α subsequently stimulates production of further cytokines, chemokines and growth factors (e.g. IL-1β, TNF-α, IL-6, IL-8/CXCL8, CCL20, CCL27, GM-CSF, vascular endothelial growth factor (VEGF)) by the neighboring cells (23, 26-28). These inflammatory molecules activate also Langerhans cells, dermal dendritic cells (DCs) and lymphocytes (23, 28). Antibodies against TNF-α were capable of inhibiting irritant reactions *in vivo* demonstrating the importance of this cytokine in ICD (23). In response to TNF-α and IL-1α, the chemokines CCL20 (MIP-3) and CXCL8 are released, which in turn can initiate infiltration of immune cells such as CCR6+ T cells and immature DCs into the

irritated skin (23). IL-1α activated fibroblasts release CXCL8, CXCL1 (GRO-α), and CCL2 (monocyte chemotactic protein-1 (MCP-1)), and TNF-α activated fibroblasts release CCL2 and CCL5 (RANTES). DCs and T cells are activated also by IL-1β which has been activated by caspase-1 (IL-1β-converting enzyme). Indeed, caspase-1 has been detected in KCs after irritation (23). KCs release also IL-6, which is chemotactic for neutrophils and T cells, as well as VEGF, which promotes angiogenesis, increases vascular permeability, and induces the expression of adhesion molecules on the endothelial cells.

In addition to inflammatory cytokines, KCs produce anti-inflammatory cytokines such as IL-10 and IL-1 receptor antagonist (IL-1RA). IL-1RA binds to the IL-1 receptor without triggering a signal cascade (25, 29). The interindividual variations in the baseline cytokine levels as well as cytokine responses after exposure to irritants have been shown to be substantial (25, 29)and the minimally invasive tape stripping method can be used to measure IL-1α- and IL-1RA –levels after irritant exposure *in vivo*(25, 30).

In chronic ICD, KCs contain significantly more IL-1α which stimulates the activation of an inflammatory cascade resulting in the production of growth factors, especially epidermal growth factor (EGF) and keratinocyte growth factor (KGF) (26).

CXCR4 and CCR7 are required for the migration of LCs from the epidermis to the dermis and the afferent lymphatic vessels. Both chemokine receptors are upregulated on LCs after allergen, but not irritant exposure (31). In an *in vitro* DC migration assay irritant-treated human MUTZ-LC have been shown to migrate towards the dermis-derived chemokine CCL5 whereas contact sensitizer treated cells upregulated CXCR4 and migrated towards CXCL12 and secreted CXCL8 (32). In a further study using human skin it was shown that in addition to CCL5, CCL2 was important for irritant induced CCR1/CCR2/CCR5 dependent migration of LCs to the dermis. The cytokine that triggered dermal fibroblasts to produce these chemokines was found to be TNF-α (33).

Several studies have shown significant overlaps but also differences in the gene expression profiles induced by irritants and contact sensitizers in human PBMC-

derived DCs (34) or auricular lymph nodes in the murine Local Lymph Node Assay (LLNA) (35). In particular, Gildea et al. stimulated PBMC-DC for 24 h with various concentrations of 11 sensitizers, 5 irritants and non-sensitizers. They selected 60 genes from microarray experiments and analyzed their expression changes by RT-PCR. 10 differentially expressed genes (DEGs) were reproducibly selective and specific for DC activation by contact sensitizers. These studies indicate that a number of genes are differentially expressed in response to contact sensitizers and irritants, and that it may be possible to identify specific gene signatures for their discrimination. Interestingly, the potent irritant SLS, known to give false positive responses in the LLNA has been shown to upregulate some of the 10 DEGs (34). These observations indicate that SLS can activate some of the pathways triggered by contact sensitizers and therefore explain on a mechanistic basis its amplifying potential with regard to sensitization to contact allergens (36).

Apart from differentiation between contact sensitizers and irritants, the results of a study by Clemmensen et al. suggest that different chemical classes of irritant substances induce different gene expression profiles in the epidermis (37). Comparing the genome-wide transcriptional changes after cumulative *in vivo* human skin exposure, the authors showed that half an hour post irritation, the non-corrosive irritant nonanoic acid induced the IL-6 pathway and the mitogen-activated signaling cascade including growth factor receptor signaling. The anionic surfactant SLS induced downregulation of the cellular energy metabolism pathways (37). Based on these results, the same group identified 23 potential biomarkers for chronic ICD that need to be further evaluated. The role of confounders such as different kinetics or concentration-dependent effects cannot be entirely ruled out, nevertheless, the findings of the study provide evidence that genetic and proteomic profiling may contribute to identification of class-specific inflammatory mediators' signatures in the future. The exact cytokine and chemokine profile of ICD remains however still unknown, but significant progress has been made (23).

Alarmins

Although ACD and ICD have different pathomechanisms, there are many similarities concerning the early keratinocyte response after contact with noxious external stimuli. As well as in ACD (5), keratinocyte-derived danger-associated

molecular pattern molecules (DAMPs or alarmins) play an important role in the pathogenesis of ICD. Irritants exert direct cytotoxic effects to the epidermal cells, activating the innate immunity and leading to release of cytokines, chemokines and alarmins.

One of the early events after exposure to irritants is the generation of low molecular weight DAMPs (ROS, uric acid) by the cells in the nucleated layers of the epidermis. The stressed keratinocytes upregulate the expression of a set of alarmins such as HMGB1, IL-33 and IL-1α, heat shock proteins, S100 proteins and antimicrobial peptides (38-40) . Upon continuous exposure to DAMPs, these primary intracellular proteins are released and continue to amplify the innate immune responses via activation of Toll-like and NOD-like receptors that activate the inflammasome and NF-κB pathways, inducing release of multiple cytokines and chemokines, including IL-1α, IL-1β, TNF-α, GM-CSF, IL-6, IL-8 and IL-18 (41). In turn these cytokines activate innate immune cells and endothelial cells, which contribute to cellular recruitments to the site of keratinocyte damage.

Most alarmins are common inflammatory mediators rather than specific, disease-associated markers. Therefore, despite their well-established role in the pathogenesis of ACD and ICD, it remains questionable whether they can be relied on as biomarkers to distinguish different inflammatory skin conditions. Nevertheless, serum and tissue levels of certain alarmins such as HMGB1 and S100A8/A9 have been shown to correlate with disease or inflammatory activity, and may be valuable future targets to monitor AD, ACD or ICD (42, 43).

Filaggrin and natural moisturizing factors

Natural moisturizing factors (NMFs) are a mixture of water soluble, small hygroscopic compounds which contribute to the retention of water in the stratum corneum (SC) (44, 45). The epidermal protein filaggrin is a dominant source of NMFs contributing to more than 50 percent of the total NMFs content (44). As proteolytic degradation of filaggrin into free amino acids occurs within the corneocytes, they contain the largest amount of NMFs in the skin, although NMFs have also been detected in eccrine sweat (46). Filaggrin expression and consequently, the levels of NMFs in the skin are influenced by both genetic and environmental factors. A major genetic

determinant of the NMFs levels are loss-of-function mutations within the filaggrin gene (*FLG*), which predispose to AD and occupational contact dermatitis (mainly ICD) (19, 20).

Estimation of profilaggrin expression and quantification of filaggrin in the skin requires biopsies, and the number of studies investigating the effect of skin irritants on profilaggrin expression and filaggrin is limited. In an experimental study by Torma et al., exposure to 1% SLS for 24 hours led to a significant decrease in profilaggrin expression 6 hours post-exposure and a compensatory increase after 4 days (47). Recently, Bandier et al. developed a new method to quantify the protein levels of filaggrin in the epidermis ex vivo (48). In a study which included healthy subjects and AD patients they showed that exposure to 0.5% SLS for 24 hours led to an immediate increase in filaggrin (1 hour after removal of the test chamber), but from 25 hours to 145 hours after removal there was a significant decrease in epidermal filaggrin (48).
As the levels of NMFs in the SC are closely correlated with the *FLG* genotype (49, 50) they might be useful as a prognostic or susceptibility marker for ICD. Recent studies (51-53) have shown that single as well as concurrent exposure to various water-soluble irritants leads to significant reduction of NMFs in the SC of healthy as well as atopic individuals. Similar findings have been reported after prolonged dermal exposure to water and organic solvents (54). Although the mechanisms by which the investigated irritants reduced NMFs are still not elucidated, the changes in the NMFs might prove useful to screen compounds for their irritating properties.

In addition to exposures to skin irritants, two recent studies (55, 56) have found that topical corticosteroid therapy leads to a significant decrease in NMFs, which might at least partly explain the deterioration of the skin barrier function associated with corticosteroid therapy. Similar, although somewhat less pronounced effects, have been found for a lipid-rich emollient (*Unguentum leniens*) suggesting that NMFs might also be applied for monitoring of the effect of different moisturizers and emollients used to treat (or prevent) ICD(56).

Based on the published literature, NMFs in the SC can be used as a biomarker in ICD for different purposes. In clinical and occupational settings, NMFs might be

useful as a susceptibility and/or prognostic parameter. As several skin irritants show pronounced effect on the NMFs it might be interesting to explore the possibility of NMFs as an early effect biomarker e.g. as an outcome in the intervention studies aiming at skin barrier protection. Next, NMFs levels can be used to study the effects of environmental insults on the barrier function facilitating development of strategies for skin barrier protection from environmental stressors. NMFs can be determined either directly in the skin by confocal Raman spectroscopy or after chemical analysis (e.g. HPLC) of the SC which can easily be collected by adhesive tapes (57).

Lipids

The SC lipids have an essential role in the formation of the epidermal barrier and the maintenance of its homeostasis. They regulate the penetration of substances across the SC and exert important functions related to cell signaling and immune cell regulation (58). In this line genetically-determined or exogenously-induced alterations in the SC lipids may affect the penetration of irritants as well as skin inflammation.

Most of the published research regarding the SC lipids focuses on the role of the main lipid classes, namely ceramides (CER), cholesterol (CHL) and free fatty acids (FFA) in the formation and maintenance of the skin barrier function. The diversity of CER and FFA due to the variable carbon chain lengths, (59) and the fact that even subtle changes in their relative composition leads to alterations of the skin barrier function, indicates that development of suitable lipid biomarkers is complex. Moreover, the lipid composition varies with location, depth of the skin, age, but also between individuals, races and seasons of the year (60), making the predictive value of such a biomarker even more complex.

In contrast to the evidence for the importance of the SC lipid composition in AD, only few studies have addressed the lipid composition in ICD. Most published investigations on the relationship between ICD and the SC lipids composition have been related to experimentally induced irritation. Di Nardo et al. showed that the amount of baseline long chain CER 1 and CER 6 correlated inversely with the clinical irritation score following SLS-exposure (61). In a study by Fulmer and

Kramer, repetitive, short-term exposure to SLS induced changes in the relative distribution of CER 2, 3 and 4, as well as, free CHL and CHL-esters (62). In the same study, the authors observed a decrease in very long chain FFA (C20 – C28) while the amount of long chain FFA (predominantly C16 and C18 saturated FFA) remained unchanged. Interestingly, in contrast to the changes in the relative composition of the structural SC lipids, no changes in the total quantity of the lipids were found. In contrast to the above mentioned experimentally induced irritation studies, in a recent publication, Jungersted et al. found no differences for any of the investigated ceramide classes or the CER/CHL ratio between patients with hyperkeratotic HE and/or ICD or ACD of the hands (63).

So far, only two studies have addressed the relationship between the CERs levels and the individual response to skin irritants. Both studies focused on skin hardening, defined as accommodation or adaptation of the skin to the cause of ICD despite continued irritant exposure (64, 65). Heinemann et al. showed enhanced production of CER1 following hardening induced by repeated SLS-exposure in healthy human volunteers (64). Interestingly, volunteers presenting a hardening phenomenon had lower baseline CER 1 in the SC as compared to the volunteers without hardening phenomenon. However, in a more recent study by Park et al., no significant association between total CER levels and skin hardening induced by repetitive irritation with SLS was found (65). Furthermore, in the same study there was no significant difference in the total CER levels at baseline between the hardened and non-hardened skin.

In contrast to cytokines and structural proteins of the epidermal barrier, so far there have been no publications for genetically determined variations in the levels of the SC lipids, associated with an increased risk for ICD. Although the SC lipids have a major impact on the regulation of various physiologic processes and disease relevant pathomechanisms, the published literature shows that their potential use as biomarkers of ICD has been poorly investigated. Lipid biomarkers could potentially be used to identify susceptible individuals and furthermore aid development of evidence-based strategies for skin care in occupations with high risk of ICD.

Tight Junctions

Tight Junctions (TJs) are intercellular junctions composed of three families of transmembrane proteins (claudins, tricellulin-associated MARVEL proteins (TAMPs) and junctional adhesion molecule (JAMs)), associated with a variety of TJ plaque proteins (e.g. MUPP1, cingulin, zonulaoccludens (ZO) proteins 1-3) (66). The TJ proteins have been shown to be involved in the skin barrier function as well as cell-cell adhesion, differentiation, proliferation, and apoptosis (67, 68).

The existence of an absorption barrier at the transition between the viable and non-viable epidermal layers was suggested as early as in the 1940s (69). In 1971, Hashimoto showed exclusion of the electron dense tracer Lanthanum in the granular cell layer at intercellular junctions morphologically compatible with TJs (70). Until then, TJs were mainly known from simple epithelia and amphibian skin in which they formed selective paracellular barriers by connecting the apical part of neighbouring cells (66). Later on, the presence of TJ structures and several TJ proteins was confirmed and identified in mammalian epidermis, namely claudins, ccluding and ZO-1 (71-76). In addition, the importance of TJs in the skin barrier function was demonstrated in murine knock-out (claudin-1) and overexpression (claudin-6) models as the mice died shortly after birth due to excessive water loss (72, 75). The exact role of TJs in the skin barrier function is complex and recently it has been found that there is an extensive interplay between TJs and other components of the skin barrier such as the SC, the microbiome and the immunological barrier (for review see 77).

The knowledge about tight junctions with regard to irritant exposure or ICD is scarce. A study in human volunteers by Clemmensen et al showed significant down-regulation of the gene-expression of the TJ protein claudin-23 by cumulative exposure to SLS or nonanoic acid (37). In murine skin it was shown that a single application of 12-O-tetradecanoyl-phorbol-13-acetate (TPA) results in a transient broader localization of claudin-6, claudin-11 and claudin-18 and in a relocalization of claudin-1 from the cell-cell borders to the cytoplasm (78).The effects of irritation on TJ proteins in parallel to TJ functionality have been studied in human corneal epithelium (HCE) models, as well as in rabbit esophagus. In HCE, a decrease of immunostaining of ZO-1 was observed in parallel to a decrease of transepithelial

resistance as a measure of the epithelial permeability after treatment with the eye irritants potassium laurate, butanol and propasol solvent P (79). In the same model, decreased immunostaining for ccluding after treatment with benzalkoniumchloride was observed (80). In addition, experimental studies in rabbits showed that damage to the esophageal epithelial barrier function induced by hydrochlorid acid or acidified sodium nitrite was accompanied by a decrease of the protein levels of ccluding whereas the levels of Cldn-1 and Cldn-4 were not influenced by the irritants (81).

Based on these observations, it may be reasonable to hypothesize that TJ proteins and TJ function are likely to be altered in ICD. However, the alterations and their biological significance need to be studied in the future, and the findings need to be compared to other types of eczema/dermatitis, notably, AD and ACD.

Antimicrobial peptides

Antimicrobial peptides (AMPs) comprise a group of endogenous, mostly cationic proteins which play an essential role in antimicrobial defense, initiation of the innate immune responses and maintenance of the skin barrier homeostasis (82). AMPs can be constitutively expressed in the skin or induced by inflammation as well as exogenous insults, including perturbation of the barrier function by tape stripping and solvent treatment (82-85). Beyond these factors, local inflammatory mediators such as TNF-α, IFN-γ, IL-6 and IL-1 are known to induce AMP expression whereas IL-4, IL-10 and IL-13 have been shown to exert opposite effects (86-88). In addition, studies in knockout mice lacking cathelicidin-related antimicrobial peptide (the murine homologue of LL-37) have shown significantly delayed barrier recovery up to 6 h after tape stripping or acetone-treatment (89).

There are few published data on the expression of AMPs in response to mechanical or chemical irritants in the human skin *in vivo*. Harder et al. showed upregulated expression of human β-defensin (hBD)-3 and increased secretion of ribonuclease 7 (Rnase 7) after barrier disruption by tape stripping in healthy skin (82). Similarly, experimentally-induced barrier disruption by tape stripping was found to result in immediate increase in psoriasin secretion that remained elevated as long as seven days after barrier damage (85).These findings have been confirmed in an *in vivo*

study by De Koning et al. showing that SLS-induced or mechanical damage to the epidermal barrier leads to increased mRNA and protein expression levels of numerous AMPs, including hBD-2, hBD-3, psoriasin and elafin in healthy volunteers as well as in clinically uninvolved skin of AD and psoriatic patients (83).

As most of the so far published studies rely on experimentally-induced skin irritation, the validity of AMPs as biomarkers for ICD remains to be examined in the future and confirmed in clinical or work-related settings.

Proteases

The epidermis is a rich source of proteases, involved in fundamental cellular and developmental processes including morphogenesis, differentiation, epithelial permeability, and/or cellular ion transport. Not surprisingly, there is a growing number of human skin pathologies caused by dysregulation of proteolytic pathways (90). Proteases mostly contained in plant extracts or fruits may act as part of complex proteolytic cascades to induce downstream targets and likely aggravate the development of ICD through their effect on the barrier function and immune response. It will be informative to identify the signals that trigger and/or maintain their activation and to prove whether proteases could be used as biomarkers in ICD *in vivo*.

Proteomics

ICD is the most common form of chronic hand eczema (CHE) (2, 91). Despite the recognition that irritant-induced impairment of the barrier function is central to disease pathogenesis (92), so far there has been limited knowledge with regard to the expression profile of the epidermal barrier proteins in hand eczema. In a recent study based on comparison of mass spectrometry data, Molin et al. identified 185 differently regulated candidate proteins in palmar skin biopsies of patients with CHE and healthy controls (93). 115 proteins were found to be less abundant and 70 proteins more abundant in eczematous compared to healthy skin. The results of the study showed altered expression of several proteins relevant to the barrier function, including down-regulation of filaggrin, filaggrin-2 and hornerin and up-regulation of the small proline-rich protein 2B and S100A11 in CHE. Furthermore, in diseased skin the desquamation-related enzymes kallikrein-related peptidase (KLK) 5 and

KLK7 as well as cystatin E/M were downregulated, whereas the antimicrobial peptides S100A7 and S100A8/A9 were upregulated. The mass spectrometry findings have been further confirmed by immunohistochemistry in biopsies from a different study population. Though the results of the study do not allow conclusions as to whether the observed changes are primary or secondary, further investigations using modern large-scale screening technologies to analyze the protein expression may be useful for identification of potential biomarkers for ICD.

This view is underlined by recent proteomic studies on human skin or human skin and immune cells applying complementary technologies such as quantitative 2D-electrophoresis coupled with mass spectrometric analyses to identify differentially regulated proteins of e.g. human keratinocytes exposed to irritants or to contact allergens (94, 95); or novel molecular studies on human skin models by applying NMR techniques (96). The data acquired in the study by Thierse et al were analyzed by using the multivariate projection method partial least squares projections to latent structures (PLS), allowing clearly to separate protein pattern reactivity of human cells stimulated by irritant chemicals or by contact allergens (97).

Transepidermal water loss (TEWL)

Transepidermal water loss (TEWL) is one of the most important parameters for assessment of the functional state of the epidermal barrier in health and disease (98). The validity of TEWL as a measure for assessment of the permeability barrier function has been proven by comparison of instrumental readings with gravimetric measurements in *ex vivo* and *in vivo* animal, as well as *in vivo* human skin models (99). The irritant-induced effects on TEWL have been shown to vary considerably, dependent on the physicochemical properties of the irritants and the exposure conditions. Surfactants such as the anionic detergent SLS or alkaline agents such as sodium hydroxide are known to cause a pronounced, concentration-dependent increase in TEWL, whereas simultaneous exposure to organic solvents or alcohols results in less pronounced effects, respectively TEWL increase (30, 51, 100, 101). Compared with exposure to a single irritant, combined exposure to multiple irritants may modify the outcomes and, dependent on the TEWL increase, result in quenching, additive or synergistic effects (1, 102). In the same context, an additive

impairment of the barrier function following combined exposure to chemical and mechanical irritants has been reported (103).

The baseline TEWL and the individual responses to one and the same irritant under identical exposure conditions are known to vary considerably within the population (104). The relevance of baseline TEWL for predicting the skin barrier response to irritants, in particular detergents, has been investigated by several groups in the past. Murahata et al. reported significant correlation between the baseline TEWL and the severity of surfactant-induced irritation in a 5-day cumulative irritation test in healthy volunteers (105). Similarly, higher baseline TEWL values were found to predict enhanced responses to 24-hour patch test exposure to SLS in healthy skin (106). These observations have been confirmed in independent studies showing that higher baseline TEWL correlates to higher post-exposure values after single as well as repeated exposure to 0.5% SLS in non-atopic individuals and suggesting that the baseline TEWL may be a sensitive indicator for the outcomes of cumulative skin irritation *in vivo* (107, 108).

Compromised barrier function and increased baseline TEWL even in clinically uninvolved skin areas are important characteristics of AD (108-112). The impaired barrier function and the epidemiological evidence that history of atopic skin disease contributes to the risk of occupational ICD have stimulated the interest in studying the changes in TEWL for assessment of the susceptibility to irritant damage in atopic individuals. Most of the published experimental studies on the barrier response to irritants in AD have been based on a single patch test application of a single chemical (113), mostly SLS or sodium hydroxide. The partly controversial results, found in the short-term irritation studies may be influenced by methodological differences as well as by further confounders such as the presence of acute inflammatory lesions at the time of investigation which are known to modify the barrier responses, respectively, the pre- as well as post-exposure readings (109, 113). Furthermore, a single irritant exposure study reflects a momentary situation and may not provide information on the outcomes of cumulative skin irritation. In contrast to short-term exposure, repeated single as well as combined exposure to multiple irritants in AD have been shown to result in a more pronounced TEWL increase compared with healthy, non-atopic control subjects (51, 108).

In the same context, experimental irritant-exposure studies in healthy aged individuals, known to have lower baseline TEWL values, have shown a delayed and less pronounced TEWL increase after single as well as repeated SLS-induced irritation (114-117). In addition, tandem repeated exposure to irritants with known synergistic effects has been shown to result in delayed and less pronounced changes, respectively significantly lower TEWL increase in aged compared to young individuals (30). The relationship between baseline TEWL and enhanced barrier damage by irritants is of considerable interest in particular in occupational settings. Nevertheless, the current knowledge has been limited mostly to surfactant- or sodium hydroxide-induced irritation and therefore, may not predict the barrier responses to other chemically unrelated primary irritants (118, 119).

Compared with other non-invasive methods, the measurement of TEWL has been found to be the most sensitive for assessing SLS-induced irritation and detection of alterations in the barrier function induced by even short-term exposure to low concentrations of the irritant (106, 120-122). In addition, a study by Fluhr et al. showed that TEWL had the best discriminatory potential to monitor changes over time in experimentally induced irritation by SLS and tape stripping (123). To minimize variations, the TEWL measurements need to be performed under controlled ambient conditions and according to the published guidelines (124, 125). Despite possible differences in the instrumental readings, dependent on the measurement principle (open chamber, closed chamber or condenser chamber), all currently available commercial devices have been shown to detect changes in the functional state of the skin barrier, respectively TEWL, following irritant damage (126-128).

Skin pH

The acidic nature of the skin surface plays a key role in maintaining the skin barrier integrity, homeostasis and antimicrobial defense (129). The relationship between increased skin surface pH and impaired barrier function has been demonstrated in numerous animal and *in vivo* human skin studies (109, 110, 129-135). The skin pH is known to be influenced by multiple endogenous as well as exogenous factors, including soaps, detergents, consumer-related or occupational irritants (136). One of the most studied endogenous factors associated with elevated pH is the presence

of *FLG* loss-of-function mutations, known as a genetic risk factor for both AD and ICD. In this line, several studies show that carriers of *FLG* mutations have an increased skin surface pH, likely due to reduced levels of filaggrin degradation products that contribute to the acidic pH of the SC (137-139).

The skin surface pH influences the permeability barrier in different ways. Numerous enzymes that are critical for the SC barrier function have pH optima within the acidic range, in particular enzymes involved in ceramides synthesis and processing of the lamellar bodies (129, 140). Studies in hairless mice have shown that neutralization of the skin pH delays the barrier recovery after acetone treatment, supporting the importance of an acidic skin environment (141). A shift towards a neutral pH due to blocking of the sodium proton exchanger NHE1 or secretory phospholipase A2 has also been shown to compromise the barrier integrity and cohesion (142-144). An elevated skin pH can also lead to increased serine protease activity with subsequent impairment of the skin barrier function and inflammation (145-147).

The alterations in the barrier function and skin pH after experimental exposure to different classes of irritants in healthy individuals have been investigated by Fluhr et al. (148). The results of the study showed that cumulative exposure to 0.5% SLS alone, as well as in a combination with 0.04% sodium hydroxide, 1.0% ammonium hydroxide, 1.0% dimethylamine or 1.5% trimethylamine, led to a significant increase in the skin pH and compromised barrier function. In the same line, several other studies have found sustained deterioration of the skin barrier function and pH after single and repetitive washing with different washing solutions (131, 149, 150).

Regarding skin pH and its role as a biomarker of ICD, studies have shown that skin pH can be used to predict the risk of an irritant reaction. A significant correlation between the baseline pH at the skin surface and in the deeper layers of the SC and TEWL after skin irritation with 1% SLS for 24 hours was found in a study on healthy volunteers (151). Furthermore, patients with sensitive skin, known to be prone to irritation, have a tendency to higher skin pH on the cheeks than healthy controls (152). Based on the published literature, the skin pH measurement might be a useful tool in investigating the irritant potency of different exposures in daily life and in occupational settings. Nevertheless, its reliability, validity and potential use as biomarker of ICD need to be evaluated in the future.

Dermal Texture Index (DTI)

Inflammatory skin conditions can lead to reduced cell size and irregular cell shape (153). Recently, by using atomic force microscopy (AFM) it has been found that diseased skin shows presence of circular nano-objects protruding approximately 300 nm above the corneocyte surface (154, 155). The number of these nano-size protrusions has been quantified and expressed as a Dermal Texture Index (DTI) (156). In patients with AD, the DTI has been found to be up to four times higher than in healthy persons in areas that had been affected during flare, but are clinically silent at the day of sampling (156). The presence of protrusions on the corneocyte surface was associated with aberrant pattern of the corneodesmosomal protein corneodesmosin, suggesting impaired corneodesmosome degradation, although the mechanisms which underlie these processes are not yet understood.

With regard to ICD, currently ongoing research demonstrates that the model skin irritant SLS increases significantly DTI in a time-dependent manner (manuscript in preparation). Although more research in that aspect is needed, the use of AFM and DTI scoring might be an interesting biomarker of ICD in the future. The method is simple, robust and the corneocytes can be collected in a minimally-invasive manner by tape stripping.

CONCLUSIONS

Biomarkers are increasingly recognized as valuable tools in both scientific research and clinical practice to study disease mechanisms, identify susceptible individuals, monitor disease severity, prognosis and treatment outcomes. Even if broadly defined (157), to be useful, biomarkers need to be evaluated both in terms of validity and clinical relevance. In contrast to other common inflammatory skin diseases such as AD or psoriasis, for which a number of skin-related or systemic biomarkers have been proposed and evaluated in clinical settings, most parameters discussed in the present review have been studied under in vitro or in vivo experimental conditions and considerably less in clinical context. Consequently, the lack of controlled studies in well-defined clinical populations at the current time point poses significant limitations to the analysis of the validity and utility of the reviewed parameters in

a systematic manner. Of all parameters, the SC NMFs and TEWL have been most extensively studied in terms of validity and relevance in both experimental and clinical settings. Several genetic markers related to polymorphisms in the genes encoding the primary pro-inflammatory cytokines IL-1α and TNF-α have been evaluated with regard to disease susceptibility and could be used in research, clinical and field studies. The correlation between the described gene polymorphisms, the levels of the respective cytokines in the SC and relevant clinical or prognostic endpoints however needs to be investigated in further studies. Assessment of the value of the known inflammatory mediators as reliable biomarkers for ICD is currently limited by the evidence of significant overlaps between ICD and ACD that requires robust evaluation in clearly defined patients' populations in the future. Most of the remaining parameters, identified as potential biomarkers of ICD by the review, have not been investigated in patients and therefore remain for use primarily in research until further evidence of validity, clinical and prognostic relevance has been published.

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CHAPTER 3

Biomarkers for contact dermatitis, experimental studies

3.1

Effect of allergens and irritants on levels of NMF and corneocyte morphology

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ABSTRACT

Background: The irritant sodium lauryl sulfate (SLS) is known to cause a decrease in the stratum corneum level of natural moisturizing factor (NMF), which in itself is associated with changes in corneocyte surface topography.

Objective: To explore this phenomenon in allergic contact dermatitis.

Methods: Patch testing was performed on patients with previously positive patch test reactions to potassium dichromate (Cr), nickel sulfate (Ni), methylchloroisothiazolinone (MCI)/methylisothiazolinone (MI), or p-phenylenediamine. Moreover, a control (pet.) patch and an irritant (SLS) patch were applied. After 3 days, the stratum corneum from tested sites was collected, and NMF levels and corneocyte morphology, expressed as the amount of circular nanosize objects, quantified according to the Dermal Texture Index (DTI), were determined.

Results: Among allergens, only MCI/MI reduced NMF levels significantly, as did SLS. Furthermore, only MCI/MI caused remarkable changes at the microscopic level; the corneocytes were hexagonal-shaped with pronounced cell borders and a smoother surface. The DTI was increased after SLS exposure but not after allergen exposure.

Conclusions: MCI/MI significantly decreased NMF levels, similarly to SLS. The altered corneocyte morphology suggests that skin barrier damage plays a role in the pathogenesis of MCI/MI contact allergy. The DTI seems to differentiate reactions to SLS from those to the allergens tested, as SLS was the only agent that caused a DTI increase.

INTRODUCTION

Allergic contact dermatitis (ACD) and irritant contact dermatitis (ICD) are common inflammatory skin diseases that pose a major problem in public health because of the widespread use of skin irritants and/or contact allergens in occupational settings and in consumer products. Although these two forms of contact dermatitis have different pathogenesis, they show similar clinical features, including erythema, fissuring, and vesicles, and, in a more severe form, bullae (1, 2). The primary step in the development of ICD is, for most skin irritants, characterized by disruption of the skin barrier, which is followed by activation of the innate immune system without involvement of T cells (3). A genetic deficiency of the epidermal protein profilaggrin is a strong predisposing factor for ICD (4, 5). The odds ratio (OR) for filaggrin gene (FLG) mutations, adjusted for atopic dermatitis (AD), was 1.61, whereas individuals with a history of AD who are also carriers of an FLG mutation have a 4.7-fold risk for ICD (6). A history of AD increases the risk for ICD threefold. It has to be noted, however, that AD patients without FLG mutations also have reduced filaggrin expression caused by T helper 2-mediated inflammation in AD (7). Filaggrin and its degradation products, which are the main constituents of natural moisturizing factor (NMF), are responsible for a number of functions concerning skin barrier function in the stratum corneum, including mechanical properties, skin hydration, and the epidermal inflammatory response (8). Recently, it has been shown that various skin irritants significantly reduce the levels of NMF (3). NMF levels, in turn, showed a strong association with corneocyte surface morphology, expressed as the Dermal Texture Index (DTI), supporting the view that alterations in the skin barrier play a major role in ICD (9).

The effect of contact allergens on the skin barrier has not been extensively studied to date, and, if so, it has been mainly assessed with skin bioengineering techniques such as transepidermal water loss measurement (10). However, skin barrier defects arising from concomitant irritant properties of an allergen may play an important role in the activation of the adaptive immune response and the development of ACD (10-13). ACD is a type IV cell-mediated immune reaction, separated into two distinct phases: the sensitization phase, in which the immune system is primed to react to a given allergen (usually molecules with a molecular weight (MW) of <500), and the

elicitation phase, following re-exposure. In this process, the impaired skin barrier may facilitate sensitization in the first place, but also the allergic response as a result of the increased penetration of contact allergens (8, 11, 12). The structural components of the stratum corneum, such as the extracellular lipid matrix, the cornified envelope, and the corneodesmosomes, are primary targets for most irritants (13). Recently, it has been shown that the model irritant sodium lauryl sulfate (SLS) affects the expression of filaggrin (14, 15). Similar morphological changes have also been seen in mice when they are exposed to 2,4,6-trinitro-1-chlorobenzene, a potent contact allergen (16). Studies on the effect of contact allergens on corneocyte morphology in humans are lacking. Therefore, in the present study, we investigated the levels of NMF, the associated filaggrin-degradation enzymes bleomycin hydrolase (BH) and calpain-1 (C-1) and stratum corneum plasmin, as an indicator of skin barrier damage and corneocyte surface topography and morphology (9, 17, 18). We focused on the effects of skin exposure to clinically relevant allergens: potassium dichromate (Cr), nickel sulfate (Ni), methylchloroisothiazolinone (MCI)/methylisothiazolinone (MI), and p-phenylenediamine (PPD), and to the model irritant SLS.

METHODS

Patients

The database of the dermatological outpatient clinic of the Zagreb University Hospital was screened for individuals with patch reactions (clinically graded according the ESCD/ICDRG guidelines, grading system for patch test reading, as 1+ or 2+) to one of four common contact allergens: Cr, Ni, PPD and MCI/MI (19). Patients with two or more 1+ or 2+ reactions to Cr, Ni, PPD or MCI/MI were preferred as multiple allergens could be tested in one individual. Patients with a 3+ reaction were not selected to avoid severe reactions which might hinder taking of tape strips. Patients with a history of atopic dermatitis were excluded. The experimental protocol followed the Declaration of Helsinki Principles and was approved by the Medical Ethics Committee of the University Hospital Centre Zagreb. Written informed consent was obtained from each participant.

Procedure

All participants were patch tested on the back with 1 or 2 allergens to which the participant had previously shown a 1+ or 2+ patch test reaction, and moreover to SLS and petrolatum. The tested substances were applied in the Van der Bend chambers (Van der Bend, Brielle, The Netherlands), namely, *p*-phenylenediamine 1% pet., potassium dichromate 0.5% pet. (Almirall Hermal, Reinbeck, Germany), nickel sulfate 5% pet. and Methylchloroisothiazolinone / methylisothiazolinone 3:1 at 0.01% aq. (Smartpractice Europe, Barsbuttel, Germany). To provoke irritant contact dermatitis, patches with 1% and 2% SLS aq. were used. A patch with the vehicle (100% petrolatum) was used as a control. Four identical patch series were applied: two series on the left and two series on right side of the upper back. Two identical series on the left side were for respective D2 and D3 assessment, enabling stripping could be performed on ''fresh'' non-stripped skin sites. The patches on the right side of the back functioned as a back-up for possible technical failures. On day (D)2 all patches were removed, patch sites were marked and the skin was allowed to rest for 30 min. On D2 and D3, respectively, the SC samples from the skin sites where the duplicate patches had been applied were collected using adhesive tapes (1.5 cm^2, D-Squame, CuDerm, Dallas, Texas, US)(20). On both D2 and D3, clinical reactions were graded according to ESCD/ICDRG guidelines (19). In total, 8 consecutive tape strips were taken from each patch application site for analysis. Different tapes were used for the various analyses; tape 3 was used for Atomic force microscopy (AFM), tape 4 for proteases and Scanning Electron Microscopy (SEM), and tape 5 for NMF analysis.

NMF and protease activity

NMF was defined as the sum of the concentrations of pyrrolidone carboxylic acid (PCA), urocanic acid and histidine. NMF was determined according to the method described in detail elsewhere (21). Briefly, the fifth tape strip was extracted with 0.5 ml 25% ammonia. The ammonia extract was evaporated and the residue dissolved in 250 μl of water before analysis by high-pressure liquid chromatography (HPLC). The NMF concentration was normalized for the SC protein amount determined with a Pierce Micro BCA protein assay kit (Thermo Fischer Scientific, Rockford, IL, USA) to compensate for the variable amount of the SC protein on the tape strips. Enzymatic activities of bleomycin hydrolase (BH), calpain-1 (C-1) and plasmin were

determined in eight randomly selected subjects who were positive either for Ni or MCI/MI and their corresponding unpatched and petrolatum test sites. Analysis has previously been described in details by Raj et al. and Voegeli et al. (17, 22-25). Briefly, buffer extracts of the tape strips (250 µl) were combined with fluorogenic peptide substrates (1.25 µl) (BH-like activity: H-Cit-AMC, C-1-like activity: Suc-Leu-Leu-Val-Tyr-AMC and for plasmin-like activity: MeOSuc-Ala-Phe-Lys-AMC) and agitated at 1000 rpm at 37°C. The reaction was stopped after 2 h by adding acetic acid (250 µl). The released AMC was quantified using reverse phase HPLC (excitation 354 nm, emission 442 nm) and results were corrected for SC protein content on the tape strips as determined by the 850 nm absorption infrared densitometer SquameScan 850A (Heiland Electronic, Wetzlar, Germany) according to the procedure described elsewhere (24).

Corneocyte morphology

Corneocytes from patients were analysed by AFM as described by Franz et al. (26). Briefly, the third consecutive tape strip was subjected to AFM measurements carried out with a Multimode atomic force microscope equipped with a Nanoscope III controller and software version 5.30sr3 (Digital Instruments, Santa Barbara, CA, USA). Silicon nitride tips on V-shaped gold-coated cantilevers were used (0.01 N/m, MLCT; Veeco, Mannheim, Germany). Imaging was performed at ambient temperature with forces less than 1 nN at one to three scan lines per second (1–3 Hz) with a resolution of 512 × 512 pixels. For texture analysis, subcellular scan areas of 20 × 20 µm2 were recorded. For a larger overview, images of 70 × 70 µm2 were recorded. Topographical data of the corneocyte surfaces were analysed with the nAnostic™ method, by the use of custom-built, proprietary algorithms (Serend-ip, Münster, Germany). The method evaluates each nanostructure protruding from the mean surface level, referred to as circular nanosize objects (CNOs). These are then automatically filtered according to their size and shape; in the present study, only structures of positive local deviational volume smaller than 500 nm in height and with an area of <1 µm2 are considered. The DTI counts these features for an area of 20 × 20 µm2 of cell surface per image (9). For MCI/MI and SLS, SEM was performed on the tape strips from 1 person. Fragments of D-Squame tapes were observed at a partial vacuum (0.133 kPa) without prior preparation of the samples (native state). Images of the removed corneocyte layers were recorded at 15 kV with the secondary electron detector of a Quanta 250 FEI scanning electron microscope.

Statistics

Data analysis was performed with graphpad prism® version 6.07 (GraphPad Software, La Jolla, CA, USA). Comparison of the NMF levels between different skin sites (allergen/irritant/unpatched) and their corresponding pet. controls in the same individual was performed with a paired two-tailed t-test. Comparison of the activity of BH, C-1 and plasmin between allergens and their pet. controls was performed with the Wilcoxon signed rank test if the distribution of data deviated from normal distribution as tested by the Shapiro–Wilk test, and with a two-tailed t-test when the data were normally distributed. The Spearman correlation coefficient was used to correlate the individual activities of the proteases with NMF levels. The Pearson correlation coefficient was used to correlate the individual patch test results (1+, 2+, and 3+) with NMF levels. One-way anova followed by Dunnett's multiple comparison post hoc test was applied to the differences in DTI between SLS and individual allergens, pet., and unpatched skin sites. Data are shown as the mean value and standard deviation (SD) when distributed normally, and as median with interquartile range when non-normally distributed.

RESULTS

Clinical response

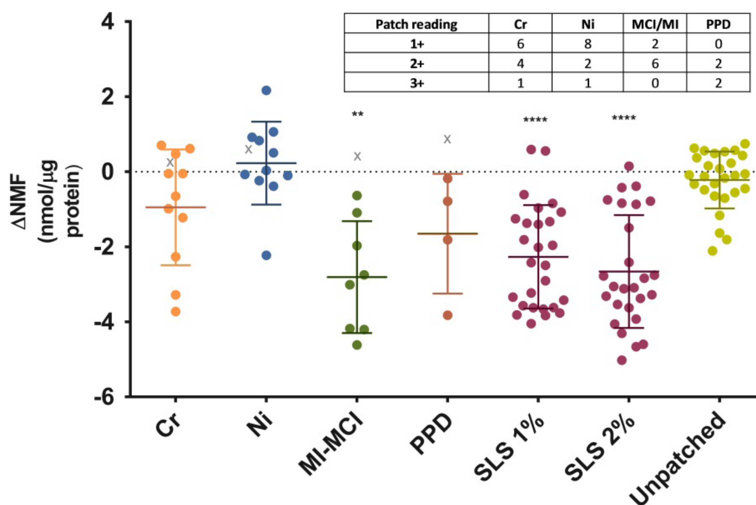
We included 27 patients (24 females), with an average age of 49.3 years (SD 12.6). A total of 34 positive 1+ or 2+, 3+ reactions to the investigated allergens were observed on D3: 11 for Ni, 11 for Cr, eight for MCI/MI, and four for PPD. Eleven patients had two positive reactions. Although selection was based on 1+ or 2+ reactions, several patients in the present study had 3+ reactions (one for Ni and Cr, and two for PPD). The clinical scores per allergen per patient are shown in Fig. 1.

Exclusion

Four patients, one for each allergen, did not show a positive reaction to the allergen to which they had a positive reaction in the past. Furthermore, one patient had a severe reaction to PPD and the tape strip samples could not be obtained. These patients (n=5) were excluded from the data analysis.

NMF

Fig. 1 shows the difference in the NMF levels (ΔNMF) between the allergens, SLS, unpatched sites, and their corresponding controls (pet.) on D3. A significant difference from the corresponding controls was observed for SLS (1% and 2%) and MCI/MI. The smallest effect was observed for Ni, for which none of the patients had ΔNMF lower than the median response after MCI/MI and SLS (Fig. 1). Although the difference with respect to the pet. control did not reach statistical significance for other allergens, several patients had negative ΔNMF values. For example, 3 patients for Ni and 1 for Cr and PPD showed NMF level decreases similar to the average decrease observed after MCI/MI and SLS (Fig. 1). Interestingly, those 5 patients had strong patch test reactions (3+ for Ni and Cr, and 2+ for PPD). To further explore possible associations between patch test readings and changes in NMF levels, we compared ΔNMF and patch test readings. The Pearson correlation coefficient amounted to -0.64 ($p < 0.001$), indicating a significant negative association between patch test reactions and decrease in NMF levels. The NMF levels after D2, determined in a limited number of patients, showed the same trend (data not shown). In each allergen group, 1 patient had no reaction to the allergen (denoted in Fig. 1. by an X symbol). As is evident from Fig. 1, the ΔNMF values in these subjects were close to those for the pet. control. There was no significant difference in the NMF levels between skin sites where no patches were applied and sites with petrolatum.



Patch reading	Cr	Ni	MCI/MI	PPD
1+	6	8	2	0
2+	4	2	6	2
3+	1	1	0	2

Fig. 1. The difference in the natural moisturizing factor levels (ΔNMF) between the skin sites tested with potassium dichromate (Cr), nickel sulfate (Ni), methylchloroisothiazolinone (MCI)/methylisothiazolinone (MI) or p-phenylenediamine (PPD), sodium lauryl sulfate (SLS), unpatched skin and their corresponding pet. controls on day 3. The individual patch test readings for each allergen are inserted as a table. As some patients had positive reactions to two allergens, the number of reactions ($n = 34$) is greater than the number of patients ($n = 27$). The ΔNMF of a positive reaction is indicated by a circle symbol, and that of a non-responder is indicated by a 'X' symbol. Non-responders were excluded from data analysis. The results are shown as mean of all subjects and standard deviation. The data of allergens, SLS and unpatched skin were compared with those of their corresponding pet. patches by use of a paired, two-tailed t-test. **$p < 0.01$; ****$p < 0.0001$.

Corneocyte surface morphology

The DTI values were determined from the AFM images of a 20×20-µm^2 area. As shown in Fig. 2, among all investigated compounds, only SLS led to a significant rise in the DTI, indicating increased numbers of CNOs, which can clearly be seen from Fig. 3g, representing a more detailed 20×20-µm2 image of an SLS-tested skin site. The CNOs in the SLS image were also shown by SEM (Fig. 4). Larger overview AFM images (70×70 µm^2) of the corneocytes from the skin sites tested with Cr, Ni, PPD, MCI/MI, SLS and pet. are shown in Fig. 3a–f. The images show that, at a microscopic level, the results for Cr, Ni and PPD resemble those for pet. MCI/MI differed, in that it caused distinct alterations in the structure; corneocytes were hexagonal-shaped and had pronounced cell borders (Fig. 3d). The surfaces were smoother, with a loss of corneocyte surface microtexture. This was also confirmed by SEM images showing loose lateral associations between the cells from the MCI/

MI-treated skin sites (Fig. 4). As indicated in Fig. 2, these microscopic alterations did not lead to an increase in cell surface CNOs; the average DTI value from MCI/MI samples was similar to that for other allergens and petrolatum.

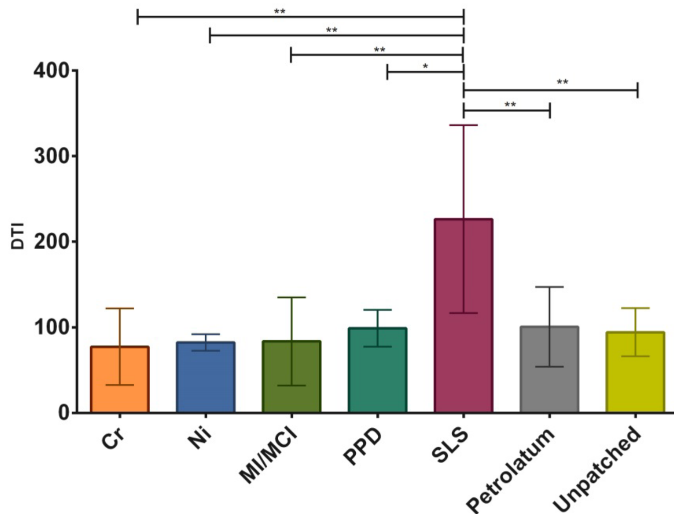


Fig. 2. The Dermal Texture Index (DTI; number of circular nanosize objects per 20-μm[2] area) measured in the stratum corneum collected on day 3. The results are averaged for all subjects, and are shown as mean values and standard deviation. The number of tested sites per group differed: Cr, n=3; Ni, n=5; methylchloroisothiazolinone (MCI)/methylisothiazolinone (MI), n=4; p-phenylenediamine (PPD), n=3; sodium lauryl sulfate (SLS), n=6; pet., n=7; and unpatched, n=4. The DTI values of allergens/pet./unpatched skin were compared with those of the SLS group; asterisks indicate level of significance. **p<0.01 (one-way anova followed by Dunnett's multiple comparisons test).

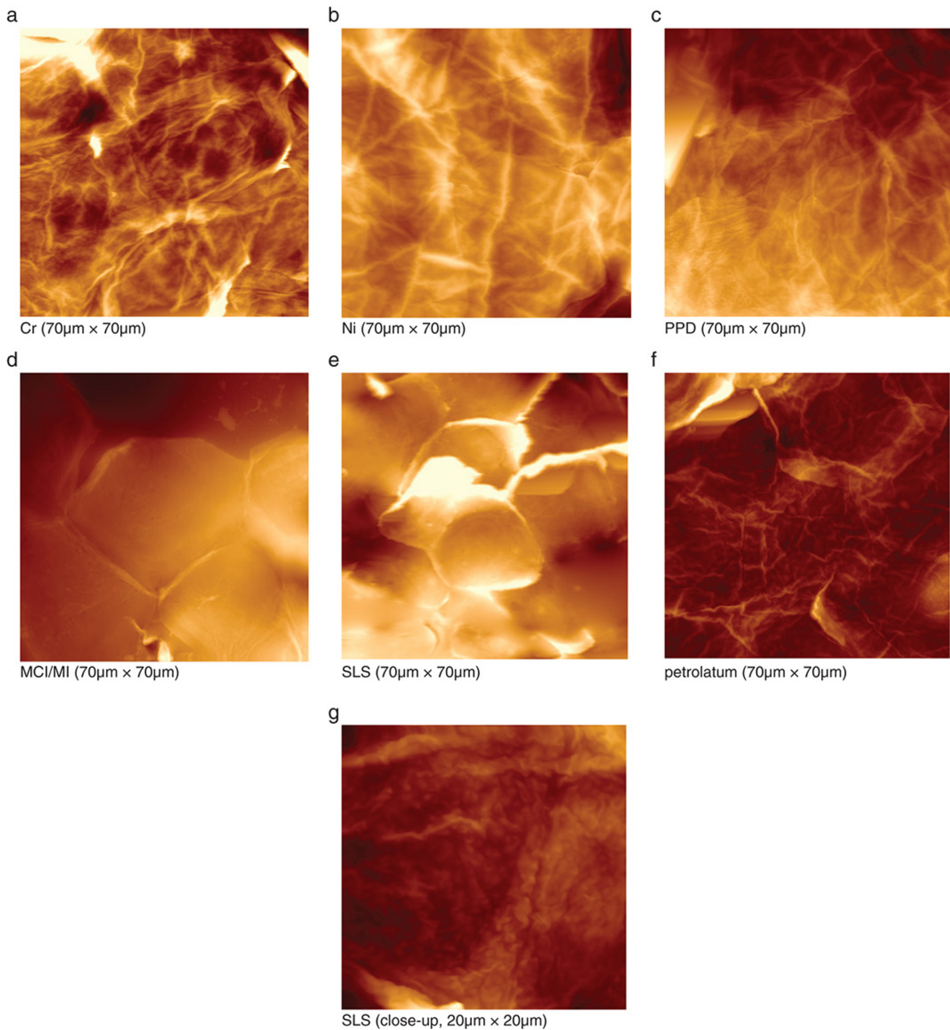


Fig. 3. (a–g) Atomic force microscopy images from stratum corneum samples collected on day 3. (a) Chromium. (b) Nickel. (c) p-Phenylenediamine. (d) Methylchloroisothiazolinone (MCI)/ methylisothiazolinone (MI). (e) Sodium lauryl sulfate (SLS). (f) Pet. (g) SLS, close-up. Images are three-dimensional representations; the brightness corresponds to the height of the imaged structures. At the microscale (70 × 70 µm), distinct morphological changes are seen for MCI/MI and SLS. On a close-up view of an SLS sample (20 × 20 µm) (g), circular nanosize objects can be distinguished on the corneocyte surface.

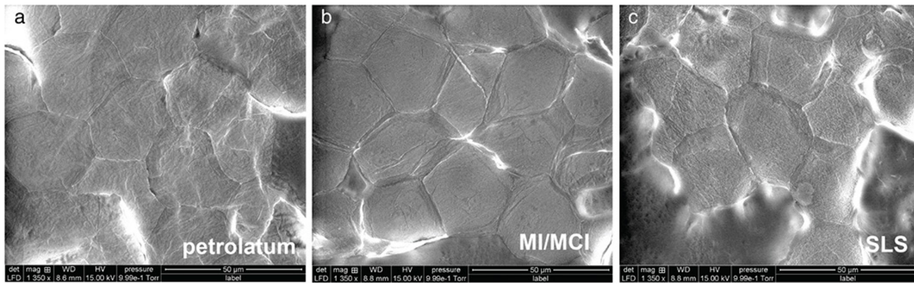


Fig. 4. Scanning electron microscopy images of corneocytes on day 3 after application of pet. (a), methylchloroisothiazolinone (MCI)/methylisothiazolinone (MI) (b) and sodium lauryl sulfate (SLS) at ×1350 magnification. Note a loose lateral association between the cells for the MCI/MI and the SLS test sites. Furthermore, circular nanosize objects can be distinguished on the corneocyte surface of the SLS test site.

Activities of stratum corneum BH, C-1, and plasmin

To explore whether allergens and SLS affect the activity of proteases that are involved in the degradation of NMF, we determined the activities of BH and C-1 in a limited number of samples. Furthermore, we included plasmin as an indicator of skin barrier damage. The activities of BH, C-1 and plasmin (Fig. 5a–c) were significantly higher in SLS-treated skin than in the corresponding pet. controls (BH, $p<0.01$; C-1, $p<0.05$; and plasmin, $p<0.01$). The allergens did not produce significant differences from the pet. controls, although MCI/MI showed a trend of increasing values for all three proteases (each $p=0.13$). The activities of all three proteases were negatively correlated with corresponding NMF levels. The respective Spearman correlation coefficients for BH, C-1 and plasmin amounted to –0.52 ($p<0.01$), –0.47 ($p<0.01$), and –0.58 ($p<0.001$).

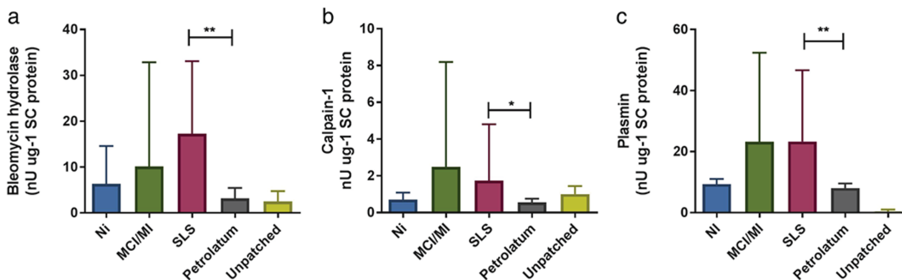


Fig. 5. Activities of bleomycin hydrolase (a), calpain-1 (b) and plasmin (c) in the stratum corneum samples of the skin sites tested with Ni (n=4), methylchloroisothiazolinone (MCI)/methylisothiazolinone (MI) (n=4), sodium lauryl sulfate (SLS) (n=8) and their corresponding pet. controls (n=8) and unpatched test sites (n=8). Data for bleomycin hydrolase are shown as median with interquartile range; those for calpain-1 (b) and plasmin (c) are shown as mean ± standard deviation. *$p<0.05$, **$p<0.01$; (a) Wilcoxon signed rank test; (b, c), paired two-sided t-test).

DISCUSSION

In the present study, we observed different effects of contact allergens and SLS on relevant properties of the epidermal barrier: the stratum corneum NMF levels, corneocyte surface morphology, and stratum corneum protease activities. NMF levels have previously been used as a skin barrier biomarker in AD and ICD. To the best of our knowledge, this is the first time that NMF levels have been investigated in ACD (27, 28). Recent experimental studies showed that skin irritants with different physicochemical properties, such as SLS, NaOH, fruit acids and aliphatic alcohols, significantly decrease the stratum corneum NMF levels (28, 29). This is in accordance with the findings from the present study, which show a significant reduction in NMF levels after exposure to 1% and 2% SLS. SLS may potentially affect NMF levels in different ways. As an alkaline compound, SLS may lead to an increase in the stratum corneum pH, which might affect the activity of stratum corneum proteases, including those involved in filaggrin degradation into NMF components. To explore this possibility, we measured, in a limited number of samples, the activity of the stratum corneum proteases BH and C-1, both of which known to be involved in breaking down filaggrin protein (30). The results suggest that it is unlikely that the decrease in NMF levels after SLS and MCI/MI treatment is caused by reduced activity of these enzymes, as their activities showed an opposite trend; protease activities were increased after SLS treatment, and an increasing trend was observed for MCI/MI ($p=0.07$). The activities of these proteases were negatively correlated with NMF levels, so the increased activity might be a feedback reaction to the reduced NMF levels. SLS is known to denature proteins of the cornified envelope, which may lead to the leakage of NMF components from the corneocytes (31). This could also occur for the proteases, causing better extraction from the corneocytes and/or intercellular lipids. As the cornified envelope acts as an attachment point for the intercellular lipids, disruption of the cornified envelope additionally affects skin barrier function (32, 33). Further evidence that the reduction in NMF levels may be caused by skin barrier damage is provided by increased plasmin activity following SLS treatment ($p<0.01$), which indicates a damaged skin barrier (18). A trend of increasing plasmin activity was also observed after MCI/MI treatment ($p=0.13$).

Among the tested allergens, only MCI/MI caused a significant reduction in NMF levels. It is not likely that the NMF decrease after MCI/MI treatment is attributable to downregulation of (pro)filaggrin, as the stratum corneum samples originate from the upper part of the stratum corneum (approximately to the upper third of the stratum corneum depth). The deeper stratum corneum layers containing potentially downregulated expression of filaggrin would require a further 14 days to reach the more superficial part of the stratum corneum from which the samples originated (34). As NMF is mainly located within the corneocyte, where filaggrin degradation occurs, it might be speculated that MCI/MI, like SLS, damages the cornified envelope, resulting in leakage of NMF. MCI (the most abundant component in the 3:1 MCI/MI mixture) is a small lipophilic compound with favourable physicochemical properties for percutaneous penetration across the membrane (Kow = 2.5; MW 111). It has been shown that MCI has corrosive properties and is retained in the epidermis, probably because of binding to the epidermal proteins (35, 36). In the present study, MCI/MI treatment resulted in dramatic changes in the microscale corneocyte structure, characterized by the smoother corneocyte surfaces, the hexagonal shape, pronounced cell borders, and the absence of apparent fibrous structures, that were distinctly different from the effects of the other three allergens. As recently reviewed by Weidinger and Novak, a compromised barrier may facilitate sensitization and increased penetration of contact allergens (37). In murine studies, the irritant effect of an allergen was shown to determine the strength of the contact hypersensitivity response (11). The decrease in NMF levels observed in this study might at least partly have been caused by irritant characteristics of MCI/MI, and this is perhaps an explanation for its high allergenic potency. Although Ni and Cr did not lead to significant changes in NMF levels, individuals with the highest clinical scoring showed the lowest NMF levels, suggesting that allergen-induced inflammation and decreases in NMF levels are associated.

At the topographical scale, quantified by the number of CNOs (expressed as the DTI), MCI/MI did not differ from the other allergens, and SLS was the only substance showing increased DTI values. Increases in DTI have recently also been found for other skin irritants, such as NaOH and lactic acid (C. Riethmuller, et al. pers. comm. 2016), suggesting that an elevated DTI is characteristic of skin irritation. The mechanisms that underlie the development of CNOs are not yet clear. In

another study by Riethmuller et al., AD patients with compound heterozygote or homozygote loss-of-function mutations in the filaggrin gene were shown to have increased numbers of CNOs (9). These patients lack filaggrin, which aggregates keratin filaments within the corneocyte and is also present in the cornified envelope. It might be suggested that, owing to the lack of filaggrin, the cornified envelope is more fragile and becomes more prone to structural changes resulting from osmotic pressure within the corneocytes caused by reduced NMF levels. Interestingly, this study shows that allergen-induced inflammation does not result in the formation of CNOs, regardless of the low NMF levels, indicating that their formation is multifactorial.

If the finding that the DTI does not change in ACD, as we show for four clinically relevant allergens, can be generalized to other allergens, the DTI might aid in differentiating ACD from ICD. However, it has to be noted that, in the occupational setting, mixed exposure to allergens and skin irritants is common, so results might be less clear than in this controlled study. Moreover, many allergens have irritant properties, so an increased DTI does not necessarily exclude ACD. Nevertheless, the investigated parameters can provide more insights in the aetiology of ICD and AD and the intrinsic irritant properties of contact allergens, which might support more targeted prevention in occupational settings.

CONCLUSION

Skin barrier characteristics, for example NMF levels and the number of nanosize objects (DTI) on the corneocyte surface, are useful for studying the effects of skin irritants and contact sensitizers on the epidermis. In contrast to the other allergens investigated, MCI/MI showed distinct effects on the skin barrier in terms of a significant decrease in NMF levels, similarly to SLS, and MCI/MI also had profound effects on corneocyte morphology; collectively, these findings suggest that skin barrier damage plays a role in the pathogenesis of MCI/MI contact allergy. The DTI seems to differentiate reactions to the tested allergens and to SLS, as the latter was the only agent that caused an increase in the DTI. Whether the effects on NMF levels and the DTI can be generalized to other skin irritants should be confirmed in further studies including irritants with different physicochemical properties.

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Stratum corneum profiles of inflammatory mediators in patch test reactions to common contact allergens and sodium lauryl sulfate

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ABSTRACT

Background: Recent studies have demonstrated allergen-specific differences in the gene expression of inflammatory mediators in patch tested skin.

Objectives: To determine levels of various inflammatory mediators in the stratum corneum (SC) after patch testing with common contact allergens and the skin irritant sodium lauryl sulfate (SLS).

Methods: In total, 27 individuals who had previously patch tested positive to nickel, chromium, methylchloroisothiazolinone/methylisothiazolinone (MCI/MI) or para-phenylenediamine were retested and then patch tested with SLS and petrolatum, with petrolatum serving as the patch test control. At 72 h, the test sites were clinically graded and the SC samples collected on adhesive tape.

Results: The levels of 18 of the 32 quantified mediators differed significantly from that of the control patches for at least one of the tested substances. SLS and MCI/MI induced the largest number of immunomediators. Interleukin (IL)-16 levels were significantly higher in patch test reactions in all allergens than they were in the controls, while no significant difference was detected for SLS. Furthermore, a strong negative correlation was found between strength of patch test reaction and IL-1α levels.

Conclusions: Cytokine profiles in the SC of patch tested skin did not show a distinct allergen-specific pattern. However, MCI/MI induced a larger and wider immune response than the other allergens, perhaps due to its potency as an irritant. The levels of IL-16 were significantly increased in patch test reactions to allergens but not to SLS; thus, they may help clinicians to differentiate between allergic contact dermatitis and irritant contact dermatitis.

BACKGROUND

Allergic contact dermatitis (ACD) and irritant contact dermatitis (ICD) are frequent, especially in occupational settings.(1-3) Although they share a similar clinical aspect, their pathogenesis and immunological mechanisms are quite different. ICD is the result of tissue damage by irritants, followed by an immunological response induced either by the direct effect of the irritant on keratinocytes or by the impairment of the skin barrier, resulting in the release of pro-inflammatory cytokines and chemokines.(4) Individuals with a skin barrier dysfunction, such as mutations in the filaggrin gene (FLG) or atopic dermatitis, are more susceptible to developing ICD(5). The barrier dysfunction facilitates the easy disruption of the stratum corneum (SC) (6). ACD is a type IV hypersensitivity reaction of the skin. It results from the activation of a previously sensitized immune system by a hapten (7). After penetrating the SC, haptens bind to epidermal proteins or peptides, which enables them to interact with dendritic cells (DCs), resulting in the activation of mainly CD4+ T cells. Metals such as nickel, cobalt and palladium are capable of directly activating DCs via Toll-like receptor-4, thereby inducing inflammatory signalling via nuclear factor-κB (8, 9). A complex range of inflammatory mediators is involved in both ICD and ACD. Many allergens can also induce skin irritation, which is associated with the activation of the innate immune system (10). It is likely that the early stages of ACD and ICD share cytokines associated with the innate immune system, such as interleukin (IL)-1 and tumour necrosis factor (TNF)-α (11-13). However, in the effector stage of ACD the adaptive immune system is mobilized and associated cytokines are upregulated (14). Vestergaard et al. have found that ICD and ACD show distinct histological responses, such as follicular spongiosis, which is present in early ICD but absent in early ACD reactions (15). Furthermore, differences in cytokines profiles between various allergens have also been reported, indicating that ACD cannot be regarded as a single entity (7). An evaluation of the differences in inflammatory profiles between ICD and ACD may give further insight into their pathogenesis and may help to differentiate between them. In addition, the detection of allergen-specific profiles may contribute to the development of more targeted anti-inflammatory drugs for treating ACD. Therefore, the aim of this study was to determine the SC profiles of inflammatory mediators induced by the common contact allergens nickel, chromium, methylchloroisothiazolinone / methylisothiazolinone (MCI/MI),

para-phenylenediamine (PPD) and an irritant frequently used in clinical research, sodium lauryl sulfate (SLS).

PATIENTS AND METHODS

Selection of patients

The experimental protocol followed the Declaration of Helsinki Principles and was approved by the Medical Ethics Committee of the University Hospital Centre, Zagreb. Patients with a previously positive (1+ or 2+) patch test reaction to either chromium, nickel, MCI/MI or PPD were identified from the database of the Department of Dermatology of the University Hospital Centre, Zagreb. Patients with a history of atopic dermatitis were excluded, and those with a positive reaction to more than one of the allergens studied were preferred over those with a single positive reaction. Eligible patients were invited to participate in the study and written informed consent was obtained from each participant. In total, 27 patients were finally included (24 female, average age 49 years).

Patch test and tape stripping

Each patient was patch tested with 8 × 8-mm Van der Bend patch test chamber® (Van der Bend, Brielle, the Netherlands) with: (i) one or two allergens dissolved in petrolatum (chromium, nickel, PPD) or water (MCI/MI); (ii) a 2% SLS solution (in water); and (iii) petrolatum. Petrolatum is used as a control although it is known that, very rarely, petrolatum can induce an allergic reaction and give a false-positive result when it is used as a vehicle.(16) An SLS concentration of 2% was chosen to ensure a strong irritant reaction, comparable with a 2+ contact allergic reaction expected in the allergens group. The following allergen preparations were used: PPD 1%, potassium dichromate 0·5% (both Almirall Hermal, Reinbek, Germany), nickel sulfate 5% and MCI/MI 0·01% (both SmartPractice Europe, Barsbuttel, Germany (MCI/MI ratio 3:1)). After 48 h the chambers were removed. The strength of the patch test reaction was graded after 72 h (1+, 2+ or 3+).(17) Each skin site was tape stripped using adhesive tape (1·5 cm2, D-Squame, CuDerm, Dallas, TX, U.S.A.).(18) Eight consecutive tapes from each skin site were collected. For the analysis, the sixth and seventh tape strips were used. The samples were extracted from the tapes

by 0·5 mL of phosphate buffered saline containing 0·05% Tween20 and sonicated for 15 min, as described previously (19). After vortexing, the extract aliquots were distributed in vials and stored at –80°C until analysis.

Multiplex analysis

The analysis of inflammatory mediators from the extracts was performed using a MESO QuickPlex SQ 120 assay (MSD, Rockville, MA, U.S.A.). The following cytokines and chemokines were included: eotaxin-1/CCL11, eotaxin-3/CCL26, granulocyte–macrophage colony-stimulating factor (GM-CSF)/CSF2, IL-1α, IL-1β, IL-1 receptor antagonist (RA), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8/CXCL8, IL-8 ha, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-16, IL-17a, IL-18, interferon (IFN)-γ-induced protein (IP)-10/CXCL10, monocyte chemoattractant protein (MCP)-1/CCL2, MCP-4/CCL13, macrophage-derived chemokine (MDC)/CCL22, macrophage inflammatory proteins (MIP)-1α/CCL3, MIP-1β/CCL4, thymus and activation regulated chemokine (TARC)/CCL17, TNF-α, TNF-β and vascular endothelial growth factor (VEGF). These inflammatory mediators were analysed using off-the-shelf panels (Human V-Plex Proinflammatory Panel 1 Kit, human IL-18, vascular and growth factor panel and a cytokine panel (MSD), according to the manufactures' instructions. All these kits use human antibodies. As the amount of SC on the tape varies, the concentrations of the mediators were normalized for the total amount of protein on the tape, which was determined with a Pierce Micro BCA protein assay kit (Thermo Fischer Scientific, Rockford, IL, U.S.A.) (20).

Statistics

Data were analysed by GraphPad prism® v. 6·07 (GraphPad Software, La Jolla, CA, U.S.A.). Data are shown as the mean ± SEM. The concentrations of the mediators on each skin site investigated were compared with the corresponding petrolatum control using a Wilcoxon matched-pairs rank test. Subsequently, a Kruskal–Wallis test followed by a Dunn's multiple comparison test was used to test the difference between allergens, which was also used to test the difference between allergens and SLS. The association between the clinical severity of the response and the levels of inflammatory mediators was assessed using Spearman's correlation test.

Power analysis
This was an exploratory study and the levels of most of the inflammatory mediators included in the SC have never previously been measured in ACD. In our previous study on cytokine and chemokine levels in patients with atopic dermatitis, we found 0·16 mean values (SD 0·18) pmol mL–1 of the proinflammatory cytokine IL-1β (21). For the power analysis, we assumed a twofold increase. On basis of these measurements, 10 participants per allergen were required for the generally recommended level of 0·80 (22).

RESULTS

Overall, 25 individuals had a total of 34 positive patch reactions: 11 to nickel, 11 to chromium, eight to MCI/MI and four to PPD. One PPD patient had a severe reaction, so that sampling was impossible. Four of the patients tested with chromium (n = 1), MCI/MI (n = 1), nickel (n = 1) and PPD (n = 1) did not show an allergic response to the relevant allergen. The average strength of the patch test reactions was 1·5 + nickel, 1·5 + chromium, 1·8 + (MCI/MI), 2·5 + (PPD) and 1·6 + (SLS). Compared with the corresponding petrolatum controls, 18 of the 32 quantified inflammatory mediators showed significant levels of change for at least one of the investigated allergens or SLS (Fig. 1 and 2). In general, the largest changes compared with petrolatum controls were observed for chemotactic cytokines and T-(memory) cell recruiters (Fig. 2). The levels of TARC/CCL17, MDC/CCL22, MCP-1/CCL2 and MIP-1β/CCL4 in patch test reactions to both SLS and allergens were elevated, although a level of significance of $P < 0·05$ was not attained in all cases. In contrast to chemokines, IL-1 cytokines and other mediators of the innate immune system showed the opposite trend. The concentration of IL-1α was significantly decreased in test reactions to SLS as well as to allergens, with the exception of PPD, to which, however, only three individuals were positive. Other mediators with decreased levels were IL-1β (SLS and nickel), IL-1RA (SLS), IP-10 (SLS), IL-2 (SLS), IFN-γ (MCI/MI) and IL-7 (MCI/MI).

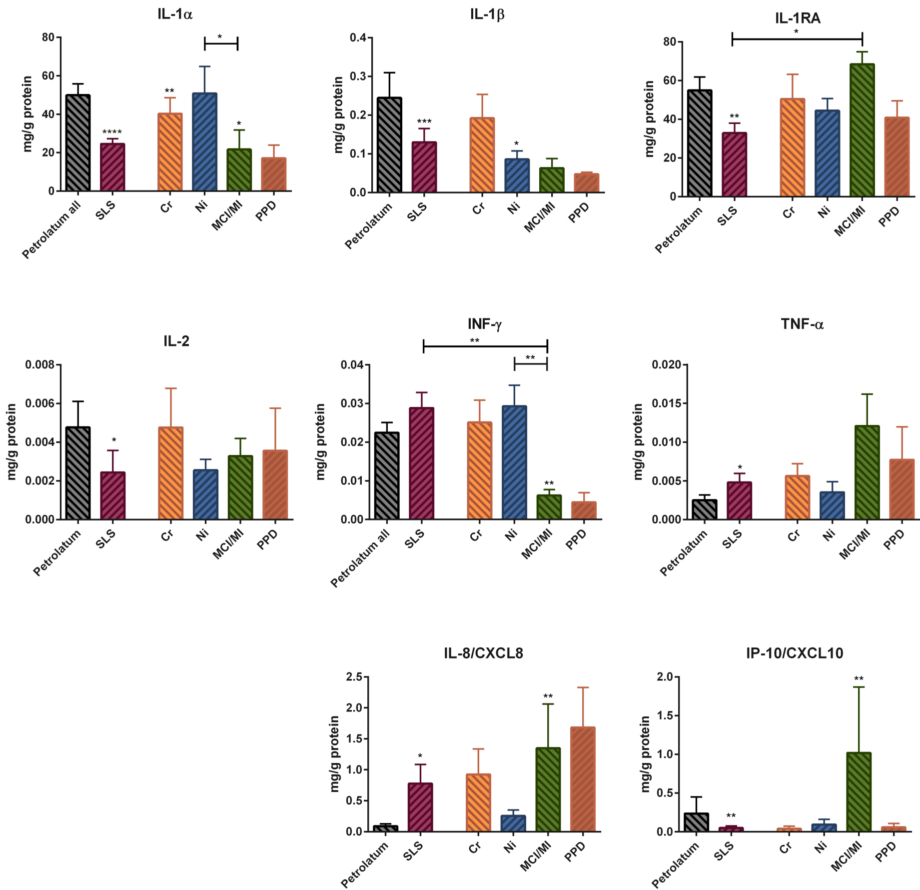

Fig. 1. Concentration of interleukin (IL)-1 and innate immunity related cytokines and chemokines of skin sites tested with chromium, nickel, methylchloroisothiazolinone/methylisothiazolinone (MCI/MI), para-phenylenediamine (PPD), sodium lauryl sulfate (SLS) and petrolatum. The results of petrolatum and SLS are averaged for all patients (n = 25). Data are given as mean with standard error of means (SEM). A Kruskal–Wallis test followed by Dunn's multiple comparison test was used to investigate differences between allergens as well as between allergens and SLS. Significant differences between allergens or SLS and their corresponding petrolatum controls, are given directly above the SEM error bar (Wilcoxon matched-pairs signed-rank test). *P < 0·05, **P < 0·01, ***P < 0·001. IFN-γ interferon-γ; TNF-α, tumour necrosis factor-α.

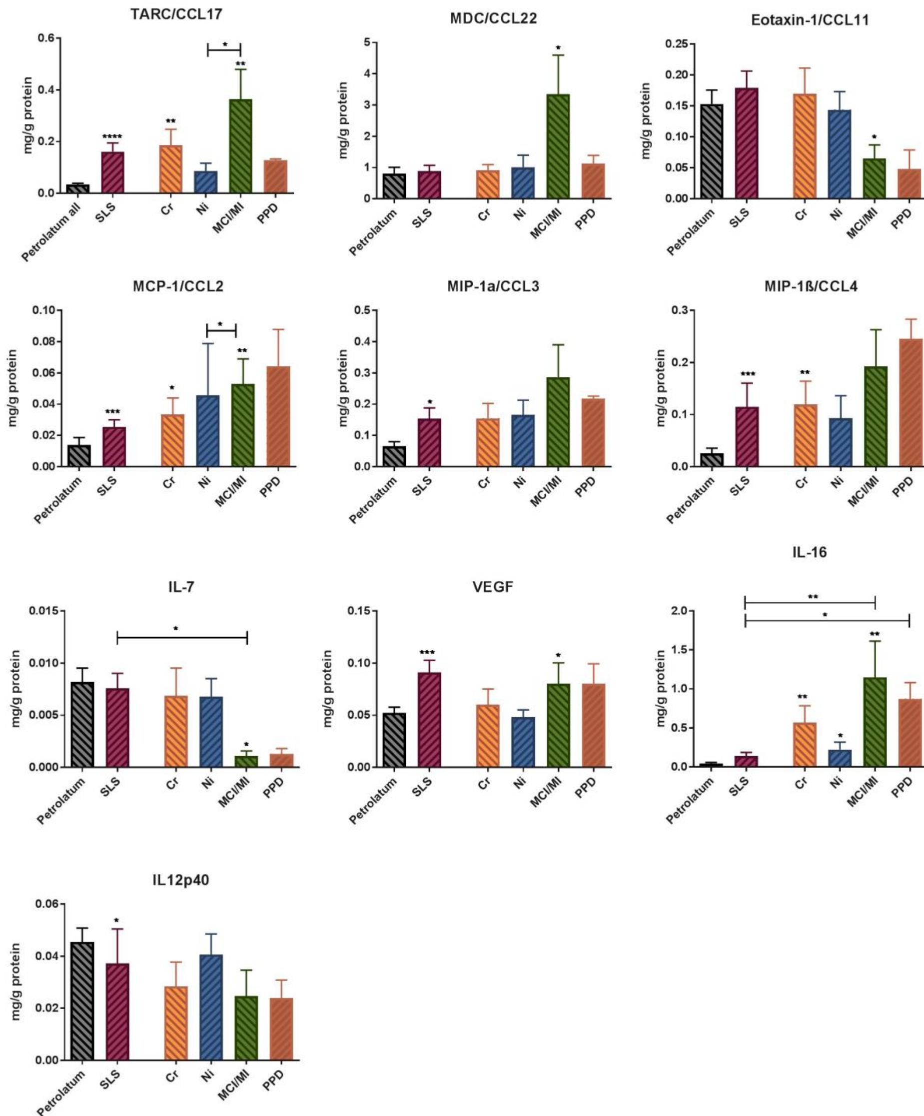

Fig. 2. Concentration of chemotactic and T-cell proliferation related cytokines and chemokines from the skin sites tested with Cr, Ni, methylchloroisothiazolinone/methylisothiazolinone (MCI/MI), para-phenylenediamine (PPD), sodium lauryl sulfate (SLS) and petrolatum. The results of petrolatum and SLS are averaged for all patients (n = 25). Data are given as mean with standard error of means (SEM). A Kruskal–Wallis test followed by Dunn's multiple comparison test was used to investigate differences between allergens as well as between allergens and SLS. Significant differences between allergens or SLS and their corresponding petrolatum controls, are given directly above the SEM error bar (Wilcoxon matched-pairs signed-rank test). *P < 0·05, **P < 0·01, ***P < 0·001. TARC, thymus and activation regulated chemokine; VEGF, vascular endothelial growth factor.

Table 1. Correlation of patch test reaction and the levels of inflammatory mediators

	Spearman's correlation coefficient, r	P-value
Eotaxin-1/CCL11	-0,30	0,09
Eotaxin-3/CCL26	-0,28	0,12
GM-CSF/CFS2	-0,29	0,10
IL-1α	-0,67	**0,0000**
IL-1RA	0,57	**0,0009**
IL-1ß	-0,07	0,71
IL-2	0,29	0,10
IL-4	0,46	**0,01**
IL-5	-0,31	0,08
IL-6	0,56	**0,001**
IL-7	-0,06	0,73
IL-8	0,74	**0,0000**
IL-8 (HA)	0,56	**0,001**
IL-10	-0,38	**0,03**
IL-12 p40	-0,64	**0,0001**
IL-12 p70	-0,68	**0,0000**
IL-13	-0,07	0,68
IL-15	-0,39	**0,02**
IL-16	0,67	**0,0000**
IL-17A	-0,23	0,19
IL-18	0,51	**0,003**
IFN-γ	-0,48	**0,004**
IP-10/CXCL10	0,57	**0,001**
MCP-1/CCL2	0,80	**0,0000**
MCP-4/CCL13	-0,36	**0,04**
MDC	0,36	**0,04**
MIP-1a/CCL3	0,42	**0,02**
MIP-1ß/CCL4	0,64	**0,0001**
TARC/CCL17	0,64	**0,0001**
TNF-α	0,60	**0,0002**
TNF-β	-0,39	**0,02**
VEGF	0,53	**0,002**

GM-CSF/CFS2:granulocyte-macrophage colony-stimulating factor, IL: interleukin, IL-1RA: IL-1 receptor antagonist,INF-γ: Interferon-γ, IP-10/CXCL10: Interferon gamma-induced protein-10, MCP-1/CCL2:Monocyte Chemoattractant Protein-1, MCP-4/CCL13: Monocyte Chemoattractant Protein-4, MDC/CCL22: Macrophage-derived chemokine MIP-1α/CCL3: Macrophage Inflammatory Protein-1 α, MIP-1β/CCL4: Macrophage Inflammatory Protein-1 β, TARC/CCL17: Thymus and activation regulated chemokine, TNF-α: Tumor necrosis factor-α, TNF-β:Tumor necrosis factor-β ,VEGF: Vascular endothelial growth factor

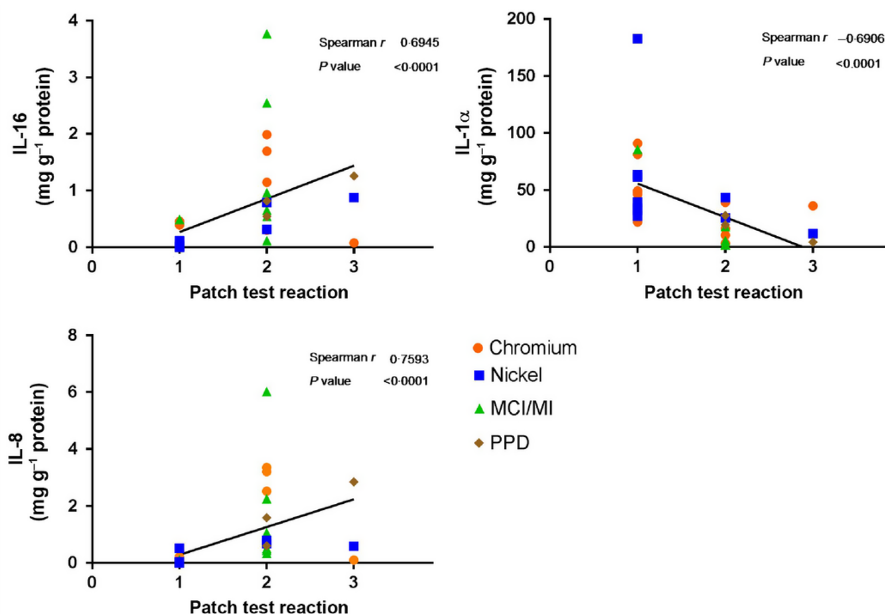

Fig. 3. Correlation between the strength of patch test reactions and the levels of IL-16, IL-1α and IL-8.

DISCUSSION

The present study shows that contact allergens and SLS induce changes in a large number of immunomediators in the SC, which can be detected by a relatively noninvasive procedure. Overall, the profiles of most mediators in the patch test reactions were similar among the investigated allergens and SLS, reflecting common inflammatory pathways. However, the levels of IL-16 seemed to be indicative for ACD, as they were significantly increased in patch test reactions to all allergens, but not to SLS. Here, it may be noted that the increase of IL-16 levels did not reach significance for PPD, probably due to small number of patients in this study (n = 3). IL-16 plays a major role in skin hypersensitivity by the chemoattraction of CD4 + T cells (23). Masuda et al. showed that IL-16 is produced by epidermal cells, especially keratinocytes, during the sensitization and elicitation phases of hapten-induced contact hypersensitivity. In agreement with the results of the present study, Masuda et al. found that the application of haptens and not of primary irritants or of the control vehicle induces IL-16 production in the skin. Interestingly, Reich et

al. showed that polymorphisms in gene-encoding IL-16 influence susceptibility to contact allergy (24).

When comparing allergens, patch test reactions to MCI/MI consistently showed the largest difference compared with the petrolatum controls, although similar patch test reactions were induced by the allergens investigated. Of the 32 mediators that could be determined in the samples, 11 showed a significant difference in MCI/MI. Most of these belong to the keratinocyte-derived cytokines (IL1-α, IL-16, TARC/CCL17, MCP-1/CCL2, IL-8/CXCL8, eotaxin-1/CCL11, IP-10/CXCL10). Among Th2-related mediators, concentrations of MDC/CCL22 and TARC/CCL17 were significantly higher than the petrolatum controls in patch test reactions to MCI/MI (MDC/CCL22) and MCI/MI and chromium (TARC/CCL17). In a recent study by Dhingra et al. increased mRNA expression of MDC/CCL22 and TARC/CCL17 was detected in biopsies from patch test reactions to nickel, rubber, fragrance mix, cobalt and potassium dichromate (7). An amplification loop has been suggested for TARC/CCL17 and MDC/CCL22, as both are regulated by IL-4 and increase their production by recruiting IL-4 releasing Th2 cells.(14) In the present study, we found an increase in levels of TARC/CCL17 in patch test reactions to MCI/MI and chromium but not to nickel, as shown by Dhingra et al (7). This discrepancy could be explained by the lower reaction severity to nickel in the present study, compared with that of Dhingra et al. (1·5+ vs. 2·1+, respectively) (7).

The levels of monocyte-derived chemotactic mediators (MCP-1, MIP-1a, MIP-1b) were increased in patch test reactions to allergens as well to SLS. Although not all these differences reached a level of significance, this may indicate there are common inflammatory pathways for allergens and irritants. While the concentrations of most mediators were increased in patch test reactions compared with their corresponding petrolatum controls, decreased values were found for several mediators, mainly those that are representatives of innate immunity, including IFN-γ (MCI/MI), IL-1α (chromium, MCI/MI, SLS), IL-1β (nickel, SLS), IL-7 (MCI/MI), IP-10 (SLS) and IL-1RA (SLS). IL-1α levels in the SC have previously been shown to decrease after exposure to various skin irritants, which is in agreement with the effect of SLS found in the present study (25, 26). The opposite, however, an increase of IL-1α, has been found in the epidermis (27). This discrepancy in the pattern of IL-1α levels

between these two skin strata should probably be sought in the contribution of the preformed pool of IL-1α in the SC. Damage of the SC causes the release of these primary cytokines stored in the corneocytes and their gradual depletion in the SC intercellular matrix. This is supported by the negative correlation between IL-1α and the strength of patch test reactions. Therefore, it may be speculated that the significant decrease of IL-1α in patch test reactions to chromium and MCI/MI may, at least partly, be caused by the inherent skin damaging properties of these allergens (28).

The magnitude of changes of the inflammatory mediators in patch test reactions to MCI/MI as compared with other allergens may also be explained by its skin damaging properties, which are associated with skin barrier damage and the induction of innate immune responses. Esser et al. have shown a correlation between the ability of an allergen to activate innate immune responses and its allergenic potency (29, 30). MCI is a lipophilic molecule and is small enough to cross the epidermal barrier (Kow = 2·5; MW 111 Da). Furthermore, in a previous study we demonstrated that MCI/MI induces morphological cell changes that are distinctly different from that of other allergens (Koppes et al., submitted for publication) suggesting it has effects on the integrity of the skin barrier.

Recently, the important role of innate immune system in ACD has been revealed. Interestingly, metals such as nickel, cobalt and palladium are capable of directly activating innate immune signalling pathways via TLR-4 and nuclear factor-κB.(8, 9) However, not all metals are able to do this, for instance, chromium and iron cannot.(9) The present study did not detect major differences between nickel and other allergens; however, this may be due to the fact that sampling was limited to the SC. To gain more insight into the mechanism biopsy samples would be more suitable.

Some limitations of the present study should be considered. The levels of inflammatory mediators are probably time and concentration dependent. For example, an initial decrease of IL-1α levels is induced by damage to the skin barrier and the gradual depletion of the preformed pool of IL-1α in the SC. However, as a consequence of inflammation, de novo synthesis of IL-1α will occur in epidermis,

which will result in an increase in IL-1α levels. Thus, the actual concentration of IL-1α depends on the kinetics of these two processes. A longer study period and the measurement of cytokine profiles at more time points and with a series of concentration dilutions would allow for the detection of possible temporal and concentration variations existing between allergens as well as between individuals. To compensate for interindividual variation in immune response to various allergens it would be interesting to study polysensitized patients.

Moreover, we looked at the concentrations in the upper part of the skin, the dead corneocytes, and not in the viable epidermis where many inflammatory mediators are formed and where they primarily exert their activity; although, as shown here, the SC sampling method offers a non-invasive approach to measuring a wide range of inflammatory mediators in vivo in the skin. Lastly, objective assessment of patch test reaction, for example, by using skin bioengineering techniques (such as transepidermal water loss, erythema meter, imaging techniques, etc.) may improve the interpretation of the results. Nevertheless, current guidelines on allergy patch testing are based on clinical scoring performed by a trained dermatologist (17).

In conclusion, this study suggests that differences in SC profiles of inflammatory mediators exist between ACD and ICD as well as between the allergens investigated, although most of them show similar patterns. MCI/MI induces larger and broader immune responses than the other tested allergens. The decreased levels of IL-1α in patch test reactions to MCI/MI and chromium may suggest their inherent irritant capacities play a role. Of the mediators investigated, IL-16 is the most promising marker in the SC for differentiating between ICD from ACD. Additional investigations are needed to evaluate the possible influence of allergen or irritant concentration and interindividual variations.

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CHAPTER 4

Biomarkers for atopic dermatitis, clinical studies

4.1

Filaggrin breakdown products determine corneocyte conformation in patients with atopic dermatitis

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ABSTRACT

Background: Loss-of-function (LOF) mutations in the filaggrin gene (FLG) are a well-replicated risk factor for atopic dermatitis (AD) and are known to cause an epidermal barrier defect. The nature of this barrier defect is not fully understood. Patients with AD with FLG LOF mutations are known to have more persistent disease, more severe disease, and greater risk of food allergies and eczema herpeticum. Abnormalities in corneocyte morphology have been observed in patients with AD, including prominent villus-like projections (VP); however, these ultrastructural features have not been systematically studied in patients with AD in relation to FLG genotype and acute and convalescent status.

Objective: We sought to quantitatively explore the relationship between FLG genotype, filaggrin breakdown products (natural moisturizing factor (NMF)), and corneocyte morphology in patients with AD.

Methods: We studied 15 children at first presentation of AD and after 6 weeks of standard therapy. We applied atomic force microscopy to study corneocyte conformation in patients with AD stratified by FLG status and NMF level. By using a new quantitative methodology, the number of VPs per investigated corneocyte area was assessed and expressed as the Dermal Texture Index score. Corneocytes were also labeled with an anti-corneodesmosin antibody and visualized with scanning electron microscopy.

Results: We found a strong correlation between NMF levels and Dermal Texture Index scores in both acute and convalescent states (respective $r = -0.80$ and -0.75, $P < .001$ and $P = .002$). Most, but not all, VPs showed the presence of corneodesmosin abundantly all over the cell surface in homozygous/compound heterozygous FLG patients and, to a lesser extent, in heterozygous and wild-type patients.

Conclusions: NMF levels are highly correlated with corneocyte morphology in patients with AD. These corneocyte conformational changes shed further insight into the filaggrin-deficient phenotype and help explain the barrier defect in patients with AD with FLG LOF mutations.

INTRODUCTION

A recent study using scanning electron microscopy (SEM) showed abnormal surface structures of corneocytes from patients with atopic dermatitis (AD) that the authors named as villus-like projections (VPs)(1). Similar structures were described as protrusions (2) and rough corneocytes (3). Bead- or nipple-like elevations have also been observed in abdominal (4), cheek, and plantar corneocytes (5), as well as in 2,4,6-trinitro-1-chlorobenzene–sensitized hairless mice (6). They seem to be absent in forearm healthy skin (2) or exclusively present in the periphery of corneocytes from the inner upper arm (5). A villous appearance with an irregular fine nodular surface pattern has also been shown in patients with ichthyosis vulgaris and in squamous cells from patients with psoriasis (7). In most of these studies, VPs were observed qualitatively, and only in one study were the VPs determined semiquantitatively (5), suggesting a correlation between VP numbers and skin barrier function, as assessed based on transepidermal water loss (TEWL). The nature and cause of VPs on the stratum corneum (SC) surface is not well understood. Several mechanistic suggestions have been proposed for the occurrence of VPs, including disturbed organization of the cytoskeleton on desmosome disruption, immature and fragile cornified envelopes (CEs), and attachment sites of desmosomes (1,2,6,8). Rankl et al. (4) showed that staining for corneodesmosin protein mostly matched the beadlike topographic features, although not all of these structures showed corneodesmosin immunoreactivity.

Because filaggrin (gene name FLG) is a component of the CE (9) and filaggrin-deficient corneocytes display gene dose-dependent alterations in CE structure,(10) we aimed to investigate the relationship between VPs on the SC with levels of filaggrin degradation products in children with AD. The filaggrin degradation products histidine, pyrrolidone-5-carboxylic acid, and urocanic acid can be used as an indirect measure of filaggrin expression that is dependent not only on FLG loss-of-function (LOF) mutations but also on other factors, including genetic factors, filaggrin degradation pathway factors (11), and both local and systemic inflammation (12, 13). Furthermore, because these products are the main source of the constituents of natural moisturizing factor (NMF) and contribute to SC hydration, their levels might influence structural conformation of the CE.

In this study we used high-resolution atomic force microscopy (AFM) to investigate the topography of corneocytes in patients with AD in relation to FLG genotype and levels of filaggrin degradation products. AFM provided nanoscale 3-dimensional resolution of native corneocytes collected by means of adhesive tape stripping. AFM involves a sharp tip at the end of a soft silicon cantilever touching and scanning the surface of a sample. Because of the change in topography, the deflection of the cantilever is transformed into a 3-dimensional image. Recently, we have developed and evaluated a software method through which VP surfaces on the corneocyte can be quantitatively determined (the Dermal Texture Index (DTI); technical manuscript in preparation, full details available on request from the authors (CR)). We measured DTI scores in corneocytes of children with active AD at first presentation and after 6 weeks of standard topical therapy with skin care regimens and appropriate topical steroids. In addition to DTI scores, we measured NMF levels; skin barrier function, as assessed based on TEWL; and severity of AD based on the SCORAD score (14). Next, we investigated the distribution of corneodesmosome remnants by using SEM and corneodesmosin immunocytochemical labeling.

METHODS

Study population

Patients with AD were recruited from a dedicated AD clinic in a tertiary referral center. An experienced pediatric dermatologist (ADI, MAM, or both) made the diagnosis and recorded the disease phenotype. All patients met the United Kingdom diagnostic criteria (15) and had moderate or severe disease. Exclusion criteria from the study included patients who had pyrexial illness in the preceding 2 weeks; those who had received immunosuppressive systemic therapy, such as oral corticosteroids, in the preceding 3 months; and those whose ancestry was not exclusively Irish (4/4 grandparents). The study was conducted in accordance with the Helsinki Declarations and was approved by the Research Ethics Committee of Our Lady's Children's Hospital, Dublin, Ireland. Full written informed consent was obtained from all patients' parents. The children were treatment naive at presentation and were assessed at first presentation and after 6 weeks of standard treatment with skin care regimens and appropriate topical steroids.

Severity assessment

The severity of a patient's AD was assessed by using the SCORAD index. A single dermatologist performed all SCORAD measurements. SCORAD is one of the most valid and reliable instruments to assess the clinical severity of AD(16). SCORAD is a composite score on a scale of 0 to 103 that incorporates both objective physicians' estimates of extent and severity and subjective patient or parental assessments of itch and sleep loss(17). SCORAD is internally consistent, responsive, and interpretable and has adequate interobserver reliability (Cohen $\kappa = 0.82$, $P < .001$) (18).

Biophysical measurements of the SC

All topical therapies, including emollients, were withheld from the patients' upper limbs for 48 hours before skin biophysical measurements were performed. All measurements were done in standardized environmental conditions (room temperature, 22°C to 25°C; humidity levels, 30% to 35%). Before testing, the patient's forearm was acclimatized to this controlled environment for a minimum of 10 minutes. All measurements were done by the same investigator and on a clinically unaffected area of skin on the volar forearm. TEWL was determined by using a Tewameter 300 (Courage and Khazaka Electronic GmbH, Cologne, Germany).

Sampling of the SC by using tape stripping

The SC was sampled by using the previously described method.19 A clinically unaffected site on the patient's volar forearm, where the TEWL measurement was also taken, was used for SC sampling. Circular adhesive tape strips (3.8 cm2, D-Squame; Monaderm, Monaco, France) were attached to volar forearm skin and pressed for 10 seconds with a constant pressure (225 g/cm2) by using a D-Squame Pressure Instrument D500 (CuDerm, Dallas, Tex). The tape strip was then gently removed and placed in a closed vial. Eight consecutive tape strips were sampled, all from the same site. The tape strips were immediately stored at –80°C until analysis.

FLG genotyping

All patients were screened for the 9 most common FLG mutations found in the Irish population (R501X, Y2092X, 2282del4, R2447X, S3247X, R3419X, 3702X, S1040X, and G1139X) from DNA extracted from a blood sample. The methods used have been previously described. 20

NMF determination

NMF analysis was performed on the fifth consecutive strip, according to methods described in detail elsewhere (19). Briefly, each tape strip was extracted with 25% (wt/wt) ammonia solution. After evaporation of the ammonia extract, the residue was dissolved in 250 μL of pure water and analyzed by using HPLC. The NMF concentration was normalized for the protein amount determined with a Pierce Micro BCA protein assay kit (Thermo Fischer Scientific, Rockford, Ill; referred to as the Pierce assay) to compensate for a variable amount of the SC on the tape.

Skin nanotexture analysis (DTI)

Corneocytes from patients were analyzed with AFM, as previously described.21 Briefly, in each case the seventh tape strip was subjected to AFM measurements carried out with a Multimode AFM equipped with the Nanoscope III controller and software version 5.30sr3 (Digital Instruments, Santa Barbara, Calif). Silicon-nitride tips on V-shaped gold-coated cantilevers were used (0.01 N/m, MLCT; VEECO, Mannheim, Germany). Imaging was performed at ambient temperature with forces of less than 1 nN and 1 to 3 scan lines per second (1-3 Hz) with 512 × 512 pixel resolution. For texture analysis, subcellular scan areas of 20 μm2 were recorded. Ten random images were analyzed from each sample. Topographic cell-surface data were analyzed with the nAnostic method, applying custom-built proprietary algorithms (Serend-ip GmbH, Munster, Germany). The principle of this method has been described elsewhere (22). Briefly, each nanostructure protruding from the mean surface level was morphometrically evaluated. These objects were then filtered by size and shape through computer vision. At this stage, only structures of positive local deviational volume smaller than 500 nm in height and with an area of less than 1 μm2 were considered. The DTI score is the count of identified objects per image (a mean value from 10 randomly recorded images).

Corneodesmosin immunolabeling

Corneocytes from 3 patients with different FLG mutation genotypes collected on D-squame discs were labeled with an anti-corneodesmosin antibody and visualized with SEM, as described elsewhere (23). Briefly, the native cells exposed to the mouse mAb to corneodesmosin (diluted 1:100; Abnova, Jhongli City, Taiwan) were immunogold labeled with the goat anti-mouse Ultra Small probe (0.8 nm, diluted

1:10; Aurion, Wageningen, The Netherlands). The labeling was silver enhanced with the BBI kit (BBI Solutions, Cardiff, United Kingdom), and the samples, after dehydration in ethanol, were observed in a partial vacuum by using secondary and backscattered electron detection modes.

Statistics

Data were checked for normality by using the Shapiro-Wilk test. The relationship between DTI scores and clinical parameters was tested either by using the Pearson correlation test or Spearman rank correlation if the variables were not normally distributed or the relationship between the variables was not linear. Because of skewed distribution, DTI scores and NMF levels were log-transformed before linear regression analysis. Differences in the investigated parameters (DTI score, TEWL, SCORAD score, and NMF level) between 2 measurement points (0 and 6 weeks) were tested by using the paired 2-sided t test (NMF and DTI score) or by using the Wilcoxon matched signed-rank test in the case of deviation from normal distribution (TEWL and SCORAD score). Differences in DTI scores among 3 FLG genotypes were tested by using the Kruskal-Wallis test, followed by Dunn multiple comparison. The relationship between the DTI score as a dependent variable versus the SCORAD score and NMF level as dependent variables was tested by using a linear regression model with SPSS software (version 22; IBM, Somers, NY). For other statistical analyses, GraphPad Prism version 5.00 software for Windows (GraphPad Software, San Diego, Calif) was used. A P value of less than .05 was considered statistically significant.

RESULTS

Demographic characteristics of the investigated populations and values of measured parameters are presented in Table 1. Fig 1 shows representative AFM images of the surfaces of corneocytes sampled from patients with AD with 3 different FLG mutation genotypes. On simple inspection, VP numbers were clearly increased in carriers of FLG mutations. The DTI score, which quantifies the number of VPs per investigated surface area, showed a trend toward higher mean values in the carriers of FLG mutations compared with FLG wild-type subjects at week 0 (427.0

and 336.2, respectively) and after 6 weeks of therapy (296.6 and 224.3, respectively), although the differences did not reach statistical significance (data not shown). At week 6, however, the DTI in the FLG–/– group was significantly higher than in the FLG+/+ group (respective median values were 496.8 and 208.2, respectively; $P < .05$, as assessed by using the 2-tailed Mann-Whitney test), whereas there was no significant difference in SCORAD scores between these 2 FLG genotype groups. When DTI scores were plotted against the NMF levels, a significant correlation was observed at both weeks 0 and 6 (respective correlation coefficients amounted to –0.80 and –0.75, $P < .001$ and $P = .002$, respectively; Fig 2, A). VP numbers reach a plateau at normal NMF levels (approximately 0.5 mmol/g protein).

Table 1. Demographic data of patients, DTI and NMF assessed at baseline (treatment naïve) and after 6 weeks of treatment

Sex	**Age (months)**	**Number of *FLG* mutations**	**SCORAD**		**TEWL (g/m² hr)**		**DTI (AU)**		**NMF (mmol/g)**	
			Week 0	**Week 6**	**Week 0**	**Week 6**	**Week 0**	**Week 6**	**Week 0**	**Week 6**
M	9.3	0	28.9	22.5	12.7	14.1	172.0	139.8	0.41	0.63
M	5.3	0	62.5	14.6	27.1	12.9	434.4	172.7	0.26	0.32
M	5	0	27.9	4.5	27.3	11.6	397.5	208.2	0.24	0.75
M	9	0	53.5	59.3	17	15.1	411.2	363.5	0.37	0.31
M	9.5	0	26.9	21.8	13.8	8.8	265.8	237.3	0.41	0.47
M	8.5	0	47.4	17.9	14.5	13.6	173.4	185.7	0.43	0.29
M	2.3	1	42.1	20.3	13.1	12.7	170.1	249.3	0.25	0.26
F	57.3	1	38.5	24.6	11.5	7.7	122.5	116.3	0.43	0.52
M	10	1	55.1	20.9	14.9	8.3	660.6	96.3	0.19	0.65
F	6	1	57.1	8.2	15.9	14.6	623.0	421.0	0.25	0.28
M	6.25	1	35.3	56.6	13.4	10.7	259.1	219.9	0.30	0.19
M	8.25	1	28.6	9.2	13.7	13.4	372.8	196.3	0.33	0.38
M	6.8	2	70.5	66.2	41.6	22.3	412.4	617.3	0.11	0.18
M	28	2	61.2	28.1	22.8	14.7	822.5	496.8	0.06	0.11
M	5.75	2	42	13.1	34.4	25.4	653.8	367.2	0.13	<LLQ*

* under the lower limit of quantification

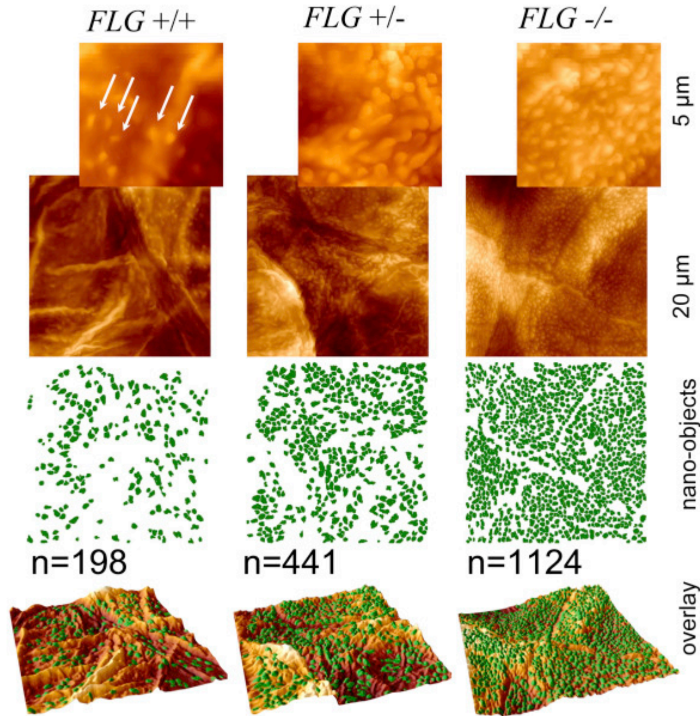

Fig. 1. Representative AFM images of the surfaces of corneocytes sampled from patients with AD with 3 different FLG mutation genotypes: +/+, wild-type homozygote; +/–, heterozygote for FLG LOF mutation; –/–, compound heterozygote or homozygote for FLG LOF mutation. On simple inspection, numbers of VPs were clearly increased in carriers of FLG mutations.

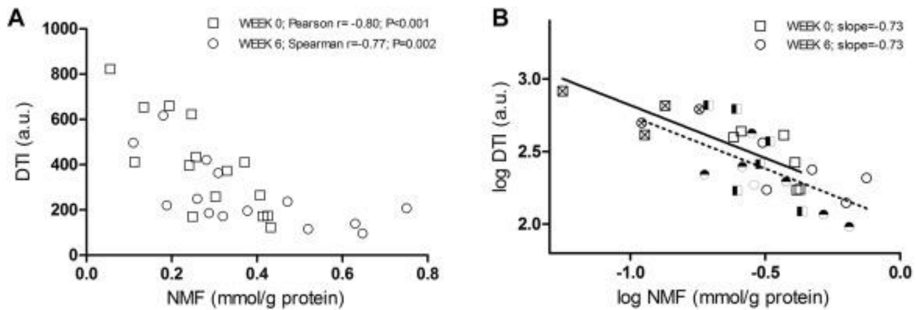

Fig 2. A. Relationship between DTI scores and NMF levels at first presentation of disease (squares) and after 6 weeks of topical therapy with skin care regimens and appropriate topical steroids (circles), with corresponding correlation coefficients (r). B, Linear regression analysis of log-transformed DTI scores and NMF levels at first presentation of disease (squares) and after 6 weeks of topical therapy with skin care regimens and appropriate topical steroids (circles). □ ○, Patients with AD wild-type with respect to FLG LOF mutations; ◓, ◨, patients with AD heterozygous for FLG LOF mutations; ⊗☒, patients with AD homozygous or compound heterozygous for FLG LOF mutations.

Regression analysis of log-transformed values of DTI scores and NMF levels showed almost identical regression coefficients for 0 and 6 weeks (–0.726 and –0.730, respectively; Fig 2, B). The relationship of DTI scores with TEWL and SCORAD scores (see Fig E1 in this article's Online Repository at www.jacionline.org) was weaker than that of DTI scores and NMF levels. Furthermore, in a linear regression model with the DTI score as a dependent variable versus the NMF level and SCORAD score, only NMF levels showed a significant effect on DTI scores (P = .005 and .015, respectively, for weeks 0 and 6; see Table E1 in this article's Online Repository at www.jacionline.org).

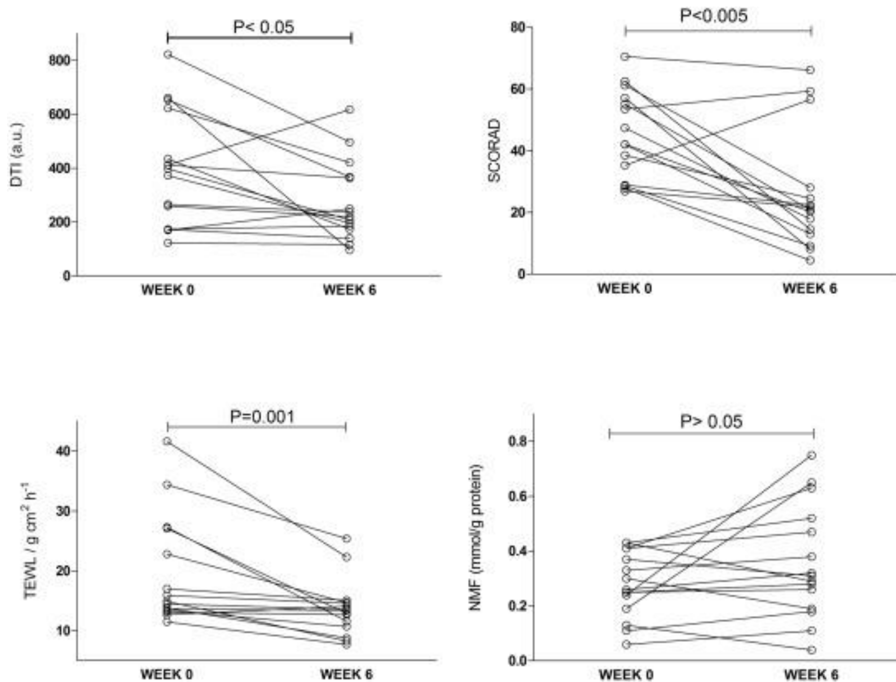

Fig. 3. TEWL, SCORAD score, DTI score, and NMF level at first presentation of disease and after 6 weeks of topical therapy with skin care regimens and appropriate topical steroids.

Changes in DTI scores, NMF levels, TEWL, and SCORAD scores measured at the first presentation of disease and after 6 weeks of standard topical therapy with skin care regimens and appropriate topical steroids are shown in Table I and Fig 3. Although the skin barrier, as measured based on TEWL and SCORAD scores, significantly improved after 6 weeks of therapy, NMF levels and DTI scores did not mirror these improvements in all patients.

Representative SEM images of D-Squames after immunogold labeling are shown in Fig 4 for 3 patients with AD of different FLG genotype status. The high abundance of VPs on the corneocytes obtained from an FLG−/− subject (Fig. 4, C) was confirmed by means of SEM. The VPs were decorated at their tips with corneodesmosin labeling, indicating the presence of disrupted corneodesmosome structures (Fig. 4, D). The corneocytes of a homozygous subject (FLG−/−) demonstrated labeling over the entire surface (Fig. 4, C). In contrast, in a patient who is wild-type with respect to FLG mutations (FLG+/+; Fig 4, A), the labeling was almost exclusively distributed on the lateral rims of the cell. In the heterozygous patient (FLG+/−; Fig 4, B) the central area of corneocytes remained largely free of the label, even though it was partially occupied by the VPs (Fig 4, B, arrows).

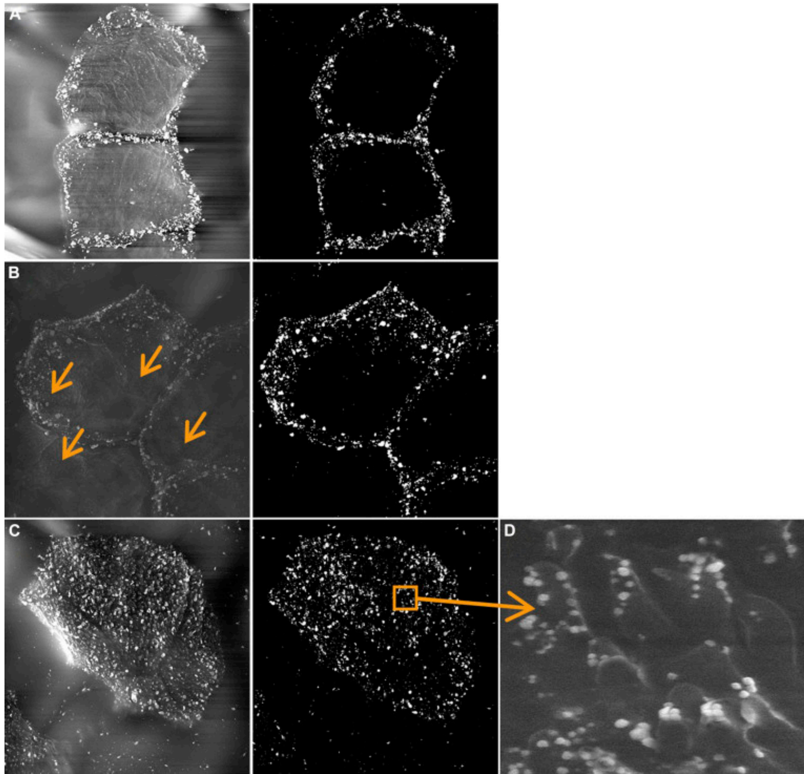

Fig. 4. SEM immunolabeling of corneodesmosin. A, Corneodesmosomes at the cell surface of a patient wild-type with respect to FLG LOF mutations (FLG+/+). B, A patient heterozygous for FLG LOF mutations (FLG+/−). C, A patient homozygous for FLG LOF mutations (FLG−/−). Insert in Fig 4, C, Corneodesmosin-expressing junctions present at the tops of the VPs in the patient homozygous for FLG LOF mutations. Arrows in Fig 4, B, show the presence of VPs (not labeled for corneodesmosin).

DISCUSSION

Filaggrin deficiency results in a definite skin barrier defect, but the pathomechanisms underlying this defect are poorly understood (11). Within the corneocytes, filaggrin aggregates intermediate keratin filaments that are linked to the corneodesmosomes, which interconnect the corneocytes, providing a physical barrier structure at the top of the skin (24 ,25). Together with keratin filaments, filaggrin has been proposed to provide a scaffold for the assembly of structural proteins, such as involucrin, loricrin, and small proline-rich proteins, which are cross-linked by several transglutaminases to form the CE (24, 25). Some CE proteins serve as an anchor for attachment of ceramides, and thus lack of filaggrin might also affect the structural organization of the intercellular SC lipid lamellae responsible for barrier function.

In the present study we demonstrate that deficiency of filaggrin is associated with altered topography of the corneocyte surface, likely caused by defects in the CE. In a recent study (2) similar villous structures were observed on the palmar skin of healthy subjects, although not on forearm skin, which is in contrast to the present study. We found that levels of filaggrin degradation products (NMF) used as a marker of filaggrin expression (12, 26) were strongly associated with corneocyte VP numbers. These corneocytes were sourced from the upper middle part of the SC (seventh strip); however, the same pattern concerning distribution of VPs was also seen in the more superficial strips (eg, strip number 4; data not shown). VP numbers were more closely related to NMF levels than to SCORAD scores, suggesting that the absence of filaggrin is important for the formation of VPs rather than inflammation per se. This is supported by similar regression coefficients of the DTI score versus NMF level relationship at weeks 0 and 6, despite the sharp decrease in SCORAD scores. Furthermore, in a linear regression model with the DTI score as a dependent variable versus the NMF level and SCORAD score as independent variables, only NMF levels showed a significant effect on DTI scores at both weeks 0 and 6. Local inflammation might have affected the presence of DTI scores indirectly by influencing NMF levels, an effect that previously has been shown in vitro and in vivo (12, 13). This might explain the lack of a significant difference in DTI scores between patients with AD with FLG LOF mutations and patients with AD without FLG LOF mutations, although the former group tended

to have higher DTI scores, and the lack of statistical significance seen here might simply be due to a lack of power in this study. Furthermore, at week 6, the FLG–/– patients, in whom inflammation is controlled and NMF levels are mainly influenced by FLG LOF mutations, had significantly higher DTI scores compared with FLG+/+ patients, despite clinical improvement, as measured based on SCORAD scores. Also of note is our observation that the relationship between TEWL and DTI scores was significant at 6 weeks (after anti-inflammatory therapy) but not at week 0 (see Fig E1). This suggests a relationship between corneocyte conformation as measured by DTI scores and barrier function (TEWL).

During the transition from the stratum compactum to the stratum disjunctum, corneocyte morphology and mechanical properties change from a "fragile" and soft to a more robust, smooth, and "rigid" phenotype (27- 29). This transition process is accompanied by loss of nonperipheral corneodesmosomes because only peripheral corneodesmosome attachments connecting consecutive layers of corneocytes remain (28, 29). Interestingly, we observed corneodesmosin on the tips of VPs, all over the cell surface in FLG–/– patients, and, to lesser extent, in heterozygous patients, which suggests changes in their maturation because of a disturbed terminal differentiation program. As discussed by Rawlings (28) and shown by Watkinson and Rawlings (30), the loss of nonperipheral corneodesmosomes and CE maturation changes seem to parallel filaggrin degradation. Lack of filaggrin in the CE and between the keratin filaments might lead to conformational changes, and the adhesive portions of the peripheral corneodesmosomes might become less accessible for degradation enzymes. In addition to the direct effect of filaggrin, the existence of VPs might also be caused by a reduction in filaggrin degradation products and reduced hydration of the SC. Matsumoto et al. (6) observed the emergence of villi on the rear surfaces of corneocytes after topical exposure to the contact allergen 2,4,6-trinitrochlorobenzene, which caused dry and inflamed skin. However, the villi disappeared after topical treatment with a moisturizer at a higher rate than after topical corticosteroid therapy. The surfactant-induced xerosis led to a considerable increase of the immature and fragile CE phenotype (29). The perturbation of CE maturation coincided with reduced hydrolysis of corneodesmosomes, which was paralleled by altered activity of transglutaminase. Recently, we have shown that exposure to sodium lauryl sulfate caused a dramatic decrease in NMF levels in the

SC,31 and therefore the changes in corneocyte maturation might have also been caused by the lack of NMF. Interestingly, also in the study of Harding et al. (29), the balance between the 2 CE phenotypes was recovered after treatment with a moisturizer, emphasizing the importance of SC hydration for the maturation process.

The size of the VPs (ie, several hundreds of nanometers: average height, 350 nm; width at half-maximum, 250-400 nm) and their high abundance is intriguing. The CE is approximately 20 nm thick, implying that considerable mechanical force lies behind its protrusion. The present results do not allow firm conclusions to be drawn regarding the relationship between VPs and retention of the nonperipheral corneodesmosomes because the presence of VPs was not always accompanied by central distribution of corneodesmosin. The persistence of VPs in both the acute and convalescent phases of AD with FLG loss-of-function mutations offers an intriguing insight into the persistent abnormalities in “normal” or “unaffected” AD skin, an area of great interest (32). The persistence of an underlying physical and structural abnormality, even in light of apparent clinical improvement, might explain why patients with AD with FLG loss-of-function mutations have more severe and persistent disease (33), why they are more likely to have eczema herpeticum,34 and why they have more food allergies (35).

In conclusion, we have shown for the first time a significant structural difference in corneocytes in patients with AD with FLG loss-of-function mutations that can be quantitatively measured. These structural changes correlate well with NMF levels and persist despite apparent clinical improvement and might explain some of the observed phenotypic differences in patients with AD with FLG loss-of-function mutations.

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The SEM images were obtained at the Centre Technologique des Microstructures, CTμ, an LBI platform of University Lyon 1.

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4 . 2

Efficacy of a cream containing ceramides and magnesium in the treatment of mild to moderate atopic dermatitis: a randomized, double-blind, emollient- and hydrocortisone-controlled trial

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ABSTRACT

The aim of this randomized controlled trial was to assess the efficacy of a cream containing ceramides and magnesium (Cer-Mg) in the treatment of mild to moderate atopic dermatitis and to compare it with hydrocortisone and a commonly used emollient (unguentum leniens; cold cream). A total of 100 patients, randomized into 2 groups, were treated for 6 weeks simultaneously (left vs. right side of the body) with either Cer-Mg and hydrocortisone (group I) or Cer-Mg and emollient (group II). The primary outcome was a reduction in severity of lesions as assessed by (local) SCORAD (SCORing Atopic Dermatitis). Levels of trans-epidermal water loss (TEWL), skin hydration, and natural moisturizing factors (NMF) were then measured. After 6 weeks, group I showed comparable significant improvement in SCORAD and TEWL, while in group II, the decrease in SCORAD and TEWL was significantly greater after Cer-Mg compared with emollient. Finally, Cer-Mg cream was more effective in improving skin hydration and maintenance of levels of NMF than hydrocortisone and emollient.

INTRODUCTION

Atopic dermatitis (AD), a chronic, inflammatory skin disease characterized by dry, pruritic and erythematous skin, affects up to 10% of adults and up to 20% of children in the Western world (1–3). Patients with mild to moderate AD are constrained for long periods to over-the-counter (OTC) emollients or, in some countries, such as the UK and the USA, to low-potency corticosteroids. However, long-term use of corticosteroids is associated with adverse side-effects, such as skin atrophy (4). Such side-effects are well known among the general public and (not always justifiable) anxiety about corticosteroids is a major factor in poor adherence to therapy (5–8). Therefore, emollient therapy is often preferred by patients and is shown to reduce corticosteroid use significantly (9). In general, emollients aim to prevent water loss from the skin, e.g. by occlusion (petrolatum) or by addition of hygroscopic compounds (e.g. glycerol and urea) and lipids (e.g. ceramides). Identification of an inherited deficiency of the epidermal protein filaggrin as a major risk factor for AD, points to the importance of the skin barrier in the aetiology of AD (10–12). The barrier is located mainly in the stratum corneum (SC), which is composed of corneocytes surrounded by lipid lamellae composed of ceramides, cholesterol and free fatty acids (13–15). Although emollients are regarded as basic therapy by the European Task Force on Atopic Dermatitis/European Academy of Dermatology and Venereology (EADV) Eczema Task Force, their efficacy in randomized controlled trials (RCT) has been insufficiently investigated (16–20). Therefore, the aim of the present double-blinded RCT was to assess the efficacy of an emollient containing ceramides and magnesium (Cer-Mg), compounds involved in the maintenance of the skin barrier (21). SC ceramide composition is altered in AD, and reduced levels of ceramides and changes in their relative composition have been shown to correlate with trans-epidermal water loss (TEWL) (12). The role of magnesium in AD is relatively unknown; however, bathing in magnesium-rich water has been shown to have a beneficial effect on the skin barrier in dry atopic skin (22). Furthermore, magnesium is known to be involved in synthesis of ceramides, regulation of epidermal proliferation and differentiation. In addition, children with AD showed a reduced level of serum magnesium (23, 24). Although there is some evidence that both ceramides and magnesium might improve barrier function in AD, their efficacy remains to be elucidated, preferably in RCTs. In the present study the efficacy of the Cer-Mg cream was compared side-by-side with 2 other creams,

which are frequently used in treatment of mild and moderate AD: a low-potency topical corticosteroid (hydrocortisone acetate 1% in petrolatum-cetomacrogol) and a commonly used OTC emollient, unguentum leniens; cold cream).

Trial population

A total of 100 patients were recruited from the outpatient clinic at VU University Medical Center Amsterdam (VUmc). Inclusion criteria were: (i) clinically diagnosed AD conforming to the Hanifin & Raijka criteria (25), (ii) mild to moderate AD, (iii) age 18–70 years, (iv) at least 2 symmetrical (i.e. left and right side of the body) skin sites with comparable AD severity. The exclusion criteria were: (i) extensive ultraviolet (UV) exposure in the last 14 days and/or expected exposure during the study, (ii) skin disease other than AD, (iii) use of antibiotics prior (at least 4 weeks) to the study and/or expected use during the study, (iv) use of systemic immuno-suppressing drugs prior (at least 4 weeks) to the study and/or expected use during the study, (v) severe disorders within the last 6 months, (vi) investigator's uncertainty about the willingness or ability of the patient to comply with the protocol requirements (e.g. mental disability). In the case of adverse health effects, such as allergic reaction or severe deterioration of the symptoms, patients were prevented from further participation. Patients could not use any AD medication for at least 2 weeks prior to participation (wash-out period). The study was approved by the medical ethics committee of the Academic Medical Centre and VUmc. All patients gave their written informed consent prior to participation.

Patients' experience

After participation patients were asked, in a short questionnaire, what their personal preferred treatment was.

Registration and medical ethics approval

The trial was registered under the number NTR 4541. Medical ethics approval was obtained on the basis of the study protocol (AMC registration number: METC 2014_090).

Randomization and blinding

The randomization list was produced prior to treatment by a random number sequence generated in Microsoft Excel™. Treatment combinations (Cer-Mg and HC or Cer-Mg and EM) were linked to a unique inclusion number. The allocation list was prepared by an investigator (SK) with no executive tasks in the trial and handed over to the VUmc pharmacy. After the enrolment of a second investigator (SAK) who had access only to the inclusion numbers, each patient was given the inclusion number and collected the creams at the pharmacy. Creams were packed in identical tubes labelled only with the 3 possible treatments (hydrocortisone, EM or Cer-Mg cream) and the body side on which to apply the cream (left or right). For safety reasons 2 investigators (TR, MFD) were given the allocation list so that in case of an adverse event medical care could be given without delay.

Intervention

Patients were randomly allocated into 2 groups. Group I was treated with Cer-Mg cream on a lesion on one side of the body and simultaneously with HC on a lesion on the contralateral side. Group II was treated simultaneously with Cer-Mg and EM (unguentum leniens, also called cold cream) contralaterally. Patients were instructed to apply one fingertip unit (approximately 1 g) of both creams twice daily for 6 weeks. Patients were instructed not to apply cream on the morning of measurements. Furthermore, patients were asked not to apply any other product on other lesions, except the study creams. Measurements were performed under the same climate conditions (21°C, controlled humidity) between September and January, by one investigator (SAK). In weeks 0, 3 and 6 the parameters were measured and samples of the SC were collected for analysis. A flow diagram is shown in Fig. 1.

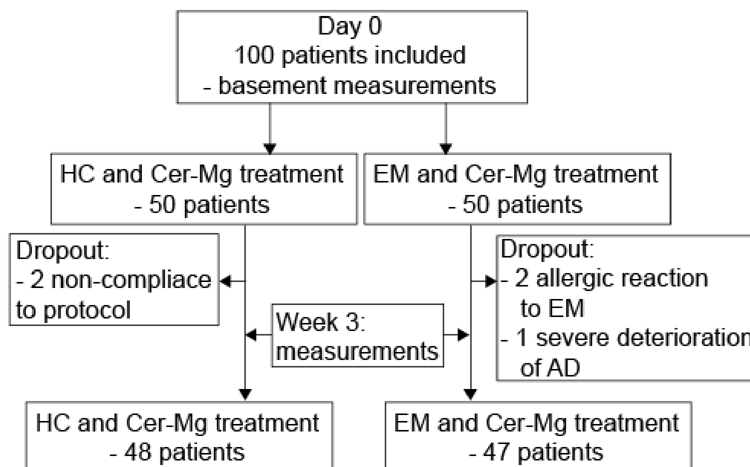

Fig. 1. Randomization flow diagram. HC: hydrocortisone; EM: emollients; Cer-Mg: ceramides and magnesium; AD: atopic dermatitis

Study material

The Cer-Mg cream (Dermalex™ Eczema, Omega Pharma, Nazareth, Belgium) contained: water, ceramide 1 (0.001%), ceramide 3 (1%), ceramide 6 II (0.5%), phytosphingosine, cholesterol, magnesium chloride hexahydrate, zeolite (the combination of magnesium and zeolites are trademarked as MagneoLiteTM), glycerol, cocoglycerides, cetyl alcohol, isopropyl myristate, emulsifiers and preservatives. The control products; hydrocortisone acetate 1% in petrolatum-cetomacrogol (HC) and unguentum leniens (EM, also called cold cream, consists of arachis oil (peanut oil), purified water, white beeswax and glyceryl monooleate) both produced by Fagron, NL, BF (Capelle aan den IJssel, the Netherlands) were, together with the Cer-Mg, packed in blinded tubes by Thiopharma (Maassluis, the Netherlands) according to the good manufacturing practice guidelines. The total lipid content of the Cer-Mg cream was 30%, of the EM 75%, and of the HC 49%.

Clinical parameters (primary outcome)

The primary outcome of the study was the comparison of the treatments based on the change in symptom severity as assessed by the difference in the SCORAD (SCORing Atopic Dermatitis) at 3 and 6 weeks from baseline. SCORAD is based on the total body surface area affected by a disease and visually apparent symptoms (erythema, oedema, excoriation, oozing/crusts, lichenification, dryness) and on 2

subjective parameters (pruritus and sleep deprivation, both measured on a visual analogue scale) (16). Due to the split-body study design a modified SCORAD (local SCORAD) was used (26). By local SCORAD, the scoring parameters were performed on the investigated skin sites and the body surface area was set to 1%.

Biophysical parameters and natural moisturizing factors (secondary outcomes)

The biophysical parameters included TEWL, skin surface pH and erythema. The measurements were conducted within a time-period of 60 min at each visit under controlled environmental conditions. TEWL was measured using a Tewameter 300 (Courage and Khazaka Electronic GmbH, Cologne, Germany) (27). Hydration was measured using a Moisture Meter SC Compact (Delfin, Inc, Kuopio, Finland). Skin pH was measured by a skin pH meter (pH900, Courage and Khazaka Electronic GmbH, Cologne, Germany) and erythema by an erythema meter (DermaSpectrometer; Cortex Technology, Hadsund, Denmark).

Natural moisturizing factors in the stratum corneum

The SC samples were collected with an adhesive tape (3.8 cm2, D-Squame, CuDerm, Dallas, Texas, USA) as described previously (12) and analysed for natural moisturizing factors (NMF) by HPLC-UV (22, 28).

Statistical analysis

Sample size was calculated using power analysis (nQuery advisor). Based on data from our pilot study (unpublished, results available on request) a difference of 5 arbitrary units (AU) (standard deviation (SD) 4.0) on the SCORAD index could be detected in a population of 39 patients (power 80%). Anticipating a drop-out percentage of 20%, we included 50 patients per group. Data analysis was performed using IBM SPSS Statistics® version 20.0. The Shapiro-Wilk test was used to check for data normality. The differences within the investigated parameters or between the 2 treatments were tested by a paired Student's t-test (normally distributed data, data are shown as the mean value and standard error of the mean (SEM)) or a Wilcoxon signed-rank test (non-normally distributed data, shown as median value with interquartile ranges). A per-protocol analysis was performed as described in the study protocol.

Online supplement contains additional information on:
Patients experience questionnaire (S1, Methods)
Registration and medical ethical approval (S1, Methods)
Randomization and blinding (S1, Methods)

RESULTS

Of 100 patients recruited between October and December 2014, 95 completed the study according to the protocol (group I: 48 patients; 16 males/32 females, median age 28.5 years (range 23.0–51.0 years) and group II: 47 patients 19 males/28 females, median age 25.0 years (range 21.0–35.0 years). Five patients were excluded during the study because of an allergic reaction to EM (n = 2), severe worsening of eczema symptoms (n = 1) or non-compliance with the study protocol (n = 2) (see Fig. 1). Due to technical failure, no reliable measurements of erythema by DermaSpectrometer could be performed; however, visual erythema was measured as a part of the SCORAD index. Furthermore, the measurement of proteins on the tapes from 3 subjects in group II could not be performed, and thus the levels of NMF in those individuals could not be determined. As the main outcome is the difference in parameter change between 2 treatments (e.g. Cer-Mg vs. HC in group I and Cer-Mg vs. EM in group II), the results will be presented separately for each group.

SCORing Atopic Dermatitis

At baseline, there was no significant difference in the (local) SCORAD between the 2 treated skin sites in either arm of the study.

Group I: HC vs. Cer-Mg. Both treatments led to clinical improvement in the test areas, as evidenced by a significant decrease in local SCORAD after week 3 and week 6 (Fig. 2A). The reduction in SCORAD from baseline (ΔSCORAD) was significantly greater for HC compared with Mg-Cer at 3 weeks; however, after 6 weeks there was no significant difference in ΔSCORAD between HC and Cer-Mg (Table I). At week 6, the ΔSCORAD amounted to −11.5 (IQR: −17.4; −5.6) for HC and −9.0 (IQR:−15.9; −5.6) for Cer-Mg.

Group II: EM vs. Cer-Mg. Cer-Mg treatment led to a significantly greater decrease in SCORAD from baseline (ΔSCORAD) compared with EM at both week 3 and week 6 (Table I). At week 6, the ΔSCORAD was −3.5 (IQR: −10.5; 3.0) for EM and −6.7 (IQR:−14.5; −2.0) for Cer-Mg.

Local pruritus (itch) intensity

Results for pruritus show a similar pattern as the SCORAD results; an extensive description can be found in online Appendix SI1.

TEWL as a marker of skin barrier

Group I: HC vs. Cer-Mg. The TEWL levels after both Cer-Mg and HC decreased significantly compared with their corresponding baseline values (Fig. 2B) reflecting an improvement of the skin barrier. The decrease in TEWL from baseline (ΔTEWL) after HC and Cer-Mg was comparable and did not significantly differ at both measurement points (Table I).

Group II: EM vs. Cer-Mg. Cer-Mg treatment did not lead to a significant change in the TEWL from baseline (Fig. 2 b), while the EM treatment showed a significant increase in TEWL at 3 weeks. The change in TEWL from baseline (ΔTEWL) was significantly greater after EM compared with Cer-Mg at both time-points (Table II).

Table 1. Change from baseline of clinical and biophysical parameters in the treatment Group I (Cer-Mg vs. HC).

	Group I: Cer-Mg versus HC					
		Cer-Mg	IQR	HC	IQR	p-value[1]
ΔSCORAD	Week 3	-6.25	(-8.40; -1)	-7.75	(-15.38; -3.63)	0.0078
(AU)	Week 6	-9.00	(-15.93; -5.63)	-11.5	(-17.38; -5.63)	0.1037
ΔPruritus	Week 3	-1.00	(-2; 0)	-1.00	(-4; 0)	0.0104
(AU)	Week 6	-2.00	(-4; 0)	-2.00	(-4; 0)	0.6123
ΔTEWL	Week 3	-4.75	(-13.66; 1.473)	-7.24	(-15.70; 2.21)	0.104
($g/m^2/h$)	Week 6	-6.28	(-12.20; 5.15)	-5.19	(-14.36; 2.21)	0.083
ΔHydration	Week 3	6.95	(0.23; 20.03)	3.90	(-1.2; 13.7)	0.0202
(AU)	Week 6	6.75	(0.83; 17.28)	3.85	(-2.9; 11.23)	0.0183
ΔNMF	Week 3	0.01	(-0.15; 0.23)	-0.02	(-0.18; 0.15)	0.209
(nmol/ug protein)	Week 6	0.08	(-0.12; 0.25)	-0.10	(-0.23; 0.06)	0.0015
ΔpH	Week 3	0.00	(-0.20; 0.28)	0.00	(-0.28; 0.40)	0.2475
	Week 6	0. 00	(-0.40; 0.20)	0.10	(-0.30; 0.40)	0.024

P-significance level of the difference in changes from baseline between two treatments (Wilcoxon signed-rank test) ; AU: arbitrary units; IQR: interquartile range; SCORAD: SCORing Atopic Dermatitis; TEWL: trans-epidermal water loss; NMF: natural moisturizing factors.

Hydration

Group I: HC vs. Cer-Mg. Treatment with HC and Cer-Mg significantly improved skin hydration (Fig. 2C). The increase in hydration from baseline (ΔHydration) after Cer-Mg was significantly greater after Cer-Mg compared with HC at weeks 3 and 6 (Table I).

Group II: EM vs. Cer-Mg. Hydration after Cer-Mg was significantly higher than the baseline values at weeks 3 and 6 (Fig. 2C), while hydration after EM treatment improved significantly only after 6 weeks. The changes in hydration from baseline (ΔHydration) were significantly larger after Cer-Mg compared with EM at week 3 (Table II).

Natural moisturizing factors

Group I: HC vs. Cer-Mg. Treatment with Cer-Mg showed a tendency of NMF increase ($p = 0.09$) (Fig. 2D). In contrast to Cer-Mg, treatment with HC resulted in a significant decrease (by 22%) of NMF levels after 6 weeks. The difference in NMF change from the baseline (ΔNMF) between HC and Cer-Mg emollient was significant at week 6 ($p < 0.05$), (Table I).

Group II: EM vs. Cer-Mg. EM treatment showed a significant decrease in NMF at week 3 (Fig. 2D). Treatment with Cer-Mg did not influence NMF levels. No significant difference in ΔNMF could be detected between the 2 treatments (Table II).

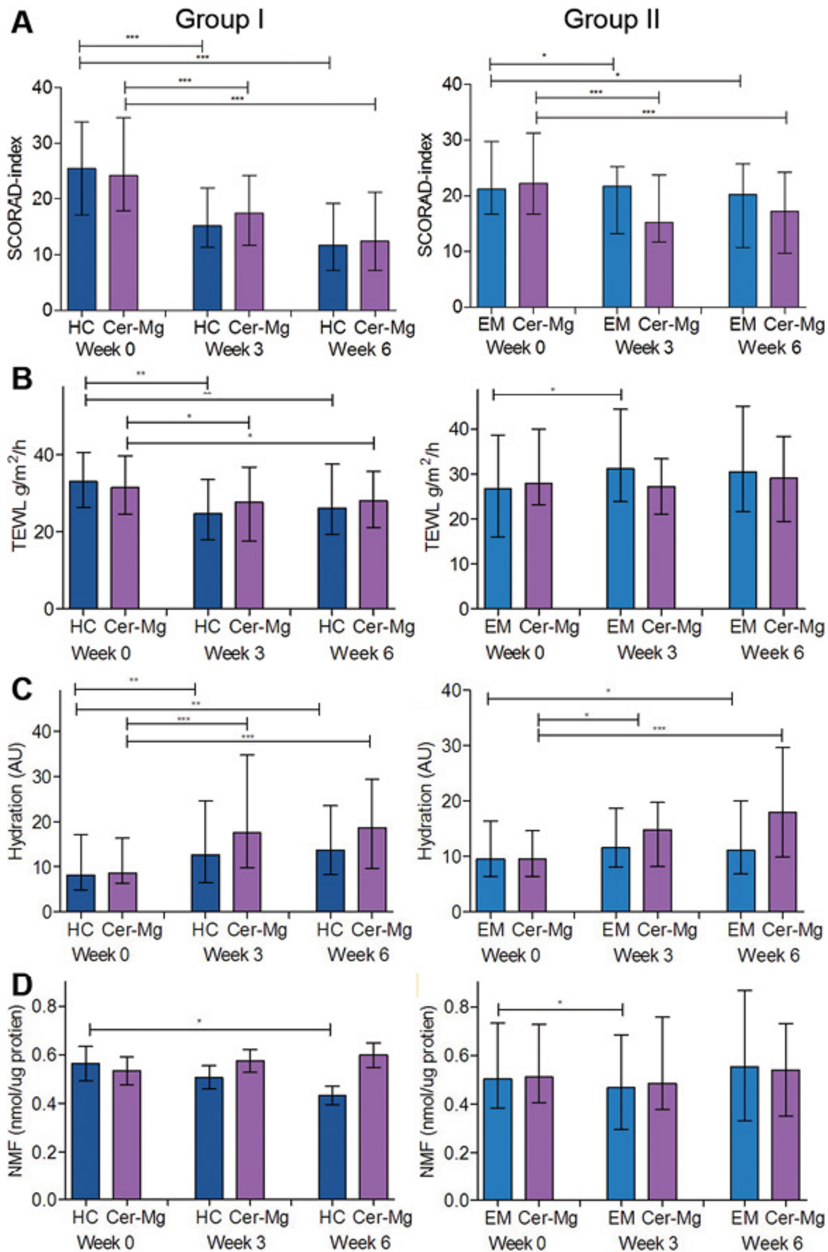

Fig. 2. (A) Local SCORAD (SCORing Atopic Dermatitis). (B) Trans-epidermal water loss (TEWL). (C) Hydration and (D) natural moisturizing factors (NMF) at baseline, after 3 and 6 weeks of treatment in group I (hydrocortisone (HC) vs. ceramides and magnesium (Cer-Mg); n = 48) and group II (emollients (EM) vs. Cer-Mg; n = 47). Results are shown as medians and interquartile ranges. Significance levels as tested by Wilcoxon signed-rank test: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Table 2. Change from baseline of clinical and biophysical parameters in the treatment Group II (Cer-Mg vs. EM).

	Group II: Cer-Mg versus Emollients					
		Cer-MG	IQR	EM	IQR	p-value[1]
ΔSCORAD	Week 3	-8.50	(-11.5; -1.5)	-3.50	(-8; 1)	0.0058
(AU)	Week 6	-6.70	(-14.5; -2)	-3.50	(-10.5; 3)	0.0056
ΔPruritus	Week 3	-1.00	(-2; 0)	0.00	(-1; 1)	0.0173
(AU)	Week 6	-2.00	(-3; 0)	0.00	(-2; 1)	0.0166
ΔTEWL	Week 3	-3.48	(-8,24; 3.66)	2.75	(-3.68; 10.07)	0.005
($g/m^2/h$)	Week 6	-3.19	(-8.57; 3.34)	4.94	(-6.97; 12.94)	0.0208
ΔHydration	Week 3	3.10	(-3.1; 9.6)	1.20	(-3.2; 6.5)	0.0401
(AU)	Week 6	9.70	(-0.7; 18.6)	1.70	(-1.5; 8.4)	0,0625
ΔNMF	Week 3	-0.02	(-0.19; 0,10)	-0.07	(-0.20; 0.09)	0.9767
(nmol/ug protein)	Week 6	-0.02	(-0.27; 0.21)	0.01	(-0.17; 0.24)	0,9767
ΔpH	Week 3	0.30	(-0.1; 0,5)	0.10	(-0.1; 0.3)	0.5189
	Week 6	0.00	(-0. 2; 0.3)	0.00	(-0.3; 0.3)	0.4739

[1]P-significance level of the difference in changes from baseline between two treatments (Wilcoxon signed-rank test) ; AU: arbitrary units; IQR: interquartile range; SCORAD: SCORing Atopic Dermatitis; TEWL: trans-epidermal water loss; NMF: natural moisturizing factors.

An extensive description of pH results can be found in the online supplementary file (S1, Results).

Online supplement contains additional information on:
- Patient characteristics (S1, results)
- Local Pruritus (itch) intensity (S1, results)
- skin surface pH (S1, results)
- Tolerability and subjective preference (S1, results)

DISCUSSION

The results of the present study show that the Cer-Mg cream is an effective approach in improving the clinical symptoms and skin barrier. Although all 3 treatments led to significant improvement in clinical symptoms after 6 weeks, only the HC and Cer-Mg cream reduced SCORAD by more than 8.7 units, which is considered clinically relevant (26). After 3 weeks of treatment HC showed slightly, but significantly, greater reduction in SCORAD than Cer-Mg (−7.8 vs. −6.3), while Cer-Mg showed significantly greater reduction than EM (−8.5 vs. −3.5). The subjective VAS-pruritus

scale and the skin barrier function parameter TEWL showed similar results: Cer-Mg and HC showed a significantly beneficial effect, which was, however, not observed after EM treatment. Overall subjective preference slightly favoured the Cer-Mg, which might be of importance in patients' adherence to therapy. Topical corticosteroids (TCS) are the first-line treatment for AD; however, their long-term use can lead to the deterioration of the skin barrier, which is an important aetiological factor in AD. Moreover, a recent study has shown that therapy with a potent TCS leads to a reduction in NMF levels, which play an important role in skin hydration, antimicrobial defence and skin inflammatory status (29, 30). Our study shows, for the first time, that a low-potency corticosteroid such as HC can lead to a significant reduction of NMF. A decrease in NMF has also been observed after EM treatment at 3 weeks, while Cer-Mg showed a tendency to increase NMF. This emphasizes the importance of this adverse side-effect of HC, as reduced NMF levels may contribute to the recurrent flares. The greatest improvement in SC hydration was observed after Cer-Mg cream that, similarly to HC, showed a decrease in TEWL, but in contrast to HC had no negative effect on NMF levels.

The Cer-Mg cream contains 2 components that might beneficially influence the skin barrier: ceramides (1, 3 and 6 II) and a complex of magnesium and zeolites (31). Huang & Chang (32) have shown that topical application of ceramide 1 and 3 reduces TEWL and increases hydration in sodium lauryl sulfate-irritated, thus beneficial effect of these ceramides, which are also present in Cer-Mg cream, might also have occurred in patients with AD in the present study. As the molecular size of the skin ceramides is > 500 Da, which is proposed as a molecular size cut-off for percutaneous penetration (33), the question arises whether and to which extent each of individual ceramides can penetrate across the SC, realizing that not only the amount, but also their balance is crucial for the skin barrier. Recently, Zhang et al. (34) demonstrated that topically applied ceramides are located mainly in the SC glyphs and that the penetration into the lipid layers is minimal. It is likely that penetration of ceramides through the impaired skin barrier is enhanced in AD; however, RCT studies on the penetration of various ceramides, and their efficacy in improvement of the skin barrier in AD, are lacking.

Another rationale candidate to explain the effectiveness of Cer-Mg cream is magnesium, which is known to be involved in synthesis of ceramides (23). Topical treatments with magnesium-rich Dead Sea salts showed a beneficial effect in dry and pruritic dermatoses (27). Whether the effect of the Cer-Mg cream could be assigned to the presence of ceramides or magnesium remains to be elucidated in a vehicle-controlled trial as some constituents of the vehicle in the Cer-Mg cream, such as glycerol, are also known to lead to improvement in the skin barrier (35, 36).

Strengths and limitations

In this RCT the efficacy of Cer-Mg cream was compared with that of 2 currently used therapeutic options for mild to moderate AD. In most RCTs the efficacy is compared only with either corticosteroid or OTC emollient. The double-blind, split-body design offers a well-paired comparison between 2 treatments, compensating partly for the heterogeneity of the disease severity among patients with AD. The inclusion of biophysical and biochemical parameters provides more insight into the target of the treatment (37). This study did not account for spontaneous resolution of the disease over the study period. However, as the primary aim was to compare the efficacy of Cer-Mg to the upper (hydrocortisone) and lower spectrum of recommended OTC therapy for mild to moderate AD, we did not include an untreated site. Finally, the study does not provide insight into the working mechanism of Cer-Mg, which needs to be confirmed in the separate vehicle-controlled clinical trial.

CONCLUSION

The present study shows that, after 6 weeks of treatment, Cer-Mg cream offers benefits over high lipid-OTC emollients and comparable clinical efficacy to hydrocortisone. In addition, in contrast to hydrocortisone, it does not influence negatively the concentration of NMF. Cer-Mg may therefore offer a non-steroid alternative for the treatment of mild to moderate AD. Furthermore, the fact that Cer-Mg might be used as a stand-alone treatment for mild and moderate AD as well as a maintenance therapy might improve adherence to AD therapy.

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4.3

Stratum corneum tape stripping: monitoring of inflammatory mediators in atopic dermatitis patients using topical therapy

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ABSTRACT

Objective: The aim of this study was to explore the tape strip sampling technique in the assessment of stratum corneum levels of inflammatory mediators in a clinical trial setting.

Methods: Thirty-eight inflammatory mediators were analyzed by a multiplex-assay in the stratum corneum, collected by adhesive tapes before and after 6 weeks of therapy, in mild and moderate atopic dermatitis (AD) patients (n = 90). Treatment was a ceramide- and magnesium-containing emollient.

Results: Twenty-four mediators could quantitatively be determined. The Th2 mediators interleukin (IL)-4, IL-13, CCL2 (monocyte chemotactic protein-1), CCL22 (macrophage-derived chemokine), and CCL17 (thymus and activation-regulated chemokine (TARC)) were significantly decreased after therapy as well as IL-1β, IL-2, IL-8 (CXCL8), IL-10, acute-phase protein serum amyloid A, C-reactive protein, and vascular adhesion molecule-1. The decrease of CCL17 and IL-8 was correlated with the decrease in disease severity in a subgroup of moderate AD individuals.

Conclusion: Stratum corneum tape stripping offers a minimally invasive approach for studying local levels of immunomodulatory molecules in the skin. CCL17 (TARC) and IL-8 were found to be the most promising biomarkers of AD and might be useful for investigating the course of skin diseases and the effect of local therapy.

INTRODUCTION

Atopic dermatitis (AD) is a chronic remitting inflammatory skin disorder. It has a complex etiology in which immunological dysregulation and skin barrier alterations play an important role (1-3). The fact that mutations in the gene encoding for the epidermal protein filaggrin predispose for AD points toward the skin barrier as an important factor in the pathophysiology of the disease (4). In addition to genetic factors, filaggrin expression is down-regulated by Th2 cytokines (5). This further emphasizes the interaction between the immune system and skin barrier in AD, particularly since flares of AD are characterized by activation of Th2 pathways (6, 7) (See Fig 1). The profile of inflammatory mediators might provide more insight into specific immunopathological pathways, offering potential targets for more personalized therapy, the sub-classification of AD and for the monitoring of therapy (8). Clinical assessment of AD lesions is often hampered by symptoms such as erythema and lichenification. Furthermore, as addressed by Mansouri and Guttman-Yasky, AD has a high placebo response rate, which might influence assessment of the efficacy of therapeutics in clinical trials (8). The profiles of inflammatory mediators in AD skin lesions during exacerbation or after treatment have been insufficiently investigated because most studies are based on blood samples, and thus only provide information on systemic profiles and not on the cutaneous microenvironment (9, 10). The lack of data on the local milieu of inflammatory mediators is partly due to the invasive and laborious nature of many procedures used to obtain skin samples, such as biopsies, harvesting of skin-derived interstitial fluid or cutaneous microdialysis (11). In contrast, the collection of the *stratum corneum* (SC), the uppermost layer of the epidermis, by adhesive tape offers the advantage of obtaining skin samples in a simple and non-invasive manner. This technique has been used to determine different skin biomarkers, such as IL-1 cytokines, enzymes, lipids and filaggrin degradation products (12-16). However, so far the poor sensitivity of the analytical methods used in this approach has hampered the analysis of inflammatory mediators relevant to AD. Recently, several multiplex assays have been introduced which are more sensitive and which also permit the determination of a broad range of inflammatory mediators in a single (tape strip) sample (17).

In the present study, we used the SC tape stripping technique to determine various inflammatory mediators in lesional skin of AD patients before and after treatment with an emollient containing ceramides and magnesium (EM). Both ingredients are involved in the maintenance of the skin barrier. Furthermore, as a control, inflammatory mediators of 20 healthy, non-AD individuals were measured. We analysed 38 different cytokines, chemokines, and vascular growth factors, most of which, to our knowledge, have not previously been determined in the SC.

METHODS

Patients

Ninety adult patients from the VU Medical Center Amsterdam outpatient clinic (average age 33 years, 57 female, 33 male) with AD as defined by the Hanifin and Rajka criteria entered the study after their written informed consent (18) was obtained. Only patients with mild to moderate AD based on the OSCORAD (Objective SCORing Atopic Dermatitis) score (range 12.5-50) were included (19, 20). Apart from the emollient used in the study, patients were not allowed to apply local steroids or other products such as calcineurin inhibitors and OTC emollients on the investigated lesions. Patients undergoing systemic immunosuppressive or antibiotic therapy were excluded. SC samples were collected before and after six weeks of topical treatment with the study emollient. A healthy population of twenty individuals without a history of AD was included as a control group.The research was conducted according to the principles of the Declaration of Helsinki and was approved by the ethics committee of the Academic Medical Center (study number: METC 2014_090).

Emollient

The AD-patients were asked to apply an emollient containing ceramides and magnesium chloride hexahydrate (Dermalex® Eczema, Omega Pharma, Nazareth, Belgium) twice daily. Other components of the emollient included water, cholesterol, zeolite, glycerol, cocoglycerides, cetyl alcohol, isopropyl myristate, emulsifiers, and preservatives. The total lipid content was 30%. The control population was asked not to use any cream for at least 3 days preceding the study.

Tape stripping of the stratum corneum

Tape stripping was performed as previously described (12, 13). Briefly, adhesive tapes (3,8 cm^2, D-Squame; CuDerm, Dallas, TX, USA) were placed on a skin site affected by AD, e.g. the volar forearm or popliteal fossa, and briefly pressed with a standardized pressure pen of 225 g/cm^2 (D-Squame pressure instrument D500, CuDerm, Dallas, TX, USA). Eight consecutive tapes were collected from the same skin site. Six weeks later, another eight consecutive tapes were taken from an adjacent AD-affected and emollient-treated area as close as possible to the skin site sampled during the first visit. For the analysis, the 6th tape was used (stored at -80°C). The tape strips of the control group were taken from the upper back of the participant and the 6th and 7th strip (1.5cm^2 D-squames) were used for analysis. These strips represent the middle part of the SC where the cytokine concentrations reach stable levels (21).

Sample preparation and analysis

The SC samples were extracted from the tapes by ultrasonification (15 min) with 0.5 ml of phosphate buffered saline (PBS) containing 0.05% Tween 20. After vortexing, the extract aliquots were distributed in vials and stored at -80°C. Analysis was performed using the MESO QuickPlex SQ 120 assay (MSD, Rockville, Maryland, USA). If more than 50% of the samples were under the level of detection, no further analysis was performed for that specific mediator (22). An overview of the inflammatory mediators can be found in Table 1 and supplement 1. All panels used human antibodies. For the analysis 50µl of the SC extract and a calibrator (provided by MSD) were incubated overnight on the sealed plate at 2-8°C. Reading was done after washing with PBS with Tween 20 and after adding the provided reading buffer. The extracts of tape 6 and 7 of the control group were pooled, as smaller D-squame tapes were used.Concentrations of all cytokines in both groups were corrected by the total protein content of the extract, which has been determined by Pierce assay (23). Samples under the limit of detection were substituted for by a value of half the level of the detection limit.

Clinical and biophysical parameters

The OSCORAD (Objective SCORing Atopic Dermatitis) was used to determine the clinical severity of AD lesions (19). Since only isolated skin sites were treated

and clinically assessed, the body surface area was set to 1% when calculating the OSCORAD. To assess the skin barrier function, transepidermal water loss (TEWL) was measured using a Tewameter 300 (Courage and Khazaka Electronic GmbH, Cologne, Germany).

Statistics

Statistical calculations were performed using Graphpad Prism 6.0 (Graphpad software, La Jolla, CA, USA). The Shapiro-Wilk test was used to check for data distribution. In the case of deviation from normal distribution, a non-parametric two-sided Wilcoxon signed-rank test was used. For normally distributed data, a two-sided t-test was applied. A p value of <0.05 was considered statistically significant. Because of the explorative character of the study, no correction for multiple testing was applied. Spearman's rank correlation coefficient was used to perform correlation analysis of individual immunomodulators, clinical and biophysical parameters.

RESULTS

In total, 90 AD patients and 20 healthy controls completed the study and their samples were analyzed. The concentrations of 24 out of 38 measured inflammatory mediators could be quantitatively determined (i.e. the concentrations were above the detection limit of the method) in the majority of the samples (Table 1). Immunomodulators of which the concentrations in majority of samples (>50%) were below the detection limit were not further analysed, a summary of these mediators can be found in Supplement 1. The levels of eleven inflammatory mediators were significantly decreased after the six week topical emollient treatment. Among them were Th2 inflammatory mediators interleukin (IL)-4 (P=0.0033), IL-13 (P=0.0016), CC chemokine ligand (CCL)2/monocyte chemotactic protein (MCP)-1 (P=0.0025), CCL22/macrophage-derived chemokine (MDC) (P=0.0153) and CCL17/thymus and activation-regulated chemokine (TARC) (P=0.0047). A significant decrease was also found for the levels of the regulatory cytokines IL-1β (P=0.0008), IL-2 (P=0.0359), IL-4 (P=0.0033), IL-8 (P=0.0011) and IL-10 (P=0.0069) as well as the acute phase proteins serum amyloid A (SAA) (P=0.0276) and C-reactive protein (CRP) (P=0.0006) and vascular adhesion molecule (VCAM-1) (P=0.0087). The

other mediators did not show a significant change over time. When compared with healthy controls most of inflammatory mediators were significantly higher in the treatment group, both before and after therapy (Table 1).

Both the clinical severity as assessed by OSCORAD and the skin barrier function as measured by TEWL improved significantly after six weeks of therapy (Table 2). The OSCORAD decreased from 14.20 to 10.70 ($p<0.0001$) and the TEWL from 29.91 to 28.2 g/m2/h ($P=0.008$). The decrease in OSCORAD was 14.0 units ($p<0.0001$) in the patients with moderate AD and 3.5 ($p<0.0001$) in patients with mild AD (Table 2). A representative picture of lesions in patients with a mild and moderate disease severity is shown in Fig. 1.

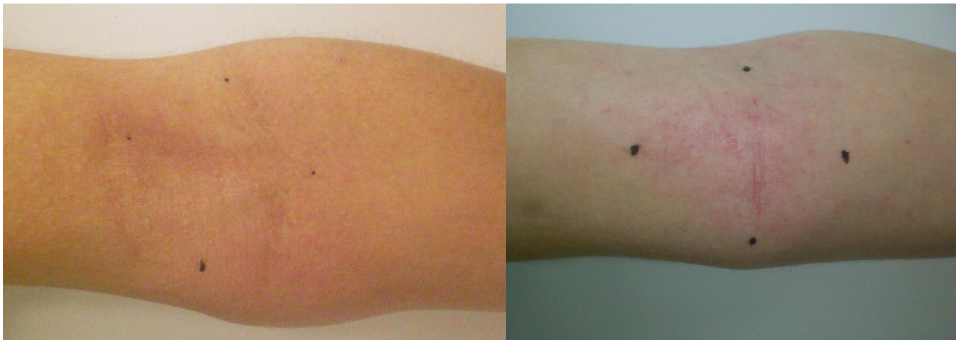

Fig. 1. Representative pictures of the studied lesions. Tape strip samples were taken in the center of the marked region. Left: mild AD, right: moderate AD.

Table 1. Concentrations of inflammatory mediators in the SC before and after topical therapy

	BEFORE THERAPY	AFTER THERAPY		HEALTHY CONTROL (n=20)	BEFORE vs. AFTER	BEFORE vs. HEALTHY	AFTER vs. HEALTHY
	M (IQR)	M (IQR)	n	M (IQR		P-value	
CRP *	3.799 (0.9;12.7)	3.516 (1.0;8.3)	75	ND	**0.0006**		
CCL11/ Eotaxin-1	270.2 (146.70;439.9)	313.5 (179.5;443.7)	75	209 (127;314.6)	0.6841	0.0721	**0.0072**
CSF2/GM-CSF	9.20 (3.60;21.58)	7.0 (2.91;19.24)	88	4.6 (2.5;11.5)	0.0705	**0.04**	0.1752
IFN-γ	49.9 (38.0;80.2)	44.3 (31.3;79.5)	57	33.4 (17.5;42.2)	0.1596	< **0.0001**	**0.0002**
IL-10	8.7 (5.6;13.9)	6.6 (4.6;12.3)	57	0.8 (0.01;1.67)	**0.0069**	< **0.0001**	< **0.0001**
IL-13	80.9 (53.7;114.8)	67.1 (45.2;94.8)	90	2.0 (1.6;8.2)	**0.0016**	< **0.0001**	< **0.0001**
IL-16	171.2 (83.4;363.3)	186.5 (71.3;334.9)	90	18.3 (1.3;42.0)	0.3914	< **0.0001**	< **0.0001**
IL-17a	74.3 (40.9;120.4)	84.5 (42.2;118.5)	88	13.7 (9.6;24.3)	0.4250	< **0.0001**	< **0.0001**
IL-1α *	2.3 (0.08;5.6)	2.4 (0.6;6.3)	88	42.1 (35.1;59.9)	0.1475	< **0.0001**	< **0.0001**
IL-1β	93.5 (37.7;243.6)	59.7 (31.8;137.6)	88	137.4 (48.3;523.5)	**0.0008**	0.2149	**0.0156**
IL-2	20.14 (12.2;56.8)	19.4 (11;39.3)	90	17 (0.04;4.0)	**0.0359**	< **0.0001**	< **0.0001**
IL-4	11.4 (8.4;16.5)	9.7 (6.1;16.0)	41	0.3(0.3;0.3)	**0.0026**	< **0.0001**	< **0.0001**
IL-5	25.0 (13.0;43.3)	26.0 (15.0;46.8)	88	10 (7.4;14.7)	0.7831	< **0.0001**	< **0.0001**
IL-8	97.8 (33.5;320.3)	46.1 (16.3;152.8)	90	14.2 (2.7;35.6)	**0.0011**	< **0.0001**	**0.0002**
IL-12_23p40	54.8 (28.7;124)	58.0 (18.9;147.1)	49	34.8 (13.2;49.8)	0.1143	**0.0009**	**0.0079**
CCL2/MCP-1	16.5 (8.4;32.8)	11.4 (6.2;21.7)	90	7.4 (3.2;13.8)	**0.0074**	< **0.0001**	< **0.0001**
CCL13/MCP-4	247.4 (166.5;472.4)	295.8 (160.2;534.1)	90	67.3 (25.0;95.6)	0.3369	< **0.0001**	< **0.0001**
CCL22/MDC	1152 (656.2;1941)	955.4 (522.1;1465)	90	716.7 (465.2;1325)	**0.0153**	0.0908	0.5992
CCL3/MIP-1a	210.8 (137.7;325.7)	245.5 (167.7;335.7)	88	5.82 (1.79;139.1)	0.7352	< **0.0001**	< **0.0001**
CCL4/MIP-1b	164.6 (102.6;335.6)	179 (94.6;318.8)	90	22.6 (7.3;29.2)	0.9669	< **0.0001**	< **0.0001**
SAA*	9.8 (102.6;335.6)	8430 (94.62;318.8)	75	ND	**0.0276**		
CCL17/TARC	85.0 (35.7;187.2)	56.3 (34.1;105.1)	90	26.1 (35.6;52.7)	**0.0047**	**0.0002**	**0.0067**
TNF-β	8,0 (4.6;20.5)	8.2 (4.3;18.4)	51	1.16 (3.41;5.3)	0.2611	< **0.0001**	< **0.0001**
VCAM-1*	3.8 (1.2;10.7)	2.4 (0.7;6.7)	57	ND	**0.0087**		

Values are expressed as medians (interquartile ranges), unless otherwise indicated. 'Before vs. after' was tested by a two-sided Wilcoxon signed rank test an 'before vs. healthy'and 'after vs. healthy and 'after vs health'by a Mann-Whitney test. n = Number of samples in which the inflammatory mediators' concentration could quantitatively be determinded; p = level of significance (significant differences are highlighted in bold). CSF2 =Colony-stimulating factor 2; GM-CSF = granulocyte-macrophage colony-stimulating factor; IFN = interferon; MCP = monocyte chemotactic protein; MDC = macrophage-derived chemokine; MIP = macrophage inflammatory protein; TNF = tumor necrosis factor; NM not determined. * All concentrations are expressed as ng/μg protein, except CRP, IL-1, SAA and VCAM-1 that are expressed in μg/μg protein.

Table 2. Clinical and biophysical parameters measured at baseline and after 6 weeks of therapy

	BEFORE THERAPY	AFTER THERAPY		
	M (IQR)	**M (IQR)**	**n**	**P**
Total OSCORAD	14.2 (10.7;21.2)	10.7 (7.2;14.2)	90	**<0.0001**
Mild OSCORAD	14.2 (10.7;17.70)	10.7 (7.2;14.20)	69	< **0.0001**
Moderate OSCORAD	28.2 (26.45;31.70)	14.2(10.7;22.95)	21	< **0.0001**
TEWL (g/m2/h)	29.91 (23.29;39.37)	28.2 (19.73;36.18)	90	**0.008**

Values are expressed as medians (interquartile ranges), unless otherwise indicated. P = Level of significance for the difference between the baseline and after therapy (two sided Wilcoxon signed-rank test).

Correlation analysis

In order to investigate relationships between the various inflammatory mediators, TEWL and OSCORAD, a correlation analysis was performed (online suppl. tables 2.1 and 2.2). A strong and significant correlation was found between the change in Th1 cytokines CCL11/Eotaxin-1 and IL-5 ($r = 0.61$, $p < 0.001$), the Th2 cytokines IL-4 and IL-13 ($r = 0.72$, $p < 0.001$), and tumor necrosis factor-β and IL-12 ($r = 0.75$, $p < 0.001$). The acute-phase proteins SAA and CRP both correlated well with VCAM-1 ($r = 0.90$; $p < 0.001$ and $r = 0.74$; $p < 0.001$, respectively). There was no correlation in changes of any of the investigated mediators with changes in OSCORAD and TEWL. However, in a subgroup analysis of moderate AD cases (OSCORAD >25), CCL17 and IL-8 showed to be significantly correlated with the decrease of OSCORAD ($r = 0.56$; $p = 0.01$ and $r = 0.45$; $p = 0.05$). Furthermore, significant correlations between the values of various inflammatory mediators and OSCORAD and TEWL at baseline as well as after the therapy (online suppl. tables 3.1 and 3.2) were found.

DISCUSSION

In the present study, we explored the potential of a minimally invasive sampling technique for the determination of inflammatory mediators in the SC of the lesional skin of AD patients with mild-to-moderate AD before and after treatment with an emollient. In total, 24 out of 38 investigated mediators could quantitatively be determined. The absolute levels of various inflammatory mediators correlated with the skin severity (OSCORAD) and skin barrier function (TEWL) at baseline and/or after therapy (online suppl. table 3).

After 6 weeks of topical therapy with an emollient containing magnesium and ceramides, both the severity of skin lesions and the skin barrier function improved. This was consistent with a significant decrease in the levels of various inflammatory mediators, mainly Th2-related cytokines and chemokines characteristic of acute AD lesions (23,24,25,26), including IL-4, IL-13, CCL2, CCL22, and CCL17. As expected, the levels of most inflammatory mediators were significantly higher in AD patients (before and after therapy) than in the healthy control group (7). Furthermore the levels of IL-4 and IL-13 were strongly correlated ($r = 0.70$). IL-4 and IL-13 are known as drivers of key pathogenic mechanisms of AD such as the survival of Th2 cells, differentiation and activation of myeloid and dendritic cells, activation of B cells, stimulation of the IgE class switch, and recruitment of eosinophils (27,28). Furthermore, they suppress lipid production, filaggrin expression, and keratinocyte differentiation, impairing the skin's barrier function (5,29). The importance of IL-4 and IL-13 in AD has been previously confirmed in clinical studies. Thus, systemic therapy of AD patients with dupilumab, a monoclonal IL-4 and IL-13 receptor antagonist, resulted in a rapid improvement of disease activity (5,30,31,32,33,34,35).

In the present study, there was no significant change in the levels of the Th1 mediator interferon-γ. However, the levels of IL-1β and IL-2 decreased significantly during the 6 weeks of therapy. The latter is associated with pruritus, a key symptom of AD (36,37,38). Apart from the Th2- and Th1-mediated molecules, significant changes were observed for the more general inflammatory mediators SAA, CRP, and VCAM-1 during therapy. Similarly, Caproni et al. (39) found a decrease of VCAM-1 in lesional skin biopsies of AD patients after local therapy with tacrolimus. Elevated CRP serum levels have previously been reported in AD (40). However, to the best of our knowledge, this is the first time that CRP was measured directly in human AD skin lesions.

Data on the local cytokine and chemokine milieu in AD are scarce (30). Morita et al. (41) showed a significant correlation between the SC levels of CCL17 and disease severity, Amarbayasgalan et al. (42) showed that IL-8 was correlated with AD-severity. Szegedi et al. (7) determined a wide range of Th1 and Th2 cytokines in dermal interstitial fluid collected from healthy subjects, and in the lesional and nonlesional skin of AD patients. IL-8 and the levels of Th2 cytokines/chemokines

IL-13, CCL2, and CCL17 were lower in healthy skin compared to lesional skin. Data on inflammatory mediators obtained from skin samples (i.e. SC tapes and interstitial fluid) are consistent with those from blood samples. Utilizing a multiplex analysis, Thijs et al. (43) found a significant decrease in the levels of 7 out of 31 investigated biomarkers, including CCL17 and CCL22, in blood samples from patients with moderate-to-severe AD who were treated with a potent corticosteroid (44). This is in line with the results of the present study in which a significant decrease in CCL17 and IL-8 was found after the therapy with the emollient containing magnesium and ceramides. CCL17 and CCL22 act via the Th2 cell chemokine receptor CCR4 (44,45,46,47). In a recent meta-analysis, serum levels of CCL17 were the most reliable available biomarker of AD disease severity, showing correlation coefficients of 0.60 (95% CI 0.48-0.70) and 0.64 (95% CI 0.57-0.70) in longitudinal and cross-sectional studies, respectively (41,43,44,48,49).

Although the levels of several immunomodulators in the present study were significantly decreased after therapy and were associated with the OSCORAD and TEWL at baseline and/or after therapy, there was no correlation between the changes in their levels and an improvement of clinical symptoms (OSCORAD) or skin barrier function (TEWL). One of the reasons for this might be the inclusion of patients with mild-to-moderate AD who showed a slight but significant reduction of OSCORAD from 14.2 to 10.7. For instance, patients in the study conducted by Thijs et al. (43) had more severe AD, and the study showed a large decrease of the severity index SASSAD ('six area, six sign AD') from 36.9 at baseline to 8.0 at the endpoint. Indeed, in the present study a subgroup analysis of moderate-AD individuals did show that declining concentrations of CCL17 and IL-8 were significantly correlated with the decrease in symptoms as assed by OSCORAD. The performance of these inflammatory mediators as biomarkers of disease activity should therefore be further investigated in patients with more severe AD. One of the limitations of the present study is that the SC samples of the control group were collected from the upper back. However, Amarbayasgalan et al. (42) have shown no regional differences for various inflammatory mediators in healthy skin, including the back, neck, and arm. Furthermore, most of the investigated cytokines are produced upon inflammation due to AD and are not constitutively present in healthy skin.

In conclusion, tape stripping of the SC is a simple, minimally invasive approach to the study of local levels of various immunomodulatory molecules relevant to AD. CCL17 (TARC) and IL-8 were found to be the most promising biomarkers for AD. These biomarkers provide not only a better understanding of the pathogenesis of AD but also information on treatment efficacy, further enabling more targeted clinical care.

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Determination of natural moisturizing factors in the skin: Raman microspectroscopy versus HPLC

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ABSTRACT

Background: Natural moisturizing factor (NMF) is used as genotypic and phenotypic biomarker in diagnostics. This study is a side-to-side comparison of two different methods to determine NMF in atopic dermatitis patients: Raman microspectroscopy and stratum corneum tape stripping followed by HPLC.

Results: Measured NMF values were significantly correlated ($R2=.61$; $p<.0001$), both methods demonstrated a concentration-depth dependence of NMF and reduced NMF levels in the carriers of filaggrin null mutations. Good agreement between measurements of left and right arms indicated robustness and good reproducibility of both methods.

Conclusions: Both methods showed comparable performance, choice of method will rather be influenced by practical consideration.

INTRODUCTION

The function of the skin barrier has gained renewed interest in dermatological research after identification of loss-of-function mutations in the filaggrin gene as a main risk factor for Atopic Dermatitis (AD), a common inflammatory skin disease (1). Filaggrin is a histidine-rich protein that is degraded into free amino acids in the uppermost layer of the skin; the stratum corneum (SC). The amino acids may be further metabolized into hygroscopic derivatives such as pyrrolidone carboxylic acid (PCA) from glutamine and urocanic acid (UCA) from histidine (His). This makes filaggrin a major source of hygroscopic compounds, which are collectively named Natural Moisturizing Factor (NMF), known to play an important role in skin hydration and barrier function (2, 3). Up to 55% of AD patients carry a filaggrin mutation; furthermore the carriers have an early onset and a more persistent and severe course of disease(1, 4) (5). We have demonstrated that filaggrin degradation products can be used as a biomarker for filaggrin genotype, which might enable sub-classification of AD (6-8). Next to genetic factors, filaggrin levels are influenced by exposure to detergents, organic solvents or topical therapy by corticosteroids(9, 10). Thus, they might also be used as a biomarker of the damaging effect of environmental exposures (10). The potential of filaggrin degradation products as a biomarker demands a feasible and reliable analytical method, which might be used for diagnostics and research purposes. Currently, for their determination the most used methods are Raman microspectroscopy (RMS) and stratum corneum tape stripping technique followed by high performance liquid chromatography (TS/HPLC). RMS is an optical method determining filaggrin degradation products via spectroscopic measurements within the SC. It is a non-invasive method, which determines, in real-time, levels of several individual filaggrin degradation products simultaneously at different SC depths (11, 12). TS/HPLC determination is based on the tape stripping technique by which the SC layers are collected in a minimally invasive manner by adhesive tapes. After extraction by acid or base, the filaggrin degradation products are determined by HPLC (6). In most published studies, RMS measurements include several free amino acids (serine, glycine, ornithine, alanine, histidine and proline) and PCA, UCA, and urea, while main TS/HPLC studies measured PCA and UCA, and in some studies also His is included(13, 14). The measured filaggrin degradation products are referred to as NMF, although taken

strictly NMF contains next to filaggrin degradation products also lactate, urea and sugars and inorganic salts (15).

So far, there is no side-to-side comparison between the two methods concerning agreement of the measurements and practical considerations. Therefore, in the present study we compared the results of both methods applied to the same AD-patient population.

METHODS

Subjects

Twenty patients with mild to moderate AD were recruited from the outpatient dermatological clinic (Waterland Hospital, the Netherlands). Diagnosis was done according to the Hanifin and Raijka criteria(16) for AD by a trained dermatologist. From all patients a buccal swab sample was collected for genotyping of the four most prevalent *Filaggrin* mutations in the European Caucasian population (R501X, 2282del4, R2447X, and S3247X)(17, 18). The experimental protocol followed the Declaration of Helsinki Principles and was approved by the Medical Ethics Committee of the Academic Medical Centre. Written informed consent was obtained from each participant.

Raman microspectroscopy (RMS)

NMF were determined on visibly non-affected volar forearm skin using confocal Raman microspectroscopy (Model 3510 Skin Composition Analyzer; RiverD International BV, Rotterdam, The Netherlands). In one subject it was difficult to find a non-affected skin site which was accessible for the RMS measurement, therefore the measurement was done on the volar aspect of the wrist. Raman spectra were recorded in the 400 - 1800 cm^{-1} spectral region using a 785-nm laser with 25 mW of laser power on the skin. Levels of NMF relative to keratin (NMF/keratin ratio in arbitrary units) are displayed in real-time by the data acquisition software included with the RMS instrument. For more detailed analysis and comparison with the TS/HPLC results, the data were further processed offline. NMF/keratin ratios were determined from the Raman spectra by classical least-squares fitting using the

SkinTools 2.0 software (RiverD International BV, Rotterdam, The Netherlands). In this analysis NMF is defined as a the sum of 7 dominant constituents of NMF: serine, glycine, PCA, ornithine, alanine, histidine (His), and proline (19). A reference spectrum of NMF has been constructed from the weighted sum of the Raman spectra of these dominant constituents and is used by the least-squares-fitting algorithm to determine the NMF/keratin ratio. The details of this method have been described elsewhere (11, 12). Possible negative values from the unconstrained classical least-squares-fit, due to low Raman signals from NMF, were replaced by value 0. NMF values from individual Raman spectra with insufficient spectral quality based on signal-to-noise ratio or insufficient quality of the fit based on too large residual between the original spectrum and the calculated fit were excluded from further analysis. The measurements were performed without any pretreatment of the skin. The subject positioned the arm on the instrument with the skin resting on the 2x4 cm^2 fused silica measurement window. After the operator had set a starting point at the skin surface, the instrument recorded a profile consisting of 8 Raman spectra from the skin surface to 28 μm below the skin surface at 4μm depth increments. The total time to record one profile of 8 Raman spectra was 48 s. A maximum of 8 profiles from different areas of the volar aspect of the left and the right forearm were measured for each patient. The NMF values measured between 2 and 8 μm from the skin surface were pooled and averaged to obtain the NMF value of each patient per location (left or right arm). This depth range overlaps with the central portion of the SC. To enable a comparison of depth-dependent NMF concentrations with the results from HPLC-TS, the NMF profiles measured with Raman spectroscopy were interpolated on 1 μm depth intervals and then pooled for all patients, resulting in the average NMF value and standard deviation in the depth range 0 to 12 micrometer. All results are presented as NMF/keratin ratio in arbitrary units.

Tape stripping/ HPLC

To enable comparison of NMF-concentrations determined with HPLC-TS and RMS, which determines NMF levels at different depths, the average concentration of three tapes (4, 6 and 8) was calculated and compared to an average value of the RMS results at corresponding position in the SC. The depth of each tape has been estimated from the cumulative amount of protein obtained by measuring optical density of the tape as described previously(20, 21).

The SC samples for TS/HPLC were taken in the vicinity (unaffected by AD lesions) of the skin sites used by RMS on the left and right volar forearm. Eight D-squame tapes (22-mm diameter, Cuderm, Dallas, TX, USA) were consecutively placed on the skin and gently pressed with a standardized pressure pen (D500 - D-Squame Pressure Instrument Cu-derm, Dallas, TX, USA). Tapes were then removed, placed in vials and stored at -20 °C. NMF components His, PCA and UCA (trans- and cis isomer) in the SC on the tape were extracted with 500 µl of 25% (w/w) ammonia solution, evaporated to dryness, and reconstituted in 500 µl of pure water. The HPLC separation was achieved by using a 250 * 3 mm reversed-phase Prevail column (Grace /Alltech, Breda, the Netherlands), with a flow rate of 0.4 mL min. The effluent was monitored by a UV / Vis detector (UV-975; Jasco, The Netherlands) set at 210 nm for PCA and His and at 270 nm for both UCA isomers. The total amount of UCA, PCA and His referred here collectively to as NMF, has been calculated by summing up their individual molar amounts. The amount of SC collected by the tapes was assessed by measurement of the optical density (OD) of each D-Squame® disc with the D-Squame Scan 850A (Monaderm, Monaco, France). The amount of SC on the tape was calculated from the OD, and expressed as the mass of protein/cm^2 according to a standardized procedure described elsewhere (20). The concentrations of NMF were corrected for the amount of protein, and expressed as nmol/µg protein.

Statistics

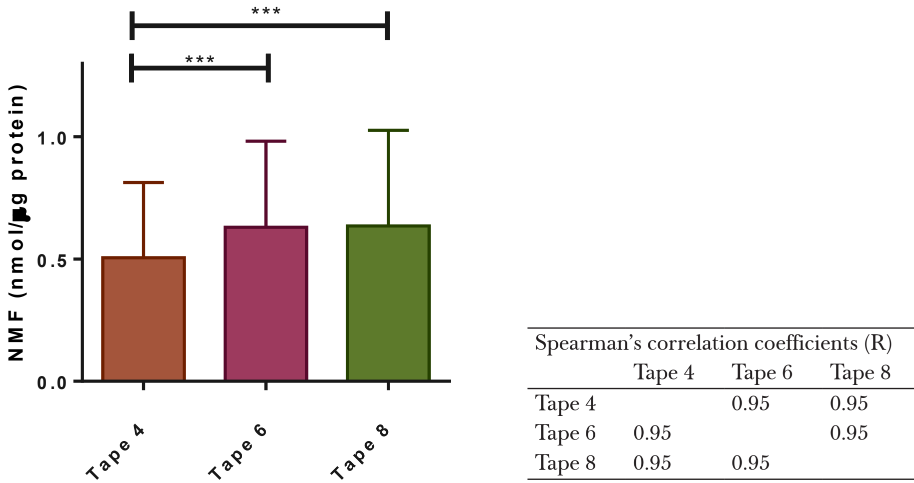
All calculations were performed by GraphPad version 6.0 (GraphPad Software, La Jolla California USA). Distribution of data was tested by Shapiro-Wilk normality test. The difference in NMF values between the tapes was tested by repeated measures ANOVA using Bonferroni correction for multiple testing. The comparison of the NMF values between RMS and TS/HPLC method was tested by linear regression model. The comparison of the NMF values between left and right arm was performed by linear regression analysis and Bland-Altman analysis. The Bland-Altman plot analysis evaluate a bias between the mean differences, and to estimate an interval, within which 95% of the differences of the second measurement, compared to the first one, fall (Giavarina, 2015).

RESULTS

Genotyping for the four most prevalent *filaggrin* mutations in the Caucasian population revealed that four patients were heterozygous and one patient was compound heterozygous carrier of a *filaggrin* loss-of-function mutation.

Depth dependence of the NMF levels in the SC

As shown in Fig. 1, the NMF concentrations on the 4th tape strip were significantly lower (paired 1-way ANOVA test with Bonferroni correction for multiple testing) than the NMF values on the 6th and 8th tapes which originate from deeper SC layers. The NMF concentrations on all tapes were very strongly correlated (Table inserted in Fig. 1). RMS shows a similar pattern of concentration-depth dependency, with maximum NMF concentrations around 2-4 μm (Fig. 2).



Spearman's correlation coefficients (R)			
	Tape 4	Tape 6	Tape 8
Tape 4		0.95	0.95
Tape 6	0.95		0.95
Tape 8	0.95	0.95	

Fig. 1. The NMF concentrations on the 4th, 6th and 8th tape and the correlations between NMF concentrations determined on the three tapes (table). The respective estimated depths of the 4th, 6th and 8th tapes were 1.9 (SD 0.32); 2.8 (SD 0.41) and 3.6 (SD 0.51) µm. P<0.0001 (ANOVA paired test with Bonferroni correction). The results are shown as mean ±SD (n=20)

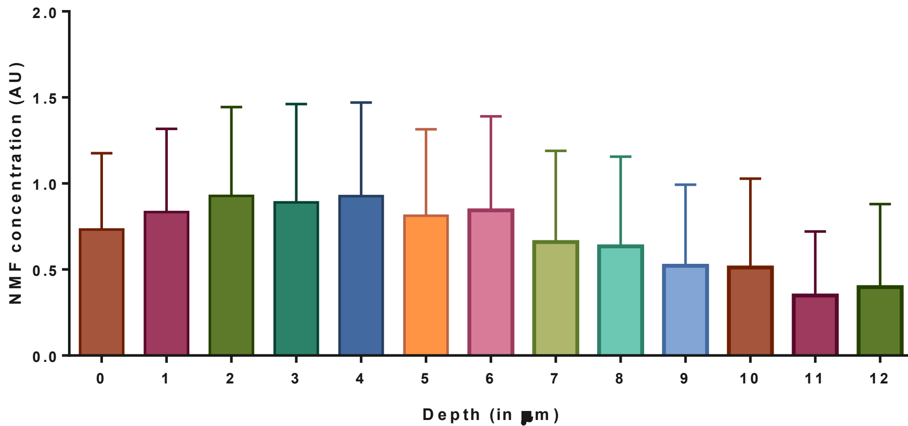


Fig. 2. The concentrations of NMF (serine, glycine, PCA, ornithine, alanine, histidine, and proline) measured by RMS, interpolated on 1 µm depth intervals and averaged for all patients. The results are shown as mean ±SD (n=20)

Comparison of NMF measured on the left and right arm

As shown in Fig. 3 (left panel), both methods showed strong correlations of NMF values between the left and right arm. Furthermore, as assessed by a Bland-Altman plot, a preference method when analyzing agreement between two measurements

of the same parameter (right panel, Fig. 3), there was a good agreement in the measured values between left and right arm with the average bias (average of the difference between left and right arm) of 0.02 for TS/HPLC and 0.005 for RMS. The fact that the average bias is close to zero indicates that the measurements on the left and right arm do not produce systematically different results.

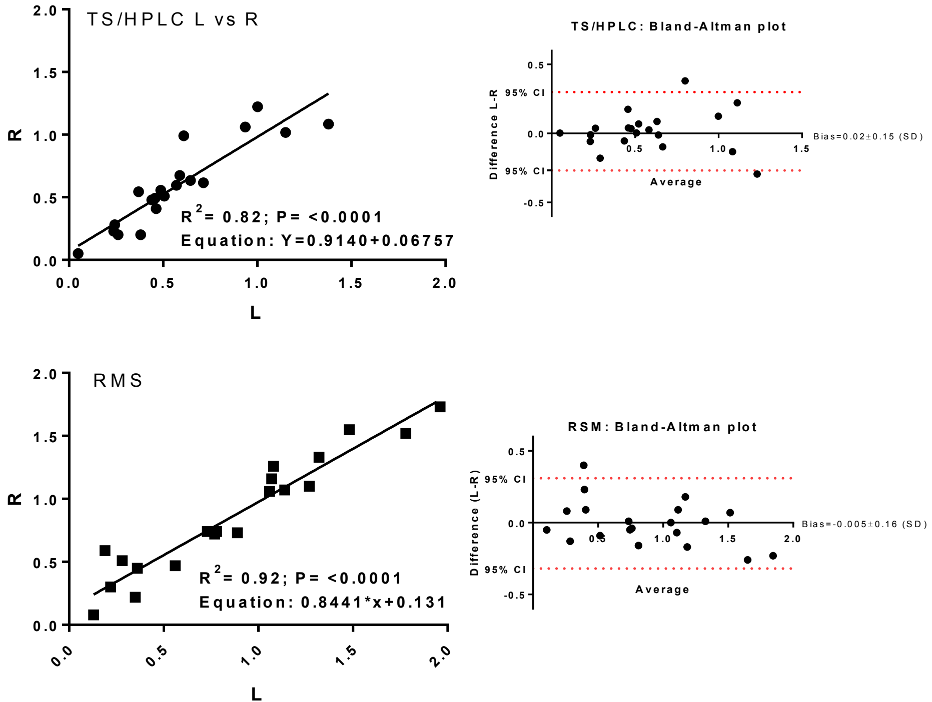


Fig. 3. Comparison of the NMF levels determined on the left and right arm by TS/HPLC (upper panel) and RMS (lower panel). In the Bland-Altman plots the horizontal lines represent the mean difference, and at the limits of agreement (mean difference plus and minus 1.96 times the standard deviation of the differences)

Correlation RMS vs TS/HPLC

As there was no systematic difference in the NMF values between left and right arm, for the comparison of the results obtained by two methods, the corresponding NMF values measured on the left and right arm were averaged. To compare the two methods linear regression analysis was performed including residual analysis. As shown in Fig. 4, both methods showed a strong correlation between the NMF values measured in the same AD patients (r^2=0.61; P<0.0001), and furthermore the

residuals showed a random distribution around the horizontal axis (Fig. 4, right panel). The average NMF value measured by the TS/HPLC method for the carriers of a *filaggrin* mutation (indicated with X in Fig.) was significantly lower than the average NMF value for the patients who were wild-type for *filaggrin* mutations (respective NMF values of 0.24 and 0.62 nmol/μg protein; P=0.005). A similar trend was found by RMS, however, the difference in NMF values between *filaggrin* mutation carriers and wild-type individuals did not reach significance (respective average NMF values were 0.53 and 0.98 AU; P=0.079).

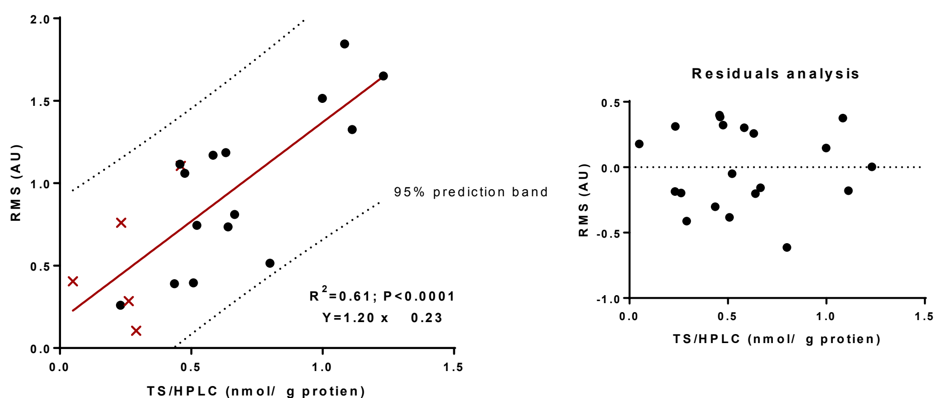


Fig. 4. The correlation between NMF concentrations determined in AD patients by TS/HPLC and RMS. The X symbol marks the patients with a filaggrin loss-of-function mutation. R^2= coefficient of determination.

DISCUSSION

NMF concentrations in the SC of AD patients as measured by of Raman microspectroscopy (RMS) and stratum corneum tape stripping method followed by HPLC (TS/HPLC) show a strong correlation despite the fact that the investigated methods did not measure the same NMF components. RMS includes in the measurement serine, glycine, PCA, ornithine, alanine, histidine and proline, while TS/HPLC includes PCA, histidine and both isomers of urocanic acid. However, all mentioned NMF components are degradation products of the same protein: filaggrin. So the choice and the number of measured NMF components seem not to be critical. This is consistent with previous data,showing that different filaggrin degradation products are highly correlated (21), (22)

Both RMS and TS/HPLC show a similar pattern in depth dependency of the NMF concentration. Although NMF concentrations on all three tapes were strongly correlated, the NMF concentrations on the 4th tape was lower than the levels on the 6th and 8th tapes (approximate SC depth of 1.9; 2.8, and 3.6 μm, respectively). Also by RMS, the NMF levels show lower concentrations in the more superficial SC layers (up to 2 μm). (14) (23)

A strong correlation and agreement in the NMF values measured on the left and right arm indicate good reproducibility and robustness of both methods.

The average NMF value in the carriers of *filaggrin* mutations measured by TS/HPLC was significantly lower than the average NMF value in the patients who were wild type for filaggrin mutations, which is in accordance with literature data [6,7]. NMF results obtained by RMS showed a similar trend although the difference in NMF values between carriers and non-carriers did not reach the level of significance (P=0.08). It has to be noted that the present study was not specifically designed to differentiate carriers of a *filaggrin* mutation from non-carriers. Especially for RMS, measurements for prediction of *filaggrin* genotype can be more accurately and rapidly performed on the palm of the hand where the SC is much thicker than on the forearm enabling more measurement points at SC depths where the NMF values level off (7). Previous studies have shown that NMF measured by RMS on the palm of the hand is a strong predictor of *filaggrin* genotype[7,](8).

On the practical level, both methods have advantages and limitations. RMS can provide a quick and real-time outcome on NMF concentration in the SC in a non-invasive manner. Due to the thorough research protocol in this study, with multiple measuring points per skin site the total period of measurement, including preparations, per patient was 15 minutes. TS/HPLC enables rapid collection of tape strip samples from the subjects (roughly half the time of RMS, including preparation), but samples require further processing and analysis. If (relatively) a quick assessment of NMF levels is required, e.g. in the clinics, RMS would therefore be the method of choice. On the other side, TS/HPLC can be implemented fast in research and clinical environments; tape stripping can be performed on-site with minimal facilities which could be advantageous in multicenter or field studies. The

used RMS hardware as a bench-top device limits the practical use to hand palm (preferably) and arms, whereas tape stripping for HPLC analysis is not limited to a specific body site which might be advantageous by monitoring of local therapy. Costs of RMS equipment are currently relatively high, but unlike HPLC RMS it does not require laboratory facilities. The current allround research RMS instrument for in vivo skin analysis, used in this study, is commercially available in various versions ranging from €195.000 to €290.000. However devices dedicated to a single specific task will have a very significantly reduced price and dedicated software for RMS and streamlining of the workflow can reduce training needed to work with RMS to a minimum. While RMS is still a specialized field, expertise in HPLC is present in most analytical laboratories and the costs of the instrument are relatively low (approximately €25.000).

An important advantage of RMS to TS/HPLC is that NMF can be measured at specified depths, although at the cost of increased measurement time. Thus the optimal SC depth (i.e. the depth where the NMF levels level off or reach maximum) can be derived from the recorded spectra. As shown in the present study, the NMF levels are depth-dependent, which is in accordance with our previous study(7) Although there was a strong correlation between all three strips, using the tapes collected from the most superficial layers would create an underestimation of the actual NMF concentration. In the present study, there was no difference between the 6th and 8th strip number which corresponds to the SC depth around 3-4 μm. Due to intra-individual variability in the SC thickness and cohesiveness the SC in other diseases or other body locations the optimal strip number might be different from AD-forearm SC and should therefore be investigated before implementation of tape stripping in a different disease or body location. Both methods normalize the NMF values with the protein levels. The RMS normalizes the NMF values with the simultaneously measured keratin levels whereas TS/HPLC uses proteins derived from the optical density (OD) of the TS which needs to be done separately. The prerequisite for using OD is that the SC cells on the tape are homogeneously distributed over the tape, which especially in diseased skin is not always the case. When SC is non-homogeneously distributed over the tape, protein concentration have to be determined by another method e.g. by weighing or by a spectrophotometric assays (21). In that case the determination of NMFs by TS/HPLC is more time consuming.

CONCLUSION

The concentrations of NMF determined by Raman microspectroscopy and tape stripping followed by HPLC show a good correlation. Good agreement between measurements obtained on the left and right arm indicate robustness and good reproducibility of both methods. The choice between these two methods therefore will likely be influenced by practical considerations such as price, accessibility, available expertise and time-constrains. RMS can provide fast and detailed results on the profile of NMF components across the SC. However the current cost and accessibility of state-of-the-art Raman equipment may be an obstacle for using this technique more widely. Development of a small portable device with user-friendly software and quick read-off would be of a great value for clinical practice. Tape stripping enables simple and quick collection of a large number of samples which can be stored for a longer period of time which is convenient for large clinical studies. However, in contrast to RMS, further processing of the samples for HPLC analysis is required, and the sampling process (i.e. the tape number) should be optimized before application in a specific population.

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CHAPTER 5

General Discussion

GENERAL DISCUSSION

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

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

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

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

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

MAIN FINDINGS

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

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

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

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

and broader difference in the immune response than the other tested allergens, suggesting that skin barrier damage caused by MCI/MI enhances its allergenic response.

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

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

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

In the second study, which was performed in patients with mild to moderate AD (**Chapters 4.2 and 4.3**), skin barrier biomarkers (NMF, TEWL, skin hydration), a large array of inflammatory mediators, and clinical symptoms (SCORAD)

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

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

METHODOLOGICAL CONSIDERATIONS

Measuring inflammatory mediators for ACD, ICD, and AD in the SC has some limitations (**Chapters 3.2 and 4.3**). In contrast to IL-1α and IL-1RA, which are present constitutively in substantial amounts in the SC, most of measured inflammatory

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

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

INTERPRETATION OF RESULTS AND RELEVANCE FOR CLINICAL PRACTICE

Biomarkers in diagnostics

Contact dermatitis (CD) is one of the most frequently seen occupational diseases(6). A major challenge for clinicians is to distinguish between the two subtypes, ACD and ICD, which have similar clinical features but different pathophysiology, treatment

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

Although MCI/MI showed a similar pattern to SLS regarding changes of NMF, proteases, and IL-1α, one of the investigated biomarkers – DTI, which expresses surface texture – showed a clear difference between ACD and ICD. DTI is a novel biomarker of skin barrier damage, which showed its value also in AD. As shown in Chapter 4.1 (a study in which DTI was introduced as a skin barrier biomarker), DTI is closely related to *FLG* LOF mutations and NMF. The group of AD patients with *FLG* mutations is shown to have a divergent characteristics compared to AD patients without *FLG* mutations. The skin barrier defect in AD patients with *FLG*

mutations, in this case represented as a high DTI, is persistent over time, even when clinical symptoms improve. Although the exact role of DTI in the pathogenesis of AD is still unclear, knowledge on the diversity within the AD patient population might aid in the stratification of AD patients and for a personalized therapeutic approach.

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

Therapy monitoring

Therapy monitoring is of importance in clinical practice, as well as in evaluating the efficacy of new interventions. Biomarkers might offer an advantage over clinical outcome scoring tools such as SCORAD or EASI, as the latter include subjective

parameters like sleep deprivation or itching, and are subject to intra- and inter-observer variability(19, 20). When biomarkers are used to evaluate therapy or assess disease severity, they are usually derived from blood samples. The invasiveness of this method makes it less suited for large cohorts of patients and poses a barrier to the implementation of biomarkers in pediatric medicine where AD is a major topic(21). Moreover, AD usually presents on certain body areas and thus the monitoring of biomarkers in the treated skin in the case of topical therapy might be more relevant than their blood levels. Biomarkers that are suggested as candidate biomarkers for monitoring or of disease severity mainly concern inflammatory mediators(22, 23). Elevated total and/or allergen-specific serum immunoglobulin IgE levels are commonly associated with AD, but only moderately correlate with disease severity and are not specific; some AD patients have normal IgE levels(24). In a recent meta-analysis by Thijs et al. on AD biomarkers, serum TARC/CCL17 levels showed the best correlation with disease severity in cross-sectional and longitudinal studies(25). TARC (thymus- and activation-regulated chemokine, also known as CCL17) is a chemokine produced by the keratinocytes in AD lesions and is important in mediation of the acute Th2 inflammatory reaction of the disease(26). Thijs et al.'s results are consistent with those from the study described in **Chapter 3.2**, which shows that the SC concentration of TARC/CCL17 is correlated with disease severity in patients with moderate AD. Furthermore, the changes in the TARC/CCL17 levels were associated with the changes in SCORAD, indicating that TARC/CCL17 is a suitable biomarker to monitor therapy efficacy as well as disease severity. The fact that TARC/CCL17 can be measured in the SC might contribute to a more feasible monitoring of disease course in research and clinical practice. SC-derived TARC/CCL17 can be used as a noninvasive tool to objectively monitor therapy success in patients who receive local therapy, including children (in which AD primarily arises). It can also be used as a surrogate endpoint in clinical trials, which would improve the comparison of study results, an option suggested earlier by Thijs et al.(27) In addition to TARC/CCL17, in the present studies several other inflammatory mediators showed a significant decrease in the SC after therapy, including Th2 and Th1 cytokines/ chemokines, regulatory cytokines, and vascular adhesion molecules. For most of these cytokines, data on their levels in the SC are lacking and their suitability for therapy monitoring should be further investigated in larger studies.

In contrast to inflammatory mediators, skin barrier biomarkers are largely neglected as biomarkers for AD with the exception of TEWL, which is mainly used in research to assess skin barrier function. This is surprising, as AD is currently regarded as disease that is at least partially caused by a skin barrier dysfunction resulting from, for example, filaggrin deficiency(28). NMF, which contains degradation products of filaggrin, has been shown to be a feasible biomarker of the *FLG* genotype, but is also affected by disease severity(29, 30). Furthermore, NMF concentrations have been shown to decrease in ICD(9, 31, 32). In the RCT presented in **Chapter 3.2**, NMF was used to monitor the effect of local therapy on the skin barrier. In this trial, despite a clinical improvement, AD patients showed a decrease in NMF when treated with hydrocortisone, which is a low-potency corticosteroid. Such an effect was also found by Danby et al. for a more potent corticosteroid, namely betamethasone valerate(33). The mechanisms that underlie this decrease are unclear. However, the implications are significant, as these creams are widely used in the treatment of inflammatory skin diseases, and in some countries hydrocortisone acetate is available as an over-the-counter product. If these treatments cause a (temporal) decrease in NMF concentration, it could be expected that this has a negative effect on the skin barrier function, making the skin more vulnerable to the effect of such external factors as skin irritants(34). NMF can be used to detect such negative effects and might also serve as an early effect biomarker. Protective measures in the form of moisturizing creams that compensate for the loss of NMF could be implemented. In a 2017 Cochrane Review on the use of moisturizers in AD, it was concluded that moisturizers are effective in prolonging time to new flares and reducing the amount of topical corticosteroids needed(35). Furthermore, the authors did not find reliable evidence that one moisturizer is more effective than another. Skin barrier biomarkers and inflammatory mediators are rarely used in the evaluation of therapy efficacy. Biomarkers could have an important role to play in such evaluations, helping practitioners to decide which moisturizer to choose.

CONCLUSION

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

Recommendations for future research

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

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

3. The predictive value of NMF as an early effect parameter of occupational irritant contact dermatitis should be evaluated preferably in a prospective cohort study in the occupational setting.

4. Evaluation of the DTI and IL-16 as biomarkers for the differentiation between irritant and allergic contact dermatitis should be performed with more allergens and skin irritants, and validation should be performed in patients with contact dermatitis.

Recommendations for future practice

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

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Summary

SUMMARY

In the present research, various biochemical and morphological parameters were determined in the stratum corneum (SC) as candidate biomarkers for contact dermatitis (CD) and atopic dermatitis (AD). CD, which can present as irritant or allergic CD (ICD and ACD, respectively), is a common occupational disease. AD is relevant for the occupational setting too, as it strongly predisposes for CD. In the workplace, ACD and ICD often coincide and diagnostics can be challenging as clinical symptoms are similar and exposure to both skin irritants and allergens often occurs. This hampers diagnosis, which is of importance as the prevention of ICD and ACD in the workplace demands different strategies and may furthermore influence the choice of therapy. Another challenge in clinical practice is the assessment of severity and the monitoring of disease; for example, scoring systems for AD and ACD are based on subjective and objective parameters, showing high inter-observer variability.

The main aim of this research was to explore skin barrier and immune response related biomarkers for ACD, ICD, and AD that are obtained from the SC. For inflammatory skin diseases like CD and AD, biomarkers obtained from the SC might be of interest, as they can easily be collected and they can provide information on both the skin barrier and the immune processes in the skin, both of which are known to play an important role in the etiology of ACD, ICD, and AD. In the SC, so far only a limited number of biomarkers have been measured, partly due to insensitive analytical techniques. Methodological advances, as well as recent insights into the key players responsible for immune response in ACD, ICD, and AD, have paved the way for a broader use of SC biomarkers in research and clinics. For this research, the following objectives and research questions were formulated.

Objective I: To gain insight into the SC biomarkers related to skin barrier and immune response in ACD and ICD

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

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

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

Research question I: Which biomarkers are known to be related to ACD and ICD, and what is their potential for use in research and clinical settings?
To answer research question I, two extensive non-systematic reviews of the existing biomarkers of ACD and ICD were conducted (**Chapter 2**). For ACD, various mediators of innate and T-cell mediated immune response have been identified as playing a crucial role in sensitization and elicitation processes. Although recent studies emphasize the importance of skin barrier impairment in the development of ACD, very few of these studies focused on skin barrier biomarkers in ACD. In addition to molecular biomarkers, several studies focused on genetic variations, and found that polymorphisms in genes involved in metabolism, immune response, and skin barrier predispose for ACD and therefore might be useful as biomarkers to identify susceptible individuals.

As skin barrier impairment underlies the development of ICD, the most commonly used biomarker is transepidermal water loss. Several studies of molecular biomarkers found reduced SC levels of IL-1α and NMF upon exposure to skin irritants; however, this was found only in experimental exposure studies and for only a limited number of skin irritants. Epidemiological studies in workers with high exposure to skin irritants identified several genetic biomarkers that modify risk for ICD, including *FLG* LOF mutations and single nucleotide polymorphisms in genes that regulate the expression of proinflammatory cytokines TNF-α and IL-1α.

So far, no biomarkers have been applied for the purpose of clinical practice, for example to differentiate ACD from ICD, or as a surrogate endpoint in patch testing.

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

In the clinical study described in **Chapters 3.1 and 3.2**, various skin barrier biomarkers and inflammatory mediators were evaluated for their suitability to distinguish ICD from ACD. Patients with confirmed contact sensitization to potassium dichromate (Cr), nickel sulfate (Ni), methyl(chloro)isothiazolinone (MCI/MI), or paraphenylenediamine (PPD) were patch tested to induce ACD. Simultaneously, patch testing with sodium lauryl sulfate (SLS) was done to induce ICD. After 72 hours, SC tape samples from the affected skin sites were obtained and analyzed for natural moisturizing factor (NMF), dermal texture index (DTI), protease activity, and a broad range of inflammatory mediators. The results showed that both SLS and MCI/MI decrease NMF concentration in the SC and therefore NMF seems not be suitable to differentiate ACD from ICD. Results on corneocyte surface texture seemed more promising, as SLS was the only substance that increased the number of circular nano-size objects (CNO), expressed as Dermal texture index (DTI). DTI could thus differentiate ICD induced by SLS from ACD induced by the tested allergens. Although not influencing DTI, MCI/MI caused morphological changes on a microscopic scale, showing pronounced cell borders and a remarkable hexagonal shape of the corneocytes. The change in corneocyte morphology and a decrease in NMF, which is similar to SLS, indicate the skin damaging potential of MCI/MI. This was also supported by the trend of increase in the activity of proteases (bleomycin hydrolase, calpain-1, and plasmin), which again is similar to SLS. Although NMF seems to be a biomarker of ICD, the differentiation between ICD and ACD by NMF might be hampered by the skin damaging properties of an allergen.

In **Chapter 3.2** the profiles of inflammatory mediators in skin sites with induced ACD and ICD were measured. In total 32 inflammatory mediators were determined and the results confirmed the exceptional mode of action of MCI/MI. In general, MCI/MI induced the largest changes in the levels of inflammatory mediators as compared to the other investigated allergens. The levels of INF-γ, IL-8/CXCL8, IP10/CXCL10, MDC/CCL22, Eotaxin-1/CCL11, IL-7, and VEGF showed significant changes from baseline for patch test reactions to MCI/MI, but not to other allergens. Interestingly, IL-1α, which is regarded as an indicator of skin barrier damage, is decreased in Cr, MCI/MI, and SLS, but not in Ni. Regarding differences between ACD and ICD,

IL-16 seemed to be indicative for ACD, as it was significantly increased in patch test reactions to all allergens (despite a trend of increase, the significance level was not reached in PPD, probably due to the low number (n=4) of patients), but not to SLS, making it the most promising immunological mediator in the SC for differentiation between ACD and ICD.

Research question III: Which skin barrier and immune response related biomarkers obtained from the SC are suitable for the monitoring of therapy in atopic dermatitis?
In order to investigate which SC-derived skin barrier and immune response related biomarkers are suited to monitor AD therapy, three clinical trials were conducted. In the first (**Chapter 4.1**), the relation between DTI and NMF was investigated in AD patients with and without a loss-of-function mutation in the filaggrin gene (*FLG* LOF). The results showed that the number of CNOs on the corneocyte surface (DTI) was associated with the presence of *FLG* LOF mutations and NMF levels. The correlation between NMF and DTI existed in both the acute and the convalescent state of disease. Furthermore, a high DTI in patients with a *FLG* LOF mutation persisted despite clinical improvement due to therapy. Most (but not all) of the CNOs appeared to contain a protein involved in corneocyte desquamation, corneodesmosin on their surface, especially in homozygote/compound heterozygote *FLG LOF* patients, suggesting an impaired cell maturation. Collectively, the results indicate that AD patients with a FLG-LOF mutation are distinctly different from patients without such a mutation, and having a FLG-LOF mutation might contribute to less favorable therapy outcomes, as found in previous studies.

In the second clinical trial (**Chapter 4.2**), various biomarkers (NMF, inflammatory mediators) and clinical and biophysical parameters (SCORAD, skin hydration and TEWL) were applied in the assessment of the therapy efficacy of three topical treatments in patients with mild to moderate AD. The evaluated treatments were an emollient containing ceramides and magnesium (Cer-Mg), a low-potency corticosteroid (hydrocortisone acetate; HC), and a lipid-rich emollient Unguentum leniens (EM). The results showed that Cer-Mg and HC led to comparable improvements in disease severity and skin barrier function as assessed by SCORAD and TEWL, respectively. Next, the decrease in SCORAD and TEWL was significantly greater in Cer-Mg compared to EM. Finally, the Cer-Mg cream

proved to be more effective in improving skin hydration compared to HC and EM. Unlike HC and EM, which decreased NMF levels, Cer-Mg maintained stable NMF levels in treated skin. **Chapter 4.3** zoomed in on the cytokine profiles of the skin treated with Cer-Mg emollient, which were determined in tape strip samples. Of the 38 analyzed mediators, 24 could be quantitatively determined. Th2 mediators IL-4, IL-13, CCL2 (MCP-1), CCL22 (MDC), and CCL17 (TARC) were significantly decreased after therapy, as were IL-1β, IL-2, IL-8 (CXCL8), IL-10, SAA, CRP, and VCAM-1. CCL17/TARC and IL-8 were found to be the most promising biomarkers, as their decrease correlated well with the decline in clinical symptoms in moderate AD cases and their concentrations were correlated with disease severity at baseline.

In the third clinical trial (**Chapter 4.4**), two techniques for the determination of NMF – namely Raman confocal microscopy (RMS) and SC tape-stripping followed by high-pressure liquid chromatography (HPLC) – were compared. The SC concentrations of NMF measured in 20 mild to moderate AD patients using the two techniques were significantly correlated (r^2=0.61; $p<0.0001$). Furthermore, both techniques demonstrated a concentration-depth dependence of NMF and reduced NMF levels in the carriers of *FLG* LOF mutations. Good agreement between measurements of left and right arms (RMS: r^2=0.92; P=<0.0001, HPLC: r^2=0.82; P=<0.0001) in both methods indicates robustness and good reproducibility. As both methods showed comparable performance, the choice of method will be influenced by practical considerations, such as study design, costs, and time constraints.

Conclusion

SC proved to be a suitable biological matrix for the determination of biomarkers of the skin barrier and immune response in ACD, ICD, and AD. Several skin barrier and immune response biomarkers have shown their potential to differentiate between ICD and ACD and to monitor topical therapy in AD. The number of circular-nano objects on the corneocyte surface (DTI) and SC-derived IL-16 seem to be promising candidate biomarkers to distinguish ACD from ICD. Whether this finding can be generalized to other contact allergens and skin irritants should, however, be confirmed in further studies. For the monitoring of AD therapy, several SC-derived biomarkers have been identified. TARC/CCL17 and IL-8/CXCL8 seem to be the most promising immunological biomarkers: baseline levels of TARC/CCL17 and

IL-8/CXCL8 correlate significantly with disease severity and, furthermore, their decrease is associated with improvement in clinical symptoms after therapy.

NMF has previously been shown to be a suitable phenotypic biomarker of filaggrin genotype. In the present study, NMF was also found to be a suitable biomarker to monitor adverse effects of topical therapy on the skin barrier in AD. In addition, the studies presented in this thesis demonstrated that NMF can be used as a biomarker to assess skin barrier alterations due to exposure to contact allergens and skin irritants

Finally, atomic force microscopy proved to be a powerful tool to assess changes in the surface topography of corneocytes caused not only by skin irritants and allergens, but also by intrinsic factors. Carriers of a loss-of-function mutation in the filaggrin gene showed an altered corneocyte surface texture that persisted even after clinical improvement. This might at least partly explain more persistent AD in these patients and contribute to a more personalized therapeutic approach.

Collectively, the findings clearly demonstrated the advantage of the simultaneous measurement of biomarkers of the skin barrier and immune response, as both play a role in the pathophysiology of both CD and AD. However, before these biomarkers are applied in the clinical setting, they require comprehensive evaluation and validation.

Recommendations for future research

More insight into the nature of CNOs and their role in the pathophysiology of atopic dermatitis and irritant contact dermatitis is needed. This could be done by studying carriers and non-carriers of *FLG* LOF mutations with and without atopic dermatitis or murine models deficient for filaggrin. In addition, the adverse effects of local therapy on the NMF and skin barrier in general, should be investigated for topical anti-inflammatory drugs and emollients of different compositions. Finally, as NMF seem to be a biomarker of skin barrier damage, it may be useful as an early effect biomarker of ICD, which could be evaluated in a prospective cohort study in workers exposed to skin irritants.

Nederlandse samenvatting

NEDERLANDSE SAMENVATTING

Onze huid vormt een barrière met de buitenwereld: ze zorgt ervoor dat stoffen het lichaam niet kunnen binnendringen en dat andere stoffen – zoals water – het lichaam niet kunnen verlaten. De barrièrefunctie van de huid wordt vooral door de bovenste laag, het stratum corneum (SC), vervuld. Deze laag bestaat uit dode, verhoornde cellen die corneocyten worden genoemd. Wanneer de huid met allergenen of irriterende stoffen in aanraking komt, kan dit leiden tot allergisch contactdermatitis (ACD) of irritatief contactdermatitis (ICD), twee vormen van contactdermatitis (CD). Bij bepaalde beroepen, zoals schoonmakers en kappers, maar ook bij werknemers in de zorg en de horeca vormt CD een groot deel van het totale aantal arbeidsgerelateerde aandoeningen. Veel van deze werknemers hebben ook atopische dermatitis (AD), een inflammatoire huidziekte waarbij de huidbarrière eveneens verstoord is. Deze mensen lopen een hoger risico om een chronische vorm van ACD of ICD te ontwikkelen en is het van belang diagnostische middelen te hebben, opdat de juiste behandeling en/of preventieve maatregelen kunnen worden ingezet. Een probleem in de huidige diagnostiek van ACD en ICD is dat klinische symptomen niet specifiek genoeg zijn om ACD van ICD te onderscheiden en dat de anamnese niet altijd duidelijkheid kan bieden. Bovendien kunnen ACD, ICD en AD tegelijkertijd bij dezelfde patiënt voorkomen. In die gevallen kunnen indicatoren van de huidbarrièrefunctie en mediatoren van het onstekingsproces mogelijk uitkomst bieden. Deze stoffen, ook wel biomarkers genoemd, kunnen niet alleen bijdragen aan de diagnostiek maar ook in worden gezet om het effect van een (lokale) therapie te monitoren. In het huidige onderzoek worden biomarkers veelal uit bloedmonsters bepaald of, om een beeld van de lokale situatie te krijgen, uit huidbiopten. Deze methodes zijn invasief, wat het gebruik in een arbeidsgeneeskundige en klinische setting in de weg staat. Daar het functioneren van de huidbarrière voor een groot deel bepaald wordt door het SC, kunnen biomarkers verkregen uit het SC een goed beeld geven van eventuele barrièrestoornissen. Bovendien zijn de ontstekingsprocessen bij ICD, ACD en AD lokaal van aard. Recente vooruitgang in de gevoeligheid van analysetechnieken en nieuwe inzichten in de mechanismen die een belangrijke rol spelen bij ontwikkeling van CD en AD hebben het mogelijk gemaakt om een grote diversiteit aan biomarkers uit het SC te bepalen. Dit proefschrift evalueert uit het SC verkregen biomarkers

voor diagnostiek, therapie en monitoring van ACD, ICD en AD. De volgende twee hoofddoelen zijn geformuleerd met de daarbij samenhangede vraagstellingen:

Doel I: Inzicht krijgen in SC-biomarkers gerelateerd aan huidbarrièrefunctie en ontstekingsreactie bij ACD en ICD
Onderzoeksvraag 1: Welke biomarkers zijn gerelateerd aan ACD en ICD en wat is de potentie van deze biomarkers in onderzoek en kliniek?

Onderzoeksvraag 2: Welke uit het SC verkregen biomarkers van de huidbarrière en lokale ontstekingsreactie kunnen worden gebruikt om ACD van ICD te onderscheiden?

Doel II: Evaluatie van de bruikbaarheid van verschillende uit het SC verkregen biomarkers voor de klinische praktijk van AD
Onderzoeksvraag 3: Welke uit het SC verkregen biomarkers van huidbarrière en lokale ontstekingsreactie kunnen worden gebruikt voor de monitoring van AD-therapie?

Onderzoeksvraag 1: Welke biomarkers zijn gerelateerd aan ACD en ICD en hoe kunnen ze gebruikt worden in onderzoek en kliniek?
In **Hoofdstuk 2** wordt twee niet-systematische reviews een overzicht gegeven van de verschillende biomarkers die bekend zijn voor ACD (**hoofdstuk 2.1**) en ICD (**hoofdstuk 2.2**).

In het onderzoeksveld van ACD ligt de nadruk op de immunologische component van de aandoening. Daarbij zijn verschillende mediatoren van het aangeboren immuunsysteem en het T-cell gemedieerde immuunsysteem geïdentificeerd die een cruciale rol spelen in het sensitisatie-proces en het elicitatie-proces. Hoewel meerdere studies wijzen op het belang van de huidbarrière bij de ontwikkeling van ACD, zijn er weinig studies bekend die hun focus hebben op biomarkers van huidbarrière bij ACD. Er zijn wel meerdere studies naar genetische variatie bij ACD, die laten zien dat bepaalde polymorphismes die van belang zijn bij metabolisme, immuun reactie en huidbarrière, predisponeren voor ACD en daarom mogelijk geschikte biomarkers zijn om gevoelige individuen te identificeren.

Huidbarrierebeschadiging is de onderliggende oorzaak van ICD en de meest gebruikte biofysische parameter om een dergelijke beschadiging te meten is trans-epidermaal waterverlies. Er zijn ook onderzoeken die laten zien dat concentraties van IL-1α en NMF, na blootstelling aan een irritatieve stof, verminderen in concentratie. Helaas is dit slechts voor een beperkte groep irritatieve stoffen onderzocht. Daarnaast is er epidemiologisch onderzoek bij werknemers die vaak in aanraking komen met irritatieve stoffen, dat laat zien dat FLG LOF en polymorfismes in de genen die verandwoordelijk zijn voor de proinflammatory cytokines TNF-α en IL-1α het riscio op het ontwikkelen van ICD beïnvloeden.

Tot nu toe heeft geen van de onderzochte biomarkers een toepassing gehad in de klinische praktijk, bijvoorbeeld om ACD van ICD te onderscheiden, of als test-uitkomst bij plak-proeven.

Onderzoeksvraag 2: Welke uit het SC verkregen biomarkers van de huidbarrièrefunctie en lokale ontstekingsreactie kunnen worden gebruikt om ACD van ICD te onderscheiden?
In een klinische studie, beschreven in **hoofdstuk 3.1 en 3.2**, is gezocht naar een biomarker die ACD van ICD zou kunnen onderscheiden. De huidbarrière-biomarker NMF, de Dermal Texture Index (DTI, het gemiddelde aantal Circular Nano-Objects (CNO) ook wel 'villous protrusions' (VP) genoemd) op het oppervlakte van de corneocyt), proteases en verschillende ontstekingsmediatoren zijn daarvoor bepaald bij ICD en ACD. Bij een groep van 27 patiënten is ACD voor vier allergenen geïnduceerd: chroom (Cr), nikkel (Ni), methyl(chloro)isothiazolinone (MCI/MI) of parafenyleendiamine (PPD) en een ICD voor één irritant (sodiumlaurylsulfaat (SLS)). SC-monsters zijn vervolgens van deze huidtestlocaties afgenomen en hieruit zijn de NMF-concentratie en ontstekingsmediatoren bepaald. Tevens is er atomic force microscopy (AFM) gebruikt om de oppervlakte van corneocyten in kaart te brengen. Abnormaliteiten in de textuur van het oppervlak van de corneocyten, de CNO's, zijn daarbij geteld en uitgedrukt in DTI. De resultaten laten zien dat MCI/MI, als enige allergeen een significante daling in NMF-concentratie bewerkstelligt vergelijkbaar met SLS. De resultaten van proteaseactiviteit en in de in **hoofdstuk 3.2** beschreven uitkomsten van IL-1α-concentratie laten gelijkenissen tussen MCI/MI en SLS zien. Op microscopische schaal vertonen de MCI/MI-monsters tevens een afwijkende morfologie (echter, zonder stijging van de DTI). De morfologische

veranderingen bij MCI/MI suggereren, samen met de overeenkomsten tussen MCI/MI en SLS wat betreft NMF, proteases en IL-1α, dat een irritatieve component deel uitmaakt van de pathogenese van MCI/MI-geïnduceerd ACD. Ten slotte lijkt het gemiddelde aantal CNO's op de corneocyten, uitgedrukt in de DTI, te kunnen differentiëren tussen SLS en de geteste allergenen; SLS is de enige onderzochte stof die een verhoging van de DTI veroorzaakt. Deze resultaten zijn veelbelovend voor onderzoek en klinische doeleinden en dienen verder te worden bestudeerd.

In **hoofdstuk 3.2** worden lokale ontstekingsmediatoren in het SC van de geïnduceerde ACD en ICD-laesies bepaald en wordt hun onderscheidend vermogen (tussen ACD en ICD) geëvalueerd. In totaal zijn er 32 mediatoren bepaald. De resultaten laten zien dat MCI/MI een uitzonderlijk profiel heeft. In zijn algemeenheid zijn de veranderingen in inflammatoire mediatoren bij MCI/MI hoger dan bij de andere allergenen; de concentraties van INF-γ, IL-8/CXCL8, IP10/CXCL10, MDC/CCL22, Eotaxin-1, IL-7 en VEGF laten significante veranderingen zien bij MCI/MI maar niet bij de andere allergenen. Met betrekking tot de verschillen tussen ICD en ACD, lijkt IL-16 indicatief te zijn voor ACD: IL-16 is significant verhoogd in test-geïnduceerde ACD-laesies van Cr, Ni, MCI/MI maar niet in SLS.

Onderzoeksvraag 3: Welke uit het SC verkregen biomarkers van huidbarrièrefunctie en lokale ontstekingsreactie kunnen worden gebruikt voor de monitoring van AD-therapie?
Om te kunnen bestuderen welke uit het SC verkregen biomarkers geschikt zijn voor het monitoren van therapie bij AD, beschrijft dit proefschrift drie klinische studies **(hoofdstuk 4).** In de eerste studie **(hoofdstuk 4.1)** is gekeken hoe NMF en DTI zich tot elkaar verhouden in een populatie van AD-patiënten waarbij meer dan de helft drager was van een filaggrin loss-of-function (FGL LOF) mutatie. Resultaten van deze studie laten zien dat NMF en DTI sterk gecorreleerd zijn, zowel voor als na behandeling (repectievelijk $r=-0.80$ en 0.77; $p<0.001$). Patiënten met een FLG LOF mutatie laten na behandeling, ondanks klinische verbetering, een persisterend hoog DTI zien. Veel (maar niet alle) CNO's hebben het eiwit corneodesmosin op het oppervlak, met name bij homozygote en (compound) heterozygote FLG LOF-patiënten. Dit laatste kan wijzen op een mogelijk probleem in de uitrijping van de corneocyt. Deze resultaten tonen aan dat patiënten met een FLG LOF-mutatie een uitgesproken corneocytmorfologie hebben, die afwijkt van die van patiënten

zonder FLG LOF-mutatie. Dit kan mogelijk de slechtere behandelingsuitkomsten bij deze groep verklaren en aanzetten tot nieuwe behandelingsstrategieën en het stratificeren van patiënten in verschillende groepen.

In **hoofdstuk 4.2** zijn verschillende biomarkers (NMF, ontstekingsmediatoren) en klinische en biofysische parameters (TEWL, SC-hydratatie, SCORAD) ingezet om, in een gerandomiseerde klinische studie, de effectiviteit van drie verschillende therapieën voor AD te beoordelen bij patiënten met milde tot matige AD. In deze studie is de effectiviteit van een crème die onder andere ceramides en magnesium (Cer-Mg) bevat, vergeleken met (1) Unguentum leniens (UL), een zalf die uit 70% vettige componenten bestaat en (2) een zwak werkend corticosteroïd, te weten hydrocortisonacetaat (HC). De resultaten laten zien dat Cer-Mg en HC vergelijkbare verbetering laten zien met betrekking tot TEWL en SCORAD. Daarnaast laat de Cer-Mg creme een significant grotere daling zien ten opzichte van UL met betrekking tot SCORAD en TEWL. Ten slotte laat de Cer-Mg creme zien dat het effectiever is in het verbeteren van huidhydratie in vergelijking met HC en UL. Anders dan HC en UL, die de NMF levels in het SC verminderen, bleef de NMF hoeveelheid in het SC onder de de Cer-Mg therapie gelijk. Een daling van NMF heeft mogelijk negatieve gevolgen op het verdere beloop van de aandoening en de gevolgen van een therapie-geïnduceerde daling zal daarom geëvalueerd moeten worden. In **Hoofdstuk 4.3** zijn de gevolgen van de Cer-Mg-behandeling voor ontstekingsmediatorenprofiel in het SC geëvalueerd. 24 (uit een totaal van 38) ontstekingsmediatoren konden vanuit SC-monsters worden gekwantificeerd; de meeste van deze mediatoren zijn niet eerder uit dergelijke SC-monsters bepaald. Van alle mediatoren blijken IL-8 en met name TARC/CCL17, het meest geschikt voor de evaluatie van de effectiviteit van de therapie en het inschatten van de ernst van de aandoening. De afname in deze ontstekingsmediatoren correleren het sterkst (respectievelijk r=0.56 en r=0.45) met de afname van klinische symptomen bij patiënten met een matig ernstige vorm van de ziekte.

In de derde klinische studie (**hoofdstuk 4.4)** worden Raman-spectromicroscopy en HPLC, twee methoden om NMF te meten, met elkaar vergeleken. Beide methoden laten zien dat NMF diepte afhankelijk is en dat lage NMF-concentraties bij patiënten met FLG-mutaties aanwezig zijn. De correlatie tussen de resultaten

van beide methodes is significant (r^2=0.61; $p<0.0001$). De overeenkomst bij beide methoden tussen metingen aan linker- en rechterarm tonen de robuustheid en reproduceerbaarheid van de methoden aan. Daar beide methoden vergelijkbare prestaties laten zien, is de keuze voor een groot deel afhankelijk van praktische overwegingen, zoals studieontwerp, kosten en tijdsrestricties.

Conclusie

Biomarkers, verkregen uit het SC, hebben potentie in diagnostiek en monitoren en evalueren van therapie bij patienten met ACD, ICD en AD. Het aantal CNO's op het oppervlakte van de corneocyt, uitgedrukt in DTI en de concentratie van IL-16 in het SC hebben de potentie om ACD van ICD te kunnen onderscheiden. Of deze resultaten generaliseerbaar zijn voor alle allergenen moet vervolgonderzoek bevestigen.

Voor het monitoren van AD-therapie zijn verschillende biomarkers geïdentificeerd. TARC/CCL17 en IL-8 hebben hierbij de meeste potentie: begin concentraties van TARC/CCL17 en IL-8 correleren significant met ernst van de ziekte en afname in de concentraties voor en na therapie zijn geassocieerd met verbetering van klinische symptomen.

NMF is een goede fenotypische biomarker voor het filagrinne genotype. Daarnaast is NMF ook een geschikte biomarker voor het monitoren van ongewenste effecten op de huidbarrière bij AD-therapie met corticosterioden. Bovendien kan NMF worden gebruikt om veranderingen van de huidbarrière door allergenen en irriterende stoffen te onderzoeken.

Atomic force microscopy kan worden gebruikt om veranderingen van het oppervlakte van corneocyten te onderzoeken. Patiënten die drager zijn van een FLG LOF mutatie laten een ander oppervlakteprofiel op corneocyten zien dan patiënten die deze mutatie niet hebben. Dit afwijkende oppervlakteprofiel persisteert, zelfs na klinische verbetering en het effect is in bijzonder zichtbaar bij de patienten met 2 FLG LOF mutaties. Dit verklaart mogelijk de moeilijker te behandelen klachten van deze patiëntengroep. Deze kennis zou kunnen helpen bij een meer persoonlijk gerichte therapie.

Concluderend, laten de resultaten de waarde zien van het meten van verschillende biomarkers van zowel de huidbarriere als de immuunreactie. Beiden spelen een belangrijke rol is in de pathofysiologie van zowel CD als AD. Voordat deze biomarkers in de klinische praktijk kunnen worden ingezet is uitgebreide evaluatie en validatie van biomarkers nodig.

Aanbevelingen voor toekomstig onderzoek

De rol van CNO's in de pathofysiologie van AD en ICD dient opgehelderd te worden. Dit kan onder andere worden gedaan door het bestuderen van dragers en niet-dragers van FLG LOF mutaties, door het bestuderen van muis-modellen met fillagrine deficientie of door de reactie van de menselijke huid op verschillende irriterente stoffen te onderzoeken. Hiernaast moeten de negatieve gevolgen van lokale therapie (bijvoorbeeld corticosterioden) op de huidbarrière in zijn algmeenheid en NMF in het bijzonder, worden onderzocht. Ten slotte, daar NMF een geschikte biomarker lijkt te zijn voor huidbarrièrebeschadiging, kan NMF worden gebruikt in prospectief onderzoek naar vroege huidbeschadiging in werknemers die veel in aanraking komen met irritatieve stoffen.

ABOUT THE AUTHOR

Curriculum vitae

Portfolio

List of publications

CURRICULUM VITAE

Sjors Arnoldus Koppes was born on November 13th, 1985 in the Dutch seaside village of Noordwijkerhout. After his secondary education (Gymnasium, Teylingen College, Noordwijkerhout) he studied biology at VU University Amsterdam. His bacherlor thesis, under supervision of prof. dr. Rozema, on plant physiology of Arctic bell-heather (*Cassiope tetragona)* was realized in cooperation with the University of Tromsø in Longyearbyen on the high arctic archipelago of Svalbard. It was there, at 78° 22 NB, that he received the news that he was admitted to the medical school of VU University. During medical internships Sjors got involved in research of prof. dr. Rustemeyer on the topic of essential oil allergies. After graduation he was introduced to dr. Sanja Kezic and prof. dr. Frings-Dresen of The Coronel Institute of Occupational Health, of the AMC and embarked on a research project on stratum corneum biomarkers. The result of this joint collaboration of the Coronel Institute of the AMC and the department of dermatology of the VU medical center is presented in this thesis. In March 2017 Sjors started his Pathology residency at the University Hospital Utrecht, under the supervision of prof. dr. Van Dijk. In his private life Sjors enjoys bird watching and modern history. Sjors lives in Amsterdam together with Ankie Lok and two cats.

PORTFOLIO

Name:	S. A. Koppes	
PhD period:	2013-2017	
Name PhD supervisor:	M. Frings-Dresen, T. Rustemeyer, S. Kezic	
1. PhD training		
	Year	**Workload (Hours/ECTS)**
General courses		
-Systematic review	2013	2.5 / 0.1
-Citation analysis	2013	2.5 / 0.1
-Biostatistics	2014	40 / 1.1
-Epidemiology	2014	30 / 1.1
-Advanced topics in epidemiology	2015	30 / 1.1
-BROK	2016	28 / 1.0
Specific courses		
-Training school 'Skin barrier', Split, Croatia	2015	40 / 1.5
-Training school 'Patch testing', Erlangen, Germany	2015	40 / 1.5
Oral Presentations		
-PhD progress, Coronel research meetings, AMC Amsterdam (2x)	2013-2015	16 / 0.6
-EDAD-results, AMC/VUmc eczema working group - Amsterdam	2015	14 / 0.5
-EDAD-study background and preliminary results presentation AMC/VUmc dermatolgy residents' educational program, Amsterdam	2015	14 / 0.5
-EDAD-study results, ISA, Munich	2015	14 / 0.5
-EDAD-study results, Omega pharma research conference Copenhagen.	2016	14 / 0.5
-Science education 'back to the future project'.	2014-2015	28 / 1.0
-CAT-study (results) Standerm working group 1, Hamburg	2016	14 / 0.5
Poster Presentations		
- EDAD-study, poster ICOH Seoul	2015	14 / 0.5
- EDAD-study, ISA Munich	2015	14 / 0.5
- CAT-Study, Annual Meeting of Amsterdam Public Health research	2016	14 / 0.5
(Inter)national conferences		
-EADV 2014 Amsterdam	2014	32 / 1.1
- ICOH 2015 Seoul	2015	32 / 1.1
-Standerm meetings Copenhagen, Amsterdam, Berlin	2013-2017	48 / 1.7
Other		
-Dublin short scientific mission	2015	32 / 1.1

2. Teaching		
	Year	**Workload (Hours/ECTS)**
Tutoring, Mentoring Klein klinisch lijnonderwijs	2014-2016	90 / 3.0
Total		21.1

LIST OF PUBLICATIONS

Koppes SA, Engebretsen KA, Agner T, Angelova-Fischer I, Berents T, Brandner J, et al. Current knowledge on biomarkers for contact sensitization and allergic contact dermatitis. Contact Dermatitis. 2017.

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Koppes SA, Hadzavdic S, Jakasa I, Franceschi N, Riethmüller C, Tončic RJ, et al. Effect of allergens and irritants on levels of NMF and corneocyte morphology Accepted for publication Contact Dermatitis 2016.

Koppes SA, Brans R, Ljubojevic Hadzavdic S, Frings-Dresen MH, Rustemeyer T, Kezic S. Stratum Corneum Tape Stripping: Monitoring of Inflammatory Mediators in Atopic Dermatitis Patients Using Topical Therapy. Int Arch Allergy Immunol. 2016

Koppes SA, Charles F, Lammers L, Frings-Dresen M, Kezic S, Rustemeyer T. Efficacy of a Cream Containing Ceramides and Magnesium in the Treatment of Mild to Moderate Atopic Dermatitis: A Randomized, Double-blind, Emollient- and Hydrocortisone-controlled Trial. Acta Derm Venereol. 2016.

Koppes SA, Ljubojevic Hadzavdic S, Jakasa I, Franceschi N, Jurakic Toncic R, Marinovic B, et al. Stratum corneum profiles of inflammatory mediators in patch test reactions to common contact allergens and sodium lauryl sulfate. Br J Dermatol. 2016.

Riethmuller C, McAleer MA, Koppes SA, Abdayem R, Franz J, Haftek M, et al. Filaggrin breakdown products determine corneocyte conformation in patients with atopic dermatitis. J Allergy Clin Immunol. 2015.

Dankwoord

DANKWOORD

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De leescommissie, bestaande uit prof. dr. T. Agner, prof. dr. E.H.D. Bel, prof dr. S. Gibbs, dr. R. Lutter en prof. dr. M.A. de Rie krijgen mijn waardering en dank voor de moeite die zij zich hebben getroost bij het lezen en beoordelen van mijn proefschrift.

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He rose, and he put down *The Yellow Book*.
He staggered – and, terrible-eyed,
He brushed past the plants on the staircase
And was helped to a hansom outside.

John Betjeman, *The Arrest of Oscar Wilde at the Cadogan Hotel* (1937), st. 9