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PhD Thesis

Effect of environmental and climatic exposures on adult skin

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This PhD-thesis is based on the following three manuscripts:

I Engebretsen KA, Kezic S, Riethmüller C, Jakasa I, Hedengran A, Linneberg A, Johansen JD, Thyssen JP. *Changes in filaggrin degradation products and corneocyte surface texture by season*. Accepted for publication in the British Journal of Dermatology.

II Engebretsen KA and Bandier J, Kezic S, Riethmüller C, Heegaard NHH, Carlsen BC, Linneberg A, Johansen JD, Thyssen JP. *Levels of filaggrin monomers, its metabolites and corneocyte surface texture in individuals with a history of atopic dermatitis and controls.* Submitted for publication in the Journal of the European Academy of Dermatology and Venereology.

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Preface

This thesis is based on scientific work performed between April 2014 and November 2017 at the National Allergy Research Centre and the Department of Dermatology and Allergy at Herlev and Gentofte Hospital. The project has received funding from the Lundbeck Foundation, the Aage Bang Foundation, the A.P. Møller foundation and the COST action TD1206 Standerm.

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Finally, I must thank my family and my boyfriend Hjalte who has made all the illustrations in this thesis. You have always believed in me, and I would not have been here today without all your love, support and encouragement.

With appreciation,

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Abbreviations

The abbreviations are listed alphabetically.

AD	Atopic dermatitis
AFM	Atomic Force Microscopy
AU	Arbitrary units
CNV	Copy number variance
DTI	Dermal Texture Index
ELISA	Enzyme-linked immunosorbent assay
FLG	Filaggrin gene
HPLC	High-performance liquid chromatography
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
NMF	Natural moisturizing factors
OR	Odds ratio
PAR	Protease-activated receptor
PCA	Pyrrolidone carboxylic acid
RH	Relative humidity
TEWL	Transepidermal water loss
SC	Stratum corneum
SCORAD	SCOring Atopic Dermatitis
SD	Standard deviation
SEB	Staphylococcal enterotoxin B
SLS	Sodium lauryl sulfate
TNF	Tumor necrosis factor
TSLP	Thymic stromal lymphopoietin
UCA	Urocanic acid
UV	Ultraviolet

Summary

Background and aims

In recent years, it has become evident that skin barrier impairment increases the risk of dermatitis and allergic disease. The epidermal protein filaggrin and its derived natural moisturizing factors (NMF) are crucial for normal skin barrier function. Filaggrin deficiency renders the skin dry, fragile and prone to inflammation, and infants with loss-of-function mutations in the filaggrin gene (*FLG*) have a 2-3-fold increased risk of developing atopic dermatitis (AD). Furthermore, recent studies have suggested that nanoscale alterations on the corneocyte surface may indicate skin barrier impairment. By using Atomic Force Microscopy, a new method has been developed which enables a quantitative measurement of these alterations, expressed as the Dermal Texture Index (DTI). Notably, a high DTI has recently been associated to pediatric AD, *FLG* mutations and reduced levels of NMF.

Importantly, limited data are available regarding the effect of internal (age, sex and inflammation) and external factors (climatic conditions, different water types, allergens, irritants and bacteria toxin) on filaggrin, its degradation products and corneocyte surface texture. Previous studies have shown that climatic factors such as cold and dry weather may have a negative effect on the skin barrier, and children born during the fall or winter have been found to have a higher prevalence of AD compared to those born during the spring or summer. Furthermore, exposure to hard domestic water during infancy and childhood has been associated to increased prevalence of AD in studies from Denmark, the UK, Spain and Belgium. Other external skin stressors such as bacteria toxin, house dust mite and cat allergen have also been associated to the development or exacerbation of AD, but their effects on the skin barrier are unclear.

The overall objective of this PhD-thesis was thus to investigate how internal and external factors affect filaggrin degradation products and corneocyte surface texture in adult skin. In more detail the aims were;

- To investigate the effect of temperate climate during winter and summer on the level of filaggrin degradation products and corneocyte surface texture in healthy, adult volunteers without AD and common *FLG* mutations (Manuscript I).
- To investigate the corneocyte surface texture, as well as the quantity of epidermal filaggrin protein and its degradation products in dermatitis patients with a history of AD and healthy adult controls (Manuscript II).

- To investigate the effect of selected daily-life skin stressors on the level of filaggrin degradation products and the inflammatory response in healthy adult volunteers without AD and common *FLG* mutations (Manuscript III).

Methods

The thesis is based on three experimental studies. In the first study (Manuscript I), 80 (40 males and 40 females) healthy volunteers, 40 aged 18 ≤ 40 years and 40 aged ≥70 years, were tape stripped on the cheek and on the dorsal aspect of the hand during the winter (January-February) and the summer (June-August). The tape strips were analyzed for NMF levels and corneocyte surface texture (DTI). Potential confounders such as use of cream or shower/bath the same day, temperature and recent UV-exposure were recorded and adjusted for in the statistical analyses.

In the second study (Manuscript II), 67 participants (47 dermatitis patients and 20 healthy controls) were included. The dermatitis patients were assessed whether they had a history of AD. All participants were tested for three common *FLG* mutations (R501X, 2282del4 and R2447X). Skin barrier function was measured (transepidermal water loss) and a skin biopsy and tape strips were analyzed for filaggrin protein, DTI and NMF, respectively.

In the third study (Manuscript III), 40 healthy volunteers (aged 18-49 years) were exposed to daily-life skin stressors, including different types of water (soft, hard, chlorinated and soap water), house dust mite, bacteria toxin, cat allergen, histamine and cooling of the skin. Skin cytokines were measured in tape strips after 24 hours for selected exposures, while NMF were measured for all exposures and an unexposed control spot after 24 and 48 hours.

Results

A significant seasonal effect on NMF and changes in corneocyte morphology, measured by DTI, was observed in skin from cheek and hands. Moreover, high self-reported UV-exposure was associated with increased DTI indicating changes in corneocyte surface texture. However, significant differences in NMF and DTI were also found with regards to age and sex, making it difficult to draw firm conclusions (Manuscript I). In relation to diseased skin, we found that the level of epidermal filaggrin and its degradation products were significantly lower in dermatitis patients compared to healthy controls, while DTI was significantly higher. Interestingly, DTI was higher in healthy aged controls compared to younger controls, suggesting a gradual change in corneocyte morphology with age (Manuscript II). Lastly, we observed that exposure to the different water types led to a significant decrease in NMF compared to the unexposed control spot. Notably, hard, but not soft, water led to a

significant increase in the AD associated cytokine IL-4. Exposure to house dust mite led to a significant decrease in NMF and increase in several pro-inflammatory cytokines, while exposure to bacteria toxin led to an increase in IL-1α, but no decrease in NMF (Manuscript III).

Conclusions

Collectively, our results demonstrate that filaggrin, its degradation products and corneocyte surface texture are significantly affected by age, sex, inflammation, changes in season and exposure to selected daily-life skin stressors. Our data help to explain why climatic and environmental factors may play a role in the development and exacerbation of AD, but also in other season and age-aggravated dermatoses. We experienced that the interplay between the different skin barrier components was complicated, likely due to influence of unmeasured factors and difference in kinetics. Our results open up for future research projects where a more extensive characterization of immunological- and skin barrier associated components are performed in one and the same study.

Dansk resume (Summary in Danish)

Baggrund og formål

Igennem de seneste år er sammenhængen mellem en nedsat hudbarrierefunktion og en øget risiko for eksem og allergi blevet klar. Hudproteinet filaggrin, og dets nedbrydningsprodukter (natural moisturizing factors, NMF), er essentielle for opretholdelse af en normal hudbarrierefunktion. Hvis huden mangler filaggrin har man en nedsat hudbarriere som er kendetegnet ved tør hud og øget forekomst af inflammation. I forhold til eksem, har børn med mutationer i genet som koder for filaggrin (*FLG*), en 2-3 gange øget risiko for at udvikle atopisk eksem sammenlignet med børn uden mutationer. Endvidere har nyere studier antydet, at morfologiske ændringer på hudcellernes overflade kan hænge sammen med en nedsat hudbarrierefunktion. Atommikroskopi kan benyttes til at kvantificere disse morfologiske ændringer og antallet af 'udposninger' på cellerne kan udtrykkes som "Dermal Texture Index" (DTI). Forskere har vist, at en høj DTI er associeret med atopisk eksem hos børn, *FLG* mutationer og en nedsat mængde af NMF.

Det er endnu uvist hvorvidt interne (alder, køn og inflammation) og eksterne faktorer (klima, forskellige vandtyper, allergener, irritanter og bakterietoksiner) har en effekt på hudcellernes morfologiske overfladestruktur eller på niveauet af filaggrin og dets nedbrydningsprodukter i huden. Klimatiske faktorer som koldt og tørt vejr er tidligere blevet associeret med en negativ indvirkning på hudbarrieren. Ligeledes er det vist at børn, som er født om efteråret og om vinteren har en højere forekomst af atopisk eksem sammenlignet med børn som er født om foråret og sommeren. I forhold til vandtyper har studier, fra Danmark, Storbritannien, Spanien og Belgien vist at børn som vokser op i områder med hårdt husholdningsvand har en øget forekomst af atopisk eksem sammenlignet med børn som vokser op i områder med blødere husholdningsvand. Andre eksterne påvirkninger som bakterietoksin og allergener fra husstøvmide og kat er også blevet associeret med udvikling eller forværring af atopisk eksem, men deres effekt på hudbarrieren er endnu ikke afklaret.

Det overordnede formål med denne PhD-afhandling var at undersøge hvordan interne og eksterne faktorer påvirker filaggrins nedbrydningsprodukter og hudcellernes morfologiske overfladestruktur De enkelte formål var;

- At undersøge effekten af klima om vinteren og sommeren på mængden af filaggrins nedbrydningsprodukter, samt hudcellernes overfladestruktur, hos voksne med normal hud uden *FLG* mutationer (Manuskript I).

- At undersøge hudcellernes overfladestruktur, samt mængden af filaggrin protein og dets nedbrydningsprodukter, hos eksempatienter sammenlignet med voksen normal hud (Manuscript II).
- At undersøge effekten af udvalgte hudstressorer på filaggrin nedbrydningsprodukter samt at karakterisere det inflammatoriske respons, der opstår som følge af eksponeringerne, hos voksne med normal hud uden *FLG* mutationer (Manuscript III).

Metode

Afhandlingen er baseret på tre eksperimentelle studier. I det første studie tog vi tape-prøver af huden på kinden og hånden hos 80 raske frivillige (50 % kvinder). 40 personer var i alderen 18 ≤ 40 år mens 40 personer var ≥70 år. Der blev både taget prøver om sommeren (juni-august) og vinteren (januar-februar). Vi analyserede tape-prøverne for NMF og hudcellernes overfladestruktur (DTI). Potentielle påvirkende faktorer som brug af fugtighedscreme, brusebad, temperatur og sol-eksponering blev registreret og justeret for i de statistiske analyser (Manuskript I).

I det andet studie inkluderede vi 67 deltagere (47 patienter med eksem og 20 raske kontroller). Patienterne blev vurderet om de havde haft atopisk eksem tidligere i livet. Alle deltagere blev testet for tre forskellige *FLG* mutationer (R501X, 2282del4 and R2447X). Vi målte hudens transepidermale vandtab, som et udtryk for hudbarrierefunktion, og tog en hudbiopsi samt tape-prøver, som vi analyserede for mængden af filaggrin protein og NMF i huden samt DTI-værdi (Manuskript II).

I det tredje studie eksponerede vi 40 raske frivillige (18-49 år) med forskellige typer vand (blødt, hårdt, vand med klor og sæbevand), bakterietoksin, histamin, allergener fra kat og husstøvmide, samt nedkøling af huden. Inflammatoriske mediatorer (cytokiner) blev målt i tape-prøver efter 24 timer for udvalgte eksponeringer, mens NMF blev målt for alle eksponeringer. Prøver fra et ikke-eksponeret kontrolpunkt blev taget efter 24 og 48 timer (Manuscript III).

Resultater

En signifikant effekt af årstid blev fundet både på niveauet af NMF og på corneocyt morfologi (DTI) i hud fra kinder og håndrygge. Endvidere, var der en sammenhæng mellem et højt niveau af selvrapporteret soleksponering og en høj DTI-værdi, hvilket indikerer at UV-stråling medfører ændret corneocyt overfladestruktur. Der blev også fundet signifikante forskelle, i NMF og DTI, i forhold til alder og køn (Manuskript I). I forhold til eksemhud fandt vi, at niveauet af filaggrin protein, og dets nedbrydningsprodukter, var signifikant lavere hos patienter med eksem sammenlignet med raske kontroller, mens DTI var signifikant højere, hvilket antyder at inflammeret hud har en nedsat

hudbarrierefunktion samt en ændret morfologi. Vores studie viste også at DTI var signifikant højere hos ældre raske kontroller sammenlignet med yngre raske kontroller, hvilket tyder på en gradvis ændring i hudcellernes overflade med alderen (Manuskript II). Slutteligt fandt vi, at eksponering med forskellige typer af vand førte til ændringer i NMF, og hudens barrierefunktion, sammenlignet med ikke-eksponerede kontrolpunkter. Vi så at hårdt, men ikke blødt, vand førte til en signifikant stigning i IL-4, et cytokin som er associeret med atopisk eksem. Derudover førte eksponering med husstøvmide til et signifikant fald i NMF og stigning i flere pro-inflammatoriske cytokiner, mens eksponering med bakterietoksin førte til en stigning i cytokinet IL-1α, men ikke et fald i NMF (Manuskript III).

Konklusion

Samlet set viser vores resultater, at filaggrin, dets nedbrydningsprodukter og hudcellernes overfladestruktur påvirkes signifikant af alder, køn, inflammation, årstid samt ydre eksponeringer man møder i hverdagen. Vores data er derfor med til at forklare hvorfor klimatiske - og miljømæssige faktorer kan spille en rolle i udviklingen og forværringen af AD, men også muligvis har en effekt på udviklingen af andre former for årstids- og aldersbetinget eksem. Vi erfarede også at samspillet mellem de forskellige komponenter i hudbarrieren var kompliceret hvilket sandsynligvis skyldes påvirkning fra andre faktorer som ikke blev målt eller taget højde for. Vores resultater åbner derfor op for fremtidige forskningsprojekter hvor man måler flere immunologiske - og hudbarrierekomponenter i et og samme studie.

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1. Introduction

1.1 The normal skin barrier and the role of filaggrin

The skin is the body's largest organ and has been referred to as 'the most important 2m^2 of your life'. The skin consists of two distinct layers, the dermis and the epidermis [1]. The dermis is composed of connective tissue and protects the body against mechanical trauma and contains specialized structures. The epidermis outside the palmar and plantar area is further subdivided into four layers; *the stratum basale*, *stratum spinosum*, *stratum granulosum* and *stratum corneum* (Fig. 1). The epidermis, and especially its outermost layer, the stratum corneum (SC), protects the organism from external insults such as allergens, chemicals and microbial colonization, but importantly, it also prevents excessive loss of water and solutes [2]. The evaporation of water from the skin surface, known as transepidermal water loss (TEWL), has traditionally been used to evaluate the skin barrier function together with other parameters [2, 3].

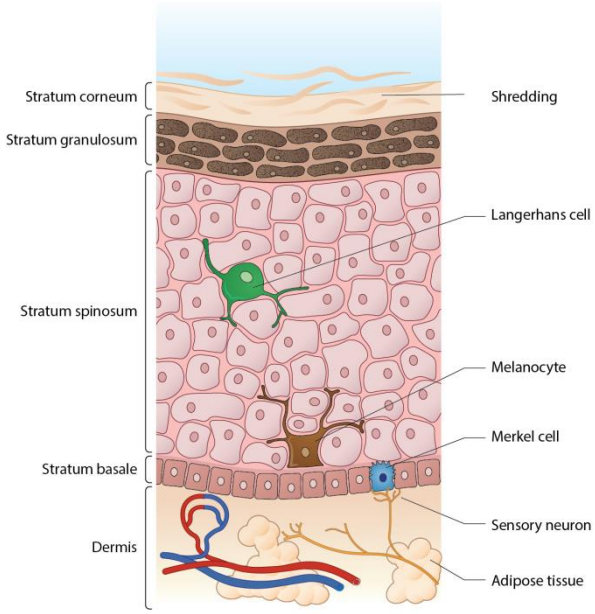

Figure 1. Structure of normal epidermis outside the palmar and plantar area.

The epidermis is constantly regenerated, and the proliferation rate in the basal layer equals the desquamation rate of the cornified layer at the skin surface [4]. The epidermal differentiation takes approximately 28 days and begins with proliferative keratinocytes in the basal layer migrating upwards into the *stratum spinosum* where the cells become connected by desmosomes [1] . When the

cells reach the granular cell layer, they acquire intracellular keratohyalin granules, which contain profilaggrin, the precursor protein of filaggrin. They also produce lipids that are secreted into the intercellular matrix and help produce a complete barrier when reaching the SC [4]. Transmembrane proteins (e.g. occludin, claudin and cingulin) collectively referred to as 'tight junctions' are also found in *stratum granulosum* [5]. They regulate paracellular properties and seal adjacent keratinocytes, thus contributing to the skin barrier function [5, 6]. Finally, the keratinocytes reach the SC where they collapse, and become anucleated corneocytes which eventually are shred off.

Filaggrin plays a key role in the epidermal differentiation [7]. During the terminal differentiation, the histidine-rich and large profilaggrin (>400 kDa) is dephosphorylated and cleaved into 10-12 filaggrin monomers (Fig. 2A) [8]. The liberated filaggrin monomers bind to keratin filaments and aggregate them into tight bundles, resulting in collapse and flattening of the corneocytes and formation of squames. Filaggrin also contributes to the formation of the cornified envelope, a rigid protein structure that surrounds the corneocytes and gradually replaces their plasma membrane [4, 8]. Lipids in the intercellular matrix get covalently bound to the cornified envelope. Together with the filaggrin attachment, the final result is a strong, multilayered structure which simplified resembles bricks (the corneocytes and the cornified envelope) and mortar (the hydrophobic lipids) [9].

In the upper layers of the SC, filaggrin is ultimately deiminated and degraded by proteases into hygroscopic amino acids, including glutamine, arginine and histidine [9] (Fig. 2B). Histidine is further metabolized to trans-urocanic acid (UCA) and glutamine to pyrrolidone-5-carboxylic acid (PCA), the two main breakdown products of filaggrin [10]. Filaggrin degradation products, together with sodium ions, lactate and urea, are collectively referred to as 'natural moisturizing factors' (NMF) and play an essential role in skin barrier homeostasis. First of all, they are potent humectants and accounts for much of the water retention and hydration of the SC [11]. They also help maintain the acidic mantle of the SC, which is important for the activity of enzymes involved in ceramide metabolism, epidermal differentiation and production of the cornified envelope [7]. Furthermore, an acidic skin surface protects the skin from microbial colonization, and it has been shown that a physiological amount of filaggrin degradation products have an inhibitory effect on the growth of *Staphylococcus aureus* [12]. Finally, it has been suggested that filaggrin degradation products might play a role in protecting the skin against ultraviolet (UV) irradiation, as trans-UCA is a major UV-absorbing chromophore [10]. However, the beneficial effect might in some way be counteracted, as UV irradiation causes photo-isomerization of trans- to cis-UCA, and the latter has been shown to upregulate genes associated with apoptosis, cell growth arrest and oxidative stress in addition to having immunosuppressive properties [10].

1.2 Filaggrin gene mutations are common and affect skin barrier function

The filaggrin gene (*FLG*) is located within the epidermal differentiation complex on chromosome 1q21 and consists of three exons and two introns [13, 14]. The third exon of *FLG* encodes the majority of the profilaggrin molecule and is one of the largest exons in the human genome [14]. Due to the large size and repetitive nature of the *FLG*, the gene was not fully sequenced before 2006 where the two first loss-of-function mutations (R501X and 2282del4) were identified in patients with ichthyosis vulgaris [15]. Since then, more than 60 different loss-of-function mutations have been described, where many are European-specific and Asian-specific [16]. *FLG* mutations can result in total absence of filaggrin in the skin (homozygous or compound heterozygous carriers) or reduced synthesis (heterozygous carriers) and approximately 10% of people with European descent are mutation carriers [17, 18]. In a large population based cohort in Denmark, the *FLG* mutations (R501X and 2282del4) were found in 8.1% of the study population (269 /3 335) [19].

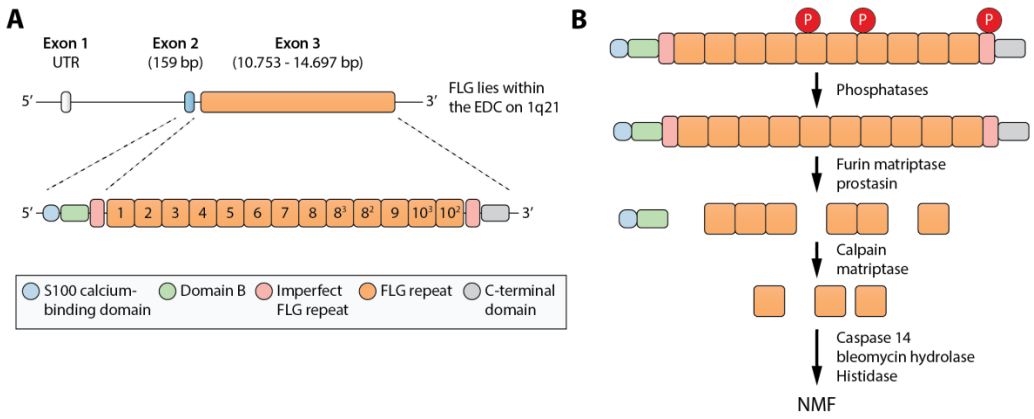

Figure 2. A) Filaggrin is encoded by the large *FLG* located on chromosome 1q21 within the epidermal differentiation complex. The profilaggrin protein has 10-12 repeats of the 37 kD filaggrin monomer. **B)** The processing of profilaggrin starts with dephosphorylation, thereafter it is cleaved into 10-12 identical filaggrin monomers. In the upper layers of the SC, filaggrin is further degraded by proteases to hygroscopic amino acids (NMF). The proteases responsible for profilaggrin and filaggrin processing have not been fully identified, and the proteases described in this figure are some of the proposed candidates. Bp, base pairs; EDC, epidermal differentiation complex; NMF, natural moisturizing factors; SC, stratum corneum; UTR, untranslated region. Figure and elements adapted from Irvine et al., N Engl J Med, 2011. 365(14): p. 1315-27 and Harding et al., Int J Cosmet Sci, 2013. **35**(5): p. 412-23.

A strong correlation between the level of filaggrin degradation products (PCA, UCA and histidine) and the *FLG* genotype has been shown in epidermal tape strips [20]. Reduced levels of filaggrin and its degradation products affect the skin homeostasis and barrier function in several ways. First of all, the decreased amount of NMF reduces SC hydration and TEWL increases [7]. The reduced level of amino acids elevates the skin pH, which in turn leads to enhanced activity of serine proteases, premature degradation of corneodesmosomes and production of pro-inflammatory cytokines (IL-1α and IL-1β) [11]. The increase in skin pH also increases the risk of bacterial colonization, amongst other due to disturbances in ceramides production [11]. Finally, filaggrin deficiency affects the intra- and extracellular architecture as it leads to disorganized keratin filaments and abnormal lipid distribution [7]. Taken together, filaggrin deficiency decreases skin barrier function and renders the skin dry, fragile and more prone to bacterial colonization, allergen penetration and inflammation.

1.3 Atopic dermatitis and filaggrin

Atopic dermatitis (AD) is a chronic, relapsing, inflammatory skin disease characterized by impaired skin barrier function , itch and dermatitis at characteristic body locations [21]. Furthermore, the disease is associated with excessive IgE sensitization and increased eosinophil numbers in inflammatory lesions and peripheral blood, shown to be correlated to disease severity [21, 22]. The incidence of AD has increased during the second half of the 20th century and now affects up to 20% of all children and up to 10% of adults in developed countries [21]. The disease can be manifested at any age, but approximately 60% develop AD within the first year of life [21], suggesting that early-life exposures play an important role in the development of the disease.

The etiopathogenesis of AD remains unclear, but two major hypotheses have been proposed. The first is the “inside-to-outside” theory where it is thought that the primary defect resides in the immune system which causes excessive IgE sensitization and inflammation, leading to a secondary dysfunctional skin barrier. The second is the “outside-to-inside” theory, which proposes that the primary defect resides in the skin barrier causing increased allergen and pathogen exposure leading to secondary excessive IgE sensitization and secondary inflammation [23, 24]. Supporting the second theory, numerous studies have shown an increased risk of AD in *FLG* mutations carriers since the discovery of the mutations in 2006, and it is considered to be the strongest genetic risk factor of AD [7]. The association between AD and *FLG* mutations has previously been evaluated in two large meta-analyses where the odd ratio (OR) was found to be 4.78 [25] and 3.12 [26]. It has additionally been

shown that the number of filaggrin units (10, 11 or 12 repeats), referred to as the copy number variance (CNV), influence the risk of developing AD, independently of *FLG* mutations. In an Irish population study, carries of the lowest CNV genotype (10 repeats, 10 repeats) had an OR of 1.67 of developing AD compared to those with the highest CNV genotype (12 repeats, 12 repeats) [27]. The same association between CNV and AD was found in a Korean study [28], and an inverse relationship between CNV and self-reported xerosis has also been described [29]. Moreover, AD patients who carry *FLG* mutations have been shown to have an earlier onset, a more severe and persistent course of disease in addition to increased levels of total IgE and allergic sensitization compared to AD patients without mutations [30-32].

Despite the strong association between AD and *FLG* mutations, a longitudinal birth cohort (n=6971) found that only 9% of children heterozygote for (R501X and 2282del4) developed AD and the majority of the AD patients were wild type [33]. This suggests that immunological and environmental factors along with other genetic variations also play a significant and important role in AD pathophysiology.

1.4 Filaggrin and skin inflammation

Skin inflammation is one of the major hallmarks of AD. Immunologically, the disease is dominated by T-helper -2 (Th2) cells in the acute phase, while Th1, Th17 and Th22 cells also contribute to the inflammation in chronic lesions [34]. Experimental studies have shown that filaggrin expression is downregulated in acute lesional AD skin, independent of *FLG* mutations [35, 36]. IL-4 and IL-13 are known to promote Th2 polarization, and AD skin is associated with an overexpression of these two cytokines [37]. Keratinocytes cultured in the presence of IL-4 and IL-13 have significantly lower *FLG* expression compared with culture in media alone [35, 36]. Furthermore, several other cytokines such as TNF-α, IL-17A, IL-22, IL-25 and IL-31 have all been shown to downregulate filaggrin expression [38]. These observations connect the two different hypotheses of AD etiopathogenesis, creating a new "outside-to-inside-and-back-to-outside" theory where a downstream immunological activation caused by a defect skin barrier provokes further secondary barrier abnormalities in AD [39]. Notably, in the study by Pellerin et al., they found that filaggrin expression was downregulated in both lesional and non-lesional AD skin [36]. Taken together, these findings suggest that Th2 driven skin inflammation reduces filaggrin expression, also independently of *FLG* mutations, and that the inflammation itself may contribute to the epidermal barrier dysfunction observed in AD patients.

1.5 Causes of epidermal filaggrin reduction and its natural variation in the skin

Although it is well established that filaggrin is reduced or completely absent in individuals with *FLG* mutations, and that skin inflammation may reduce filaggrin levels, we have limited knowledge regarding its natural variation according to age and sex and after exposure to exogenous skin stressors. In AD patients, several factors are known to cause exacerbation, such as cold and dry weather, microbial colonization, wool fabrics, exposure to skin irritants and allergens and emotional stress [40, 41]. It would be of great value to identify external factors that reduce filaggrin and NMF levels in the skin, as avoidance of these factors could possibly prevent AD flares or even influence the development of the disease.

1.5.1 Effect of age and sex

The effect of age on filaggrin and its degradation products is unclear. A transcriptional downregulation of epidermal filaggrin has been found in old skin (59-74 years) compared to young skin (1-10 years) [42] in an Austrian study, while another study found a similar expression of profilaggrin in the skin of aged (60-81 years) and young (18-29 years) participants [43]. In the latter study, old participants had lower levels of epidermal filaggrin protein and higher levels of SC amino acids, suggesting an increased turnover of filaggrin. Increased NMF levels in cheek and forearm skin was also found in aged (59-76 years) participants compared to young participants (22-40 years) in a Japanese study [44]. The same study found no difference in NMF levels between female and male participants.

1.5.2 Climatic conditions

The effect of climatic conditions on AD development and prevalence has previously been investigated in several large epidemiological studies. In the US, children living in regions with low humidity, low temperatures, low UV exposure, increased precipitation and increased indoor heating had a higher prevalence of AD compared to those living in regions with more favorable climatic conditions [45]. Furthermore, children born during the fall and winter have a higher prevalence of AD compared to those born during spring and summer [46-50]. The mechanisms behind these observations are not fully understood, but it has been hypothesized that the first months spent in a dry and cold climate might negatively influence the skin barrier function and increase the risk of dermatitis [51].

Low humidity has been shown to reduce skin hydration, elasticity and desmosome degradation, and increase the release of inflammatory mediators [52-55]. The skin surface becomes rough and scaly, the skin barrier function decreases, and it is more susceptible to mechanical stress [56-59]. In an experimental animal study, filaggrin was accumulated throughout the whole SC in late fetal rat skin, but after birth when the relative humidity (RH) declined, filaggrin degradation was immediately initiated. Furthermore, the degradation of filaggrin could be blocked if the humidity was maintained at 100% RH, mimicking the aqueous environment *in utero*, suggesting that filaggrin degradation is controlled by SC water amount [60]. Another study showed that mice transferred from a humid (RH>80%) to a dry (RH <10%) environment had a reduced expression of filaggrin and content of free amino acids and increased TEWL compared to mice transferred from normal conditions [61]. Low temperatures have a negative effect on lipid production and delay the recovery rate after barrier disruption [62, 63]. Furthermore, it decreases skin hydration and leads to xerosis and increased sensation of itch [64-67]. Workers in the fish processing industry were shown to have low TEWL values immediately after work, suggesting a positive effect on the skin barrier. However, after the skin temperature returned to normal, TEWL increased significantly compared to unexposed controls [68].

1.5.3 Bacterial colonization

Another well-established aggravating factor in AD patients is microbial colonization of the skin, especially with *Staphylococcus aureus (S. aureus)* [69]. A possible link between the observed flares in AD patients and colonization with *S. aureus* is Staphylococcal enterotoxin B (SEB). The bacteria toxin is produced by most strains of *S. aureus* isolated from atopic skin [70, 71] and acts as a superantigen, bypassing the normal and controlled activation of the immune system [72]. Application of SEB on intact skin induces dermatitis in both healthy individuals and patients with AD [73]. Furthermore it induces release of the pro-inflammatory cytokine IL-1β [74] and a significant T-cell accumulation in the skin [75].

1.5.4 Allergens and histamine

The role of house dust mite in AD pathogenesis is debated. The two most common house dust mites are the European *Dermatophagoides pteronyssinus* (Der p) and the American *Dermatophagoides farina* (Der f) [76]. Application of house dust mite on non-lesional AD skin provokes eczematous lesions, also in AD patients with a negative skin prick test and without specific IgE against house dust mite [77, 78]. One study showed that healthy controls with and without specific IgE against house dust mite may

also react, although less intensively and frequently than AD patients [79]. Taken together, this suggests that immunological mechanisms independent of specific IgE against house dust mite might initiate skin inflammation, or that the house dust mite itself may initiate skin inflammation.

Another major source of airborne allergens is cats and cat dander [80]. Neonatal cat exposure has also been linked to an increased risk of infant AD in *FLG* mutation carriers, however the mechanism behind this association is unknown [81].

Itch is one of the hallmark symptoms of AD and is among other mediated through histamine [82]. Histamine is released from mast cells [83], and AD patients have been shown to have increased numbers of mast cells compared to healthy controls [84]. Interestingly, experimental studies have shown that corneocytes cultured in the presence of histamine have a significant decrease in filaggrin expression [83, 85], suggesting that filaggrin deficiency in AD patients among other could be mediated through elevated histamine levels.

1.5.5. Water

Water is a known skin irritant [86], and in a previous study by Törmä et al. they found that exposure to water lead to a 50% reduction in profilaggrin expression compared to the control site [87]. Of notice, the hardness of water has been shown to influence the irritation potential of washing regimes. Skin washed and rinsed in hard water became significantly more dry end red compared to washing with soft water [88]. Water is considered hard when the mineral content is high and soft when it is low [89]. Calcium and magnesium are the principal ions, and the most common source of calcium and magnesium are the sedimentary rocks limestone and chalk [90, 91]. Several, but not all, epidemiological studies have found a higher prevalence of AD in children living in regions with hard domestic water compared to those living in regions with softer water [92-97]. Hard water requires more soap to create lather, and the increased use of soap and detergent may irritate the skin and cause inflammation [98]. Furthermore, it may produce irritant soap salt residues deposited on the skin and clothes that are difficult to rinse off [99, 100]. Chlorinated water is also considered a skin irritant, and excessive exposure has been associated to skin complaints and dermatitis [101].

1.5.6 Sodium lauryl sulfate

Sodium lauryl sulfate (SLS) is a common anionic detergent widely used in cosmetics, detergents, shampoo, soap and other cleaning agents [102]. In dermatological research, SLS is often used as a model irritant, and its action is assumed to be mediated through lowering of the surface tension between non-mixable substances [103]. Several studies have shown that experimental exposure to SLS reduces skin barrier function and induces skin inflammation [104, 105]. Exposure to SLS has also been shown to decrease the quantity of epidermal filaggrin protein and NMF levels [106-108] and could therefore serve as a positive control in studies investigating the effect of external skin stressors on filaggrin degradation in the skin.

1.6 Corneocyte surface texture and filaggrin

It has been suggested that nanoscale alterations of the corneocytes surface, described as "villus-like projections" or "circular nano-objects", might influence skin barrier function and indicate skin disease [109, 110]. Previously, these changes have been visualized by electron microscopy, but a novel method using atomic force microscopy (AFM) and pattern recognition software has recently been developed [110].The number of nano-scale alterations in 10 randomly selected images is averaged and gives a score called the Dermal Texture Index (DTI). The nature of the nanoscale alterations is not fully understood, but the DTI has been found to be significantly higher in both lesional and non-lesional AD skin and in carriers of *FLG* mutations [110, 111]. Moreover, a strong negative correlation between DTI and filaggrin degradation products has been observed, suggesting that the altered corneocyte morphology might be associated to filaggrin deficiency [111]. Although available data is limited, no differences in DTI have been found with regards to age or skin type (pigmentation) [110].

2. Objectives

This PhD-thesis is based on three experimental studies.

The overall objective of the thesis was to investigate the effect of internal (age, sex, inflammation) and external (daily-life skin stressors and season) factors on filaggrin degradation products and corneocyte surface texture in adult skin.

In more details, the aims were:

Aim I: To investigate the effect of temperate climate during winter and summer on the level of filaggrin degradation products and corneocyte surface texture in healthy adult volunteers without AD and common *FLG* mutations (Manuscript I).

Aim II: To investigate the corneocyte surface texture, as well as the quantity of epidermal filaggrin protein and its degradation products in dermatitis patients with a history of AD and healthy adult controls (Manuscript II).

Aim III: To investigate the effect of selected daily-life skin stressors on the level of filaggrin degradation products and the inflammatory response in healthy adult volunteers without AD and common *FLG* mutations (Manuscript III).

2.1 Manuscript I

Engebretsen KA, Kezic S, Riethmüller C, Franz J, Jakasa I, Hedengran A, Linneberg A, Johansen JD, Thyssen JP. *Changes in filaggrin degradation products and corneocyte surface texture by season*. British Journal of Dermatology. Accepted for publication 2017.

Changes in filaggrin degradation products and corneocyte surface texture by season

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What is already known about the topic?

- Low temperature and humidity, as well as high-dose ultraviolet B irradiation, impair the skin barrier and cause xerosis.
- Skin on the cheeks and dorsal aspects of the hands have altered skin barrier function compared to other sites due to different skin composition and likely changing exposure to climatic conditions.
- Epidermis with decreased levels of filaggrin degradation products contain corneocytes with increased numbers of nano sized protrusions.

What does this study add?

- In normal adult skin, the levels of filaggrin degradation products and number of corneocyte surface protrusions changed between winter and summer on cheek and hand skin.
- High self-reported UV-exposure increased the number of corneocyte surface protrusions.

What is the translational message?

- Seasonal changes in filaggrin degradation products and corneocyte surface texture suggests an influence of climatic factors on a biochemical and ultrastructural level.
- This study adds new pieces to the puzzle of why many experience seasonal aggravation of dermatitis and xerosis.

Abstract

Background: During the winter in Northern countries, the risk of dermatitis is increased due to low temperature and humidity. Dermatitis is particularly common on weather-exposed skin such as the cheeks and hands. Recently, increased numbers of unidentified nano-sized protrusions on the corneocyte surface were associated with dermatitis and deficiency of natural moisturizing factors (NMF).

Objective: To investigate the effect of season on NMF levels and corneocyte surface texture in cheek and hand skin from normal adults.

Methods: 80 (40 males and 40 females) healthy volunteers were recruited; 40 aged 18-40 years and 40 aged ≥70 years. Cheek and dorsal hand skin was tape-stripped in the winter and summer. Analysis for NMF and corneocyte surface texture was done (Dermal Texture Index, DTI). Potential confounders were registered and adjusted for.

Results: In cheek skin, NMF levels were reduced and DTI elevated during the winter compared to the summer. Old participants had higher NMF levels compared to young participants. In the summer, DTI level was dependent on self-reported UV-exposure. In hand skin, NMF levels were higher during the winter compared to the summer, and female participants had higher NMF levels compared to male participants.

Conclusion: Seasonal effects on NMF and DTI on the cheeks and hands were found, suggesting an influence of climatic factors on biochemical and ultrastructural level. Significant variations were also observed regarding age and sex, making it difficult to draw firm conclusions. Our study adds new pieces to the puzzle of seasonal differences in xerosis and dermatitis.

Introduction

In recent years, there has been a tremendous interest in the pertinent role of a competent skin barrier against the risk of dermatitis and allergy.[1] For example, infants with mutations in filaggrin gene (*FLG)* develop xerosis and have a 2-3 fold increased risk of developing atopic dermatitis (AD).[1-3]

Low temperatures and humidity have a negative impact on skin barrier function and increase the risk of dermatitis.[4] Along that line, we recently showed that fall and winter birth in Denmark, a time with low indoor humidity, cold temperatures and deprivation of ultraviolet (UV) light was significantly associated with AD.[5,6] Interestingly, skin on the cheek and hands is more exposed to changing climatic conditions than other skin regions, as it is rarely covered by clothes. While this could explain the increased occurrence of dermatitis on these specific sites, and in particular in *FLG* mutation carriers,[7-9] the skin barrier on the face and dorsal hands is also markedly different from that on other anatomical areas.[10] Cheek skin has small corneocytes, thin cell layers, reduced levels of natural moisturizing factors (NMF), but increased total lipid and ceramide levels.[11-13]

Recently, a morphometric parameter was established to account for characteristic nanoscale objects on corneocytes. Their density is called Dermal Texture Index (DTI), and the DTI is elevated in skin collected from both non-lesional and lesional AD skin, as well as children with *FLG* mutations compared to controls.[14,15] Although the function or exact composition of these structures remains unknown, their numbers correlate inversely with NMF and therefore seem to represent a biomarker for skin barrier impairment.[15] Since there is still little insight in factors that affect NMF levels, and in particular the number of corneocyte surface protrusions, we examined these parameters in skin samples collected from cheeks and hands in a cohort of adult Danes during winter and summer to study the effects of season.

Material and methods

Study participants

A total of 80 healthy Danish volunteers (76 Caucasian and 4 non-Caucasian) were recruited by advertisement; 40 participants in the young age group (18≤40 years; mean age 29.2 ± 4.4 years) and 40 participants in the old age group (≥70 years; mean age 76.7 ± 4.2 years) (female/male ratio = 1:1). The participants were ineligible if they had a history of AD, psoriasis, systemic inflammatory diseases, asthma and rhinitis which demanded treatment, or if they were pregnant or breastfeeding. The first sampling was performed in the winter of 2016 (18th of January to the 11th of February). Follow-up sampling was performed during the summer of 2016 (7th of June to the 15th of August). The study was

powered at 80% (P=0.05) to detect a difference in NMF levels of 15% compared to wild-type NMF levels.[16] All participants gave informed and written consent, and the study was conducted in accordance with the Declaration of Helsinki principals. The protocol was approved by the regional ethics committee (H-15011396) and the Danish Data Protection Agency.

Assessment of potential confounders

At baseline and follow-up, all participants were asked if they had taken a shower or bath the current day, and if they had applied moisturizers to their face or hands. In the winter, the lowest temperature on the sampling day was recorded. During the summer, participants were asked if they had spent more than one hour in direct sunlight within the last week and whether they had applied sunscreen, and if so, how many times (< 2 times or ≥2 times).

Genotyping

At the first sampling, all participants were screened for three prevalent *FLG* mutations found in the Northern European population (R501X, 2282del4 and R2447X) as previously described.[17]. Samples were obtained from buccal swabs (Isohelix, Harrietsham, UK). Participants with *FLG* mutations were excluded from the study.

Sampling of the stratum corneum

The stratum corneum was sampled from all participants by a tape stripping technique.[16] Round, adhesive tapes (3.8 cm^2, D-Squame, CuDerm, Dallas, TX, USA) were attached to normal skin of the dorsal part of the right hand and the right cheek and pressed on for 5s with a standardized force (D500 – D-Squame Pressure Instrument, CuDerm, Dallas TX, USA).[18] The same skin location was tape stripped 5 consecutive times, and the three first tapes were discharged. Tape 4 was used for NMF analysis and tape 5 for corneocyte surface texture.

Determination of filaggrin degradation products in the stratum corneum

To extract the NMF components (histidine, PCA and UCA (trans- and cis-isomer)), 500 µL 25% (w/w) ammonia solution was added to each vial, followed by 2 hours shaking and subsequent evaporation to dryness. The residues were dissolved in Millipore water, and an aliquot of each sample was analysed by HPLC. The total amount of protein on each tape strip was measured, and the NMF levels were expressed as mmol/g protein.[19] A detailed description of the analysis can be found in Supplementary Materials.

Corneocyte surface texture

Atomic force microscopy (AFM) was used to evaluate nano-scale alteration on corneocytes (Figure 1). The number of nano-objects can be expressed as the Dermal Texture Index (DTI).[14,20] Due to high costs, DTI was only determined for a subgroup of 10 participants in the young age group and 10 participants in the old age group (female/male ratio = 1:1) during the winter and summer, all randomly chosen. The method is described in detail elsewhere[21], but a summary can be found in Supplementary Materials.

Statistics

The NMF levels and DTI are presented as mean values ± one standard deviation (SD). The Shapiro-Wilk test was used to determine normality of the data. DTI values for the hand during the winter were log transformed to obtain normal distribution, and thus tested with log transformed values of DTI for the cheek during the winter and hand during the summer. For comparison of mean values between winter and summer, we used the two-sided paired sample t-test. For comparison of mean values between covariates and possible confounders, we used the two-sided independent-samples t-test. Our a priori hypotheses were that the NMF levels would be lower and DTI higher during the winter compared to the summer and that the NMF levels would be lower and DTI higher in aged skin compared to young skin. We did not expect any difference with regards to sex. The mean NMF levels during the winter were adjusted for age group, sex, shower/bath the current day, use of cream and the minimum temperature on the day of the sampling in a multiple linear regression model. In the summer, we adjusted for age, sex, shower/bath the current day, use of cream and the cis/trans ratio of UCA (objective measurement of recent sun exposure).[22] In subanalyses, we included self-reported sun exposure instead of the cis/trans ratio (exposure to direct sunlight and use of sunscreen). The significance level was set at $P < 0.05$. The DTI data were not adjusted due to a small sample size (n=20). Statistical analyses were performed with IBM SPSS Statistics 22 (IBM, Armonk, NY, USA) and GraphPad Prism 7.0 (GraphPad software, La Jolla, Calif., USA).

Results

Of 80 participants, six participants (7.5%) were excluded due to heterozygous loss-of-function mutations in *FLG* and seven were lost during follow-up, resulting in 67 participants included in both the winter and the summer measurements. The mean values of NMF and DTI stratified by anatomical localization, age group, sex and possible confounders are shown in Table 1 (winter samples) and Table 2 (summer samples). Overall, the NMF levels were lower on the cheeks compared to the hands during the winter and summer (Figure 2), however, only significantly during the winter. For DTI, the levels on

the cheek and hand were similar during the winter, while in the summer DTI was higher on the hands (P=0.020) (Figure 3).

NMF and DTI in cheek skin

Season

The NMF levels were lower on the cheek during the winter (0.44 ± 0.17 mmol/g protein) compared to the summer (0.50 ± 0.18 mmol/g protein) (P=0.030) (Figure 2). DTI was higher on the cheek during the winter (172 ± 64) compared to the summer (128 ± 52) (P=0.008) (Figure 3).

Covariates and potential confounders

NMF and DTI levels were similar in both genders. Old participants had higher NMF levels on the cheek than young participants both during the winter (0.50 ± 0.18 vs. 0.39 ± 0.14 mmol/g protein, P=0.005) and summer (0.54 ± 0.19 vs. 0.45 ± 0.15 mmol/g protein, P=0.026). When adjusting for potential confounders (shower/bath, use of cream, temperature/sun exposure), the difference remained significant only during the winter (β-coefficient 0.11 mmol/ g protein, P =0.012). DTI was similar in the two age groups. Self-reported showers/baths, or application of cream, had no effect on the NMF levels during the winter (Table 1). In the summer, those who had applied cream to the face had lower NMF levels on the cheek compared to those with no use of cream in the unadjusted (0.43 ± 0.18 vs. 0.55 ± 0.15 mmol/g protein, P=0.004) and adjusted analyses (β-coefficient -0.14 mmol/g protein, P=0.004). Lower NMF levels on the cheek were found for temperatures below the freezing point (<0°C) in the unadjusted (P=0.011), but not the adjusted analyses. In the summer, self-reported UV-exposure or application of sunscreen did not influence the level of NMF, but participants who had been exposed ≥2 times to direct sunlight for ≥1 hour had higher DTI on the cheek compared to those who had been exposed <2 times (144 ± 52 vs. 90 ± 30, P=0.031).

NMF and DTI on the dorsal aspect of hands

Season

The NMF levels on the hands were higher during the winter compared to the summer (0.75 ± 0.19 vs. 0.55 ± 0.20, P<0.0001) (Figure 2), and the difference remained significant after stratification for age and sex (Table S1). There was no seasonal difference in DTI for the hand overall, but young participants had higher DTI during the winter compared to the summer (196 ± 89 vs. 146 ± 57, P=0.037) (Table S2).

Covariates and potential confounders

Female participants had higher NMF levels on the hands compared to the male participants in adjusted analyses during the winter (β-coefficient 0.11 mmol/g protein, P=0.030), and summer (β-coefficient 0.16 mmol/g protein, P=0.001). For DTI, no significant difference was found during the winter, but in the summer, female participants had lower DTI on the hand compared to male participants (124 ± 49 vs.194 ± 47 mmol/g protein, P=0.004). No age-related differences in NMF levels or DTI on the hand were found during the summer or winter. The NMF levels and DTI on the hands did not differ according to shower/bath the same day, use of cream, temperature during the winter or UV-exposure during the summer.

Discussion

Main findings

In this exploratory study, we observed seasonal effects on corneocyte surface texture (DTI) and amino acid content (NMF levels). Namely on the cheek, DTI was found to be higher during the winter compared to the summer. Individuals reporting recent UV-exposure exhibited elevated DTI-values, suggesting an influence on barrier function. Reduced NMF levels were observed on the cheeks during winter and on the hands during summer, but significant variations were also observed regarding gender and age, making it difficult to draw firm conclusions.

Interpretation

The anatomical sites hands and cheeks were chosen in this study as they represent light and weather exposed skin areas with distinct skin barrier profiles.[10-13] NMF levels on the cheek were significantly lower in the winter compared to the summer, while the opposite was observed for the hands. The difference in response suggests that the NMF levels in cheek skin and hand skin could be differently regulated and affected by climatic conditions, but other day-to-day factors not accounted for should also be considered such as use of gloves, hand wash, and soap usage. Interestingly, a similar decrease in NMF on the cheek during the winter was found in a Japanese study.[11] In Denmark, the winters are relatively cold (average temperature 1.6°C), with low ambient humidity and few hours of daylight compared to the summer. Low temperature and low humidity may have a negative effect on skin barrier function and increase the risk of dermatitis.[4,23] The degradation of filaggrin increases when environmental humidity decreases.[24] This could explain why the NMF levels on the hands were higher during winter compared to the summer. The reason for the reduced levels of NMF on cheek skin is unknown, but could be explained by a more or less complete depletion of filaggrin, or a compensatory increase in lipid production.

Although speculative, the increase in NMF levels on the cheek during the summer could be explained by reduced skin barrier function and stratum corneum hydration as a result of UV irradiation.[25,26] The reduced skin hydration could in turn initiate filaggrin degradation. Furthermore, UCA is excreted in sweat,[27] and it is possible that the higher temperatures during summer and increased sweating could contribute to the higher NMF levels. We observed that participants who had applied cream to the face on the day of the sampling had significantly lower NMF levels on the cheek during the summer. This supports the assumption that filaggrin degradation, and hence NMF levels, is decreased in well-hydrated skin.

Interestingly, DTI, which was measured only in a subgroup, varied significantly with season and self-reported UV-exposure. Thus, DTI was higher in the winter compared to the summer. Furthermore, DTI levels on the cheek were elevated in participants who reported a high exposure to direct sunlight, suggesting that winter climate, but also a high UV exposure may influence corneocyte surface texture. The exact cause of these alterations in corneocyte surface texture represented by a high DTI is, however, unknown. It has been hypothesized that the nanoscale alterations on corneocytes could be due to a disorganized cytoskeleton, an immature or fragile cornified envelope, or that they are related to cell-to-cell adhesion.[28,29] Filaggrin plays an essential role in the aggregation of keratin filaments and the development of the cornified envelope,[30,31] and it is thus plausible that filaggrin deficiency might play a role in the development of the alterations.

Previous studies have shown that DTI is higher in both non-lesional and lesional AD skin compared to healthy skin, and in individuals with *FLG* mutations,[14,15] suggesting that a high DTI may be associated with an impaired skin barrier function or dry skin. A positive correlation between transepidermal water loss (TEWL) and DTI has been reported, supporting this assumption.[15] Irradiation with high doses of broadband UVB negatively affects the intercellular lipids and corneodesmosomes, leading to decreased integrity of the skin and impaired skin barrier function.[26] Furthermore, UVB dosage has been found to correlate linearly with skin barrier disruption.[32] The elevated DTI observed after high self-reported UV-exposure in the current study could be explained by UV-induced xerosis. Along that line, experimentally induced xerosis and inflammation by exposure to the contact allergen 2,4,6-trinitrochlorobenzene, led to protrusions on the corneocyte surface which disappeared after treatment with moisturizers,[33] supporting this hypothesis. However, a high self-reported UV-exposure did not influence the NMF levels in the current study. This might indicate that UV-irradiation affects filaggrin degradation and corneocyte surface texture differently, or at least the time frame from exposure to induction of changes.

The difference in filaggrin levels and its metabolites in young and old skin is unclear. An Austrian study showed a transcriptional down-regulation (3.3-fold) of epidermal filaggrin in 59-74 year olds compared to 1-10 year olds,[34] whereas a Japanese study found a similar expression of profilaggrin between aged (60-81 years) and young (18-29 years) subjects.[35] Notably, in the latter study, as shown by immunohistochemical analyses, filaggrin levels were decreased, and the amount of stratum corneum amino acids increased in the old age group, suggesting increased filaggrin degradation.[35] A similar increased level of NMF was observed in aged skin compared to young skin on the cheek and forearm.[11] The observed epidermal increase of NMF in aged skin could be explained by longer stratum corneum transit time and larger cell surface areas,[36] suggesting decreased epidermal turnover as well as more mature cells.[37,38] This would result in decreased need for profilaggrin expression since NMF levels would be higher in absolute numbers. Alternatively, aged skin has been shown to have significantly lower levels of all major lipids.[39] which might lead to increased TEWL and reduced skin hydration. To compensate for this, increased degradation of filaggrin could in turn lead to increased NMF levels.

Strengths and limitations

The main strength of this study is the large study population and the paired design with a high participation rate. We are the first to investigate the effect of season and self-reported UV-exposure on nanoscopic morphology. Sampling and assessments of the participants was performed by the same investigator, preventing interpersonal variability. A limitation is that since we had no information about profilaggrin expression and filaggrin levels in the epidermis, we cannot determine the kinetics and dynamics of NMF synthesis. Both increased degradation of filaggrin into NMF as a response to xerosis or UV-exposure, and an overall increased expression of profilaggrin and hence filaggrin in the stratum corneum could influence NMF levels. Concomitant data on profilaggrin expression and filaggrin levels, however, would have required invasive skin biopsies. Information about NMF derived from other sources than filaggrin (e.g. lactate and urea), other proteins and lipids in the epidermis would have improved interpretation of observed differences. Moreover, a non-exposed control site would have enhanced interpretation of our findings and the association with UV exposure and climate. Due to the small sample size, DTI data were not adjusted. Decreased skin hydration has previously been suggested to affect corneocyte morphology, and thus the possibility to adjust cream usage the same day would have been preferred. Finally, several of the potential confounders were self-reported, potentially influencing the accuracy of the adjusted analysis.

Conclusion

In this explorative study, we observed seasonal effects on NMF levels and corneocyte surface texture (DTI) on both the cheeks and the hands, thereby detailing the influence of climatic factors. Significant differences were also observed regarding sex and age, making it difficult to draw firm conclusions. Our findings help explain why many people experience seasonal variations of dermatitis or senile xerosis. The multiparametric study design highlights the many factors that determine the epidermal barrier function.

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Tables

Table 1. Total levels of NMF (mmol/g protein) and DTI in the winter, stratified by age group, sex and potential confounders

Population characteristics	NMF[†]			DTI		
	Total (%) (n/ntotal)	Cheek[‡] mean (± SD)	Hand[‡] mean (± SD)	Total (%) (n/ntotal)	Cheek[‡] mean DTI (± SD)	Hand[‡]* mean DTI (± SD)
Age group	n= 74			n = 20		
≤ 40 years	50.0 (37/74)	0.39 (± 0.14)	0.77 (± 0.15)	50.0 (10/20)	193 (± 46)	196 (± 89)
≥70 years	50.0 (37/74)	0.50 (± 0.18)	0.72 (± 0.22)	50.0 (10/20)	151(± 74)	168 (± 71)
p-value		***0.005***	*0.211*		*0.139*	*0.571*
Sex						
Male	50.0 (37/74)	0.46 (± 0.15)	0.72 (± 0.22)	50.0 (10/20)	175 (± 59)	210 (± 75)
Female	50.0 (37/74)	0.43 (± 0.18)	0.78 (± 0.15)	50.0 (10/20)	169 (± 72)	153 (± 78)
p-value		*0.407*	*0.175*		*0.820*	*0.067*
Shower or bath the current day						
No	41.9 (31/74)	0.49 (± 0.16)	0.75 (± 0.19)	30.0 (6/20)	158 (± 49)	162 (± 93)
Yes	58.1 (43/74)	0.41 (± 0.17)	0.74 (± 0.19)	70.0 (14/20)	178 (± 70)	190 (± 75)
p-value		*0.055*	*0.957*		*0.528*	*0.276*
Applied cream to the face the current day						
No	48.6 (36/74)	0.48 (± 0.15)	-	50.0 (10/20)	159 (± 52)	-
Yes	51.4 (38/74)	0.41 (± 0.18)	-	50.0 (10/20)	185 (± 75)	-
p-value		*0.066*			*0.378*	
Applied cream to the hands the current day						
No	68.9 (51/74)	-	0.76 (± 0.19)	70.0 (14/20)	-	185 (± 75)
Yes	31.1 (23/74)	-	0.72 (± 0.20)	30.0 (6/20)	-	173 (± 97)
p-value			*0.379*			*0.573*
Lowest temperature at the day of sampling						
Below freezing point	51.4 (38/74)	0.40 (± 0.15)	0.72 (± 0.16)	65.0 (13/20)	176 (± 75)	190 (± 90)
Above freezing point	48.6 (36/74)	0.49 (± 0.17)	0.77 (± 0.21)	35.0 (7/20)	165 (± 40)	166 (± 58)
p-value		***0.011***	*0.319*		*0.730*	*0.707*

DTI, Dermal Texture Index; NMF, natural moisturizing factors; SD, standard deviation

[†] mmol/g protein

[‡]Independent-samples t-test was used to test the statistical difference between the groups within each population characteristic

* DTI values for the hand during winter were log transformed to obtain normal distribution

Highlighted in bold are the significant results (P<0.05)

Table 2. Total levels of NMF (mmol/g protein) and DTI in the summer, stratified by age group, sex and potential confounders

Population characteristics	NMF†			DTI		
	Total (%) (n/ntotal)	Cheek‡ mean (± SD)	Hand‡ mean (± SD)	Total (%) (n/ntotal)	Cheek‡ mean DTI (± SD)	Hand‡ mean DTI (± SD)
Age group	n = 67			n= 20		
≤ 40 years	49.3 (33/67)	0.45 (± 0.15)	0.54 (± 0.22)	50.0 (10/20)	139 (± 52)	146 (± 57)
≥70 years	50.7 (34/67)	0.54 (± 0.19)	0.55 (± 0.18)	50.0 (10/20)	117 (± 53)	172 (± 62)
p-value		***0.026***	*0.832*		*0.363*	*0.344*
Sex						
Male	52.2 (35/67)	0.49 (± 0.17)	0.48 (± 0.18)	50.0 (10/20)	140 (± 52)	194 (± 47)
Female	47.8 (32/67)	0.50 (± 0.18)	0.62 (± 0.20)	50.0 (10/20)	115 (± 53)	124 (± 49)
p-value		*0.902*	***0.004***		*0.291*	***0.004***
Shower or bath the current day						
No	52.2 (35/67)	0.52 (± 0.16)	0.56 (± 0.18)	45.0 (9/20)	146 (± 58)	181 (± 66)
Yes	47.8 (32/67)	0.47 (± 0.19)	0.53 (± 0.22)	55.0 (11/20)	113 (± 44)	141 (± 49)
p-value		*0.271*	*0.543*		*0.153*	*0.142*
Applied cream to the face the current day						
No	56.7 (38/67)	0.55 (± 0.15)	-	55.0 (11/20)	119 (± 61)	-
Yes	43.3 (29/67)	0.43 (± 0.18)	-	45.0 (9/20)	139 (± 40)	-
p-value		***0.004***			*0.416*	
Applied cream to the hands the current day						
No	83.6 (56/67)	-	0.55 (± 0.21)	75.0 (15/20)	-	154 (± 65)
Yes	16.4 (11/67)	-	0.51 (± 0.16)	25.0 (5/20)	-	175 (± 42)
p-value			*0.569*			*0.500*
Exposure to direct sunlight >1 hour within the last week						
< 2 times	34.3 (23/67)	0.54 (± 0.18)	0.57 (± 0.22)	30.0 (6/20)	90 (± 30)	122 (± 47)
≥ 2 times	65.7 (44/67)	0.48 (± 0.17)	0.53 (± 0.19)	70.0 (14/20)	144 (± 52)	175 (± 58)
p-value		*0.168*	*0.544*		***0.031***	*0.068*
Use of sunscreen within the last week						
< 2 times	76.1 (51/67)	0.51 (± 0.17)	0.54 (± 0.21)	70.0 (16/20)	122 (± 51)	163 (± 59)
≥ 2 times	23.9 (16/67)	0.44 (± 0.17)	0.56 (± 0.18)	30.0 (4/20)	152 (± 59)	144 (± 67)
p-value		*0.171*	*0.767*		*0.308*	*0.571*

DTI, Dermal Texture Index; NMF, natural moisturizing factors; SD, standard deviation

† mmol/g protein

‡Independent-samples t-test was used to test the statistical difference between the groups within each population characteristic

Highlighted in bold are the significant results (P<0.05)

Figures

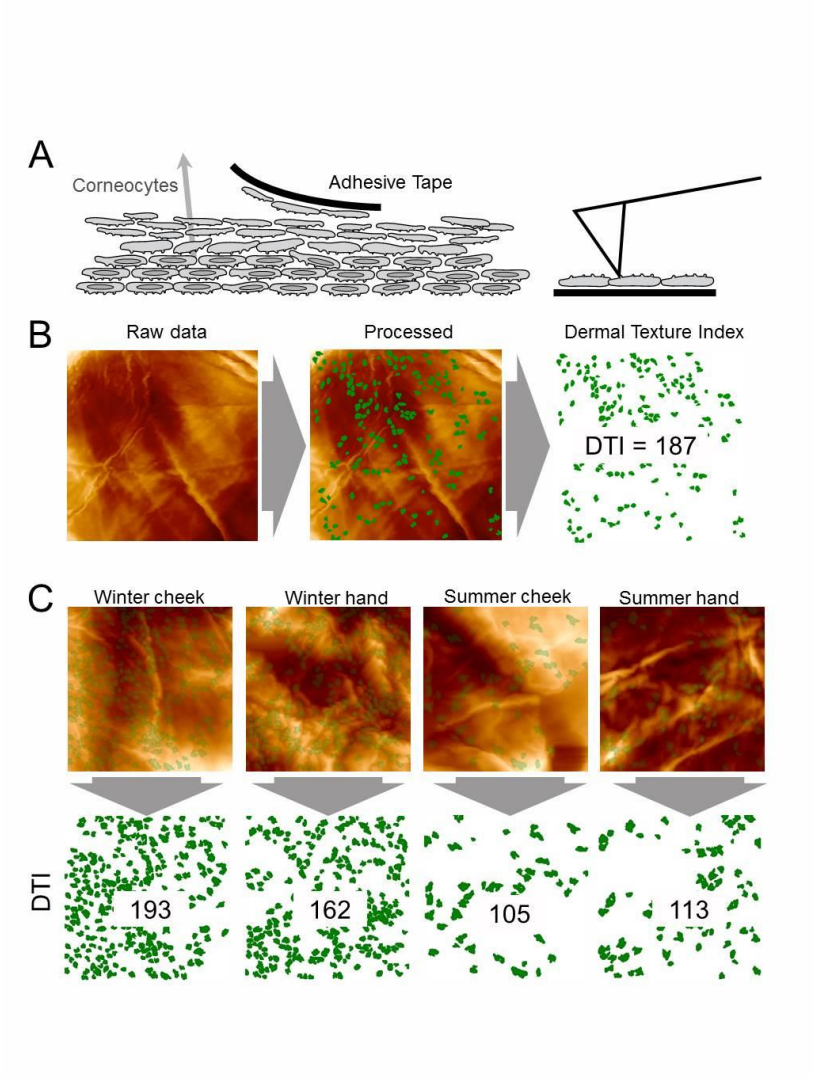

Figure 1. Nanostructures on human corneocytes. **A)** Surficial corneocytes were obtained by the dermatological tape stripping method. The basal (bottom) face of corneocytes adhering to the tape was imaged by scanning force (AFM). **B)** 20 μm^2 scans are analyzed by computer vision to count circular structures (= Dermal Texture Index, DTI). **C)** 4 samples of one individual taken in winter and summer from cheek and hand. DTI values presented are the mean of 10 images. AFM, Atomic Force Microscopy.

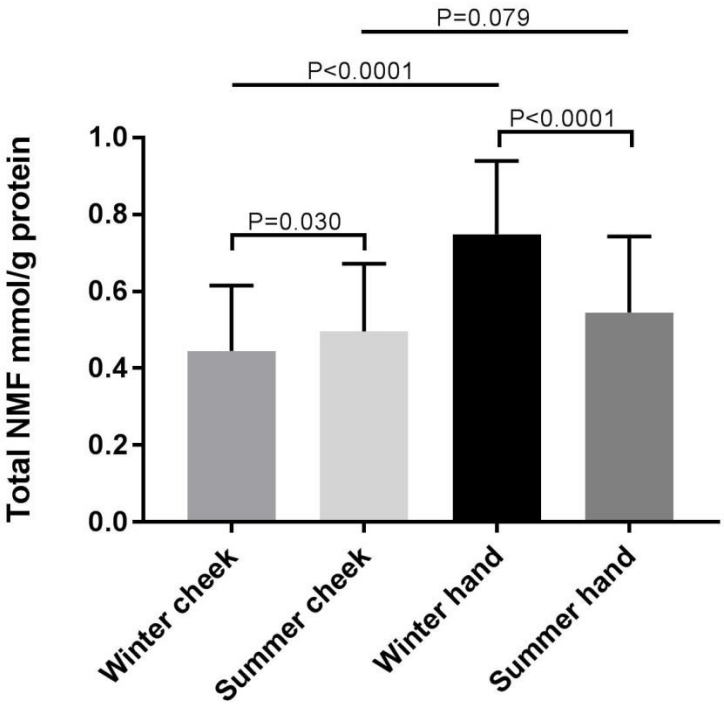

Figure 2. Total levels of natural moisturizing factors (NMF) (mmol/g protein) according to anatomical location and season, not stratified for age or sex, (n=67). Paired sample t-tests were used to test the statistical difference between the anatomical localizations and between the winter and summer samples for both the cheek and the hand.

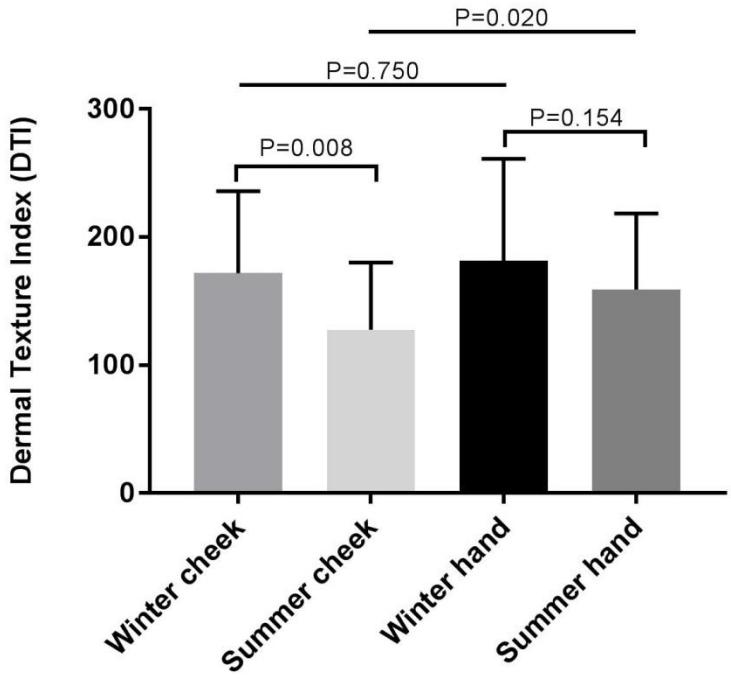

Figure 3. Dermal Texture Index (DTI) according to anatomical location and season, not stratified for age or sex, (n=20). Paired sample t-tests were used to test the statistical difference between the anatomical localizations and between the winter and summer samples for both the cheek and hand. DTI values for the hand during the winter was log transformed to obtain normal distribution and tested with log transformed values of DTI for the cheek during the winter and hand during the summer.

Supplementary Materials

Determination of filaggrin degradation products in the stratum corneum

To extract the NMF components histidine (His), 2-pyrrolidone-5-carboxylic acid (PCA) and UCA (trans- and cis-isomer), 500 µL 25% (w/w) ammonia solution was added to each vial.[1] The extracts were shaken for 2 hours (IKA-Vibrax Model 2200, IKA-works Inc, Wilmington, NC, USA), transferred to a new vial and evaporated to dryness at 60°C (Eppendorf Concentrator 5301, Eppendorf AG, Hamburg, Germany). The residues were dissolved in 500 µl of Millipore water and an aliquot of the sample was analysed by HPLC. To compensate for the variable amount of protein on each tape strip, the total amount of protein was determined. The extraction of protein by ammonia was incomplete and therefore a second extraction with 0.1 M KOH was performed. The protein amount in each extraction was determined by the Pierce Micro BCA protein assay kit (Thermo Fischer Scientific, Rockford, IL, USA), and the levels of NMF were expressed as mmol/g protein.

Corneocyte surface texture (DTI)

The fifth consecutive tape was used for the analysis. A Multimode AFM equipped with the Nanoscope III controller and software version 5.30sr3 (Digital Instruments, Santa Barbara, CA, USA) with silicon-nitride tips on V-shaped gold-coated cantilevers were used (0.01 N/m, MLCT, VEECO, Mannheim, Germany). For each sample, the topographic cell surface data were analysed by using the nAnostic method (Serend-ip GmbH, Munster, Germany).[2] Nanostructures protruding from the mean cell surface were evaluated morphometrically and filtered by size and shape through computer vision. For each sample, 10 randomly selected images were assessed, and the mean value of the identified objects gave the final DTI score.

1 Dapic I, Jakasa I, Yau NL *et al.* Evaluation of an HPLC method for the determination of natural moisturizing factors in the human stratum corneum. *Anal Lett* 2013; **46**: 2133-44.

2 Thoelking G, Reiss B, Wegener J *et al.* Nanotopography follows force in TGF-beta1 stimulated epithelium. *Nanotechnology* 2010; **21**: 265102.

Supplementary tables

Table S1. Total levels of NMF (mmol/g protein) according to seasonal variation, stratified by age and sex

		Cheek (mean NMF† ± SD)				Hand (mean NMF† ± SD)			
			Winter	Summer	p-value‡		Winter	Summer	p-value‡
All		n = 67	0.44 (± 0.17)	0.50 (± 0.18)	***0.030***	n = 67	0.75 (± 0.19)	0.55 (± 0.20)	**<0.0001**
Age group									
	≤ 40 years		0.39 (± 0.14)	0.45 (± 0.15)	***0.010***		0.77 (± 0.15)	0.54 (± 0.22)	**<0.0001**
	≥70 years		0.50 (± 0.18)	0.54 (± 0.19)	*0.374*		0.72 (± 0.22)	0.55 (± 0.18)	**<0.0001**
Sex									
	Male		0.46 (± 0.15)	0.49 (± 0.17)	*0.359*		0.72 (± 0.22)	0.48 (± 0.18)	***<0.0001***
	Female		0.43 (± 0.18)	0.50 (± 0.18)	***0.015***		0.78 (± 0.15)	0.62 (± 0.20)	***<0.0001***

NMF, natural moisturizing factors; SD, standard deviation

† mmol/g protein

‡Paired sample t-test was used to test the statistical difference between the winter and summer samples

Highlighted in bold are the significant results (P<0.05)

Table S2. DTI according to seasonal variation, stratified by age and sex

		Cheek (mean DTI ± SD)				Hand (mean DTI ± SD)			
			Winter	Summer	p-value‡		Winter	Summer	p-value*
All		n = 20	172 (± 64)	128 (± 52)	*0.008*	n = 20	182 (± 80)	159 (± 59)	*0.154*
Age group									
	≤ 40 years		193 (± 46)	139 (± 52)	***0.043***		196 (± 89)	146 (± 57)	**0.037**
	≥70 years		151 (± 74)	117 (± 53)	*0.107*		168 (± 71)	172 (± 62)	0.870
Sex									
	Male		175 (± 59)	140 (± 52)	*0.154*		210 (± 75)	194 (± 47)	*0.678*
	Female		169 (± 72)	115 (± 53)	***0.025***		153 (± 78)	124 (± 49)	*0.139*

DTI, Dermal Texture Index; SD, standard deviation

‡Paired sample t-test was used to test the statistical difference between the winter and summer samples.

*Paired sample t-test with log transformed values of DTI was used to test the statistical difference between the winter and summer samples.

Highlighted in bold are the significant results (P<0.05)

2.2 Manuscript II

Engebretsen KA and Bandier J, Kezic S, Riethmüller C, Heegaard NHH, Carlsen BC, Linneberg A, Johansen JD, Thyssen JP. *Levels of filaggrin monomers, its metabolites and corneocyte surface texture in individuals with a history of atopic dermatitis and controls.* Journal of the European Academy of Dermatology and Venereology. Submitted 2017.

Levels of filaggrin monomers, its metabolites and corneocyte surface texture in individuals with a history of atopic dermatitis and controls

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† *This work is dedicated to our dear friend and colleague, Niels H. H. Heegaard, who suddenly and sadly passed away.*

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Abstract

Background: Atopic dermatitis (AD) is characterized by skin barrier dysfunction. Notably, a high number of nano-scale protrusions on the surface of corneocytes, which can be expressed by the Dermal Texture Index (DTI), was recently associated with pediatric AD, loss-of-function mutations in filaggrin gene (*FLG*), and reduced levels of natural moisturizing factors (NMF). No study has so far examined the association between these parameters and monomeric filaggrin levels in adults.

Objective: To determine DTI, monomeric filaggrin and NMF in healthy controls and a group of patients with controlled dermatitis.

Methods: A total of 67 adults (20 healthy controls and 47 dermatitis patients) were included. In the patient population, a personal history of AD was diagnosed by the U.K. Working Party's Diagnostic Criteria. All participants were tested for *FLG* mutations (R501X, 2282del4, R2447X). Transepidermal water loss, monomeric filaggrin, DTI and NMF were measured.

Results: In the patient population, 78.7% (37/47) had a history of AD and 59.5% (28/47) had *FLG* mutations. Patients had significantly higher levels of DTI and significantly lower levels of monomeric filaggrin and NMF compared to the 20 healthy controls. Among patients, reduced level of monomeric filaggrin and NMF correlated with the presence of *FLG* mutations and clinical phenotypes such as xerosis, palmar hyperlinearity and AD. Among healthy controls, DTI was significantly higher in the oldest age group compared to the two younger age groups.

Conclusion: A significant difference in DTI, monomeric filaggrin and NMF levels was found when comparing dermatitis patients with healthy controls. These findings suggest that even mild dermatitis or non-visible inflammation has a significant and negative effect on the skin barrier as inflammation is known to reduce filaggrin levels. DTI was significantly increased in aged individuals in the healthy control group, suggesting a gradual change in corneocyte morphology with age.

Introduction

Human stratum corneum (SC) consists of multiple layers of protein-enriched, flattened and smooth corneocytes in a matrix of hydrophobic lipids, resembling 'bricks and mortar'.[1] In case of skin barrier impairment and atopic dermatitis (AD), nano-scale protrusions on the corneocyte surface may appear (Figure 1).[2, 3] These protrusions have in the past been described as 'villus-like projections' or 'circular nano-objects', but neither their function, nor their composition has been identified.

Recently, a novel method was developed to accurately count these corneocyte protrusions, the Dermal Texture Index (DTI), was established to enable comparative studies [3]. Importantly, DTI was higher in both non-lesional and lesional skin from pediatric AD patients and correlated with the presence of common loss-of function mutations in filaggrin gene (*FLG*).[3, 4] Furthermore, a strong inverse correlation between DTI and the degradation products of filaggrin, natural moisturizing factors (NMF), was observed.[4]

Since AD, and other forms of dermatitis, is characterized by primary or secondary skin barrier impairment, there is a need for additional studies that examine the difference in various novel biomarkers between dermatitis patients and controls. For example, cytokines that are active in innate and acquired immune response of AD down-regulate *FLG* expression and reduce filaggrin monomer levels.[5] In the current study, we determined DTI as well as the quantity of epidermal monomeric filaggrin and NMF in healthy controls and dermatitis patients with controlled disease.[6]

Material and methods

Study population

A total of 67 Caucasian individuals aged 18-68 years (20 healthy controls and 47 dermatitis patients with controlled disease) participated in the study between October 2011 and March 2012. Healthy controls were recruited by online advertisement. The patient population was recruited by reviewing medical charts of patients with dermatitis at the Department of Dermatology and Allergy, Herlev and Gentofte Hospital, Denmark. Participants were invited in connection with a previous study by Bandier et al. [6] That study demonstrated a dose-dependent correlation between epidermal monomeric filaggrin levels and *FLG* mutations and included both homozygous and heterozygous carriers of common *FLG* mutations.[6] Patients were ineligible for participation if they had active or widespread dermatitis (mild eczema was accepted), other chronic inflammatory diseases besides eczema (i.e. rheumatoid arthritis, Crohn's disease, ulcerative colitis, systemic lupus erythematous or psoriasis), or underwent ultraviolet (UV) irradiation 3 weeks before study start, had used topical corticosteroids

within 2 weeks, or used systemic immunosuppressants. The healthy controls had no current or past history of dermatitis, otherwise the same exclusion criteria applied for the healthy controls as for the patient population. Blood samples were collected from the healthy controls for *FLG* genotyping. The participants were instructed not to use moisturizers at the day of measurements and skin sampling.

Atopic dermatitis and filaggrin genotype

In the patient population, a personal history of AD was diagnosed by the U.K. Working Party's Diagnostic Criteria and represented 'AD ever'.[7] Current AD was not assessed. All participants were tested for three of the most common *FLG* loss-of-function mutations among Northern European populations (R501X, 2282del4 and R2447X) by multiplex PCR analysis.[8, 9]

Clinical assessment of the participants and history of atopic diseases and symptoms

J.Bandier assessed and registered clinical skin features such as xerosis, palmar hyperlinearity and keratosis pilaris. Furthermore, participants were asked if they had a history of asthma or hay fever, if they ever had experienced fissures on their hands and feet and questions related to the diagnosis of AD according to the U.K. Working Party's Diagnostic Criteria.[7]

Transepidermal water loss

TEWL was measured with the MPA5 (Multi Probe Adaptor system, Courage and Khazaka electronics GmbH, CK electronics, UK) at the inner surface of the left upper arm (opposite side of where the punch biopsy for epidermal filaggrin monomer quantification was taken). TEWL (g/m^2)/h) was measured for 30 seconds and the average of the 10 last seconds of steady state was calculated and used in the statistical analyses.

Corneocyte surface morphology and DTI

Corneocytes were sampled by a tape stripping technique where round, adhesive tapes (3.8 cm^2, D-Squame, CuDerm, Dallas, TX, USA) were attached to healthy or non-lesional skin on the volar forearm. Tapes were applied for 5s by the use of a pressure applicator (D500 – D-Squame Pressure Instrument, CuDerm, Dallas TX, USA),[10] gently removed by tweezers, and stored in a closed vial until analysis. The same skin location was tape-stripped five consecutive times, and the fourth tape was analyzed by Atomic Force Microscopy (AFM) as described in details elsewhere.[11] Briefly, a Multimode AFM equipped with the Nanoscope III controller and software version 5.30sr3 (Digital Instruments, Santa Barbara, CA, USA) and silicon-nitride tips on V-shaped gold-coated cantilevers were used (0.01 N/m, MLCT, VEECO, Mannheim, Germany). The topographic cell surface data was analyzed by using the nAnostic method with custom-built proprietary algorithms (Serend-ip GmbH, Munster, Germany).[12]

Computer vision was used to evaluate each nanostructure morphometrically and to filter them by size and shape. For each sample, 10 randomly selected images of 20 μm^2 were assessed, and the mean value of the identified objects in these 10 images gave the final DTI.

Determination of filaggrin degradation products in stratum corneum

Corneocytes were collected by the same tape stripping technique as described previously to determine the amount of filaggrin degradation products, and the analyses were also performed on the fourth consecutive tape.[13] Briefly, the NMF components histidine (His), 2-pyrrolidone-5-carboxylic acid (PCA) and urocanic acid (UCA) (trans- and cis isomer) were extracted from each tape by adding 500 µL 25% (w/w) ammonia solution. After 2 hours of continuous shaking (IKA-Vibrax Model 2200, IKA-works Inc, Wilmington, NC, USA), the extracts were evaporated to dryness at 60°C (Eppendorf Concentrator 5301, Eppendorf AG, Hamburg, Germany) and the residue was dissolved in 500 µL Millipore water and analyzed by HPLC. To compensate for the variable amount of stratum corneum protein on the tape strips, the total amount of protein was determined. Due to incomplete extraction of proteins by ammonia, a second extraction with 0.1 M KOH was performed. Proteins in both extractions were measured by a Pierce Micro BCA protein assay kit (Thermo Fischer Scientific, Rockford, IL, USA), added together, and the levels of NMF were expressed as mmol/g total protein.[13]

Quantification of epidermal monomeric filaggrin in the skin

Epidermal monomeric filaggrin was quantified in 4 mm skin biopsies taken from the inner surface of the upper arm.[6] Biopsies were stored in Eppendorf vials containing storage buffer (10 mM potassium phosphate, 2mM Na_2EDTA, pH 7) and placed at -80°C until analysis. Epidermis was peeled off after incubation at 56°C for 10 min and then the epidermal protein was extracted by the use of mincing, extraction buffer, grinding, sonication, centrifugation, delipidation and dialysis. Quantification of the monomeric filaggrin level was performed by an in-house enzyme-linked immunosorbent assay (ELISA) as previously described.[6] The level of monomeric filaggrin was expressed as AU/mg epidermis.

Statistics

To determine whether the data were normally distributed, we used the Shapiro-Wilk test. The DTI and levels of monomeric filaggrin and NMF are presented as median values (25th/75th percentiles). In the patient population, NMF and DTI data were log transformed to obtain normal distribution. The independent sample t-test was used to compare two groups, while one-way ANOVA followed by Tukey post hoc test was used for multiple comparisons. For monomeric filaggrin levels, the Mann-Whitney U test and Kruskal-Wallis test with pairwise Mann-Whitney U-test post hoc tests were used for

comparisons of two groups and multiple groups, respectively. In the healthy controls, data on DTI, monomeric filaggrin and NMF were log transformed to obtain normal distribution and, the independent samples t-test or the one-way ANOVA followed by Tukey post hoc test was used. The influence of possible confounders, respectively, age, sex, AD and *FLG* mutations, was assessed by using multiple linear regression models, where the log-transformed data of DTI, filaggrin and NMF were used as dependent variables. The exponentiated coefficient was reported as % change from the reference variable in the linear regression. Our a priori hypothesis was that DTI would be higher, and the level of monomeric filaggrin and NMF lower, in dermatitis patients compared to healthy controls. Furthermore we expected that patients with *FLG* mutations and a history with AD would have a higher DTI, and lower levels of monomeric filaggrin and NMF, compared to patients without these features. P-values <0.05 were considered to be statistically significant. Due to the explorative and experimental design of the study, no correction of multiple comparisons was performed. All statistical analyses were performed with IBM SPSS Statistics 22 (IBM, Armonk, NY, USA) and GraphPad Prism 7 (GraphPad Software, Inc, San Diego, Calif).

Results

The patient population consisted of 18 males and 29 females, and the median age was 41 years (range 18-67). The control group had 10 male and 10 female participants, and the median age was 29 years (range 20-68 years). No statistical difference in age was found between the patient population and the healthy controls (P=0.553). In the patient population, 78.7% (37/47) had a history of AD and 59.6% (28/47) had at least one *FLG* mutation (20 heterozygotes and 8 homozygotes). Furthermore, 61.7% (29/47) had a history of asthma or hay fever, 29.8% (14/47) had clinical xerosis, 66.0% (31/47) had keratosis pilaris, 63.8% (30/47) had palmar hyperlinearity and 44.7% (21/47) had a history of fissures on hands and feet. In the control group 15.0% (3/20) had a history of asthma or hay fever, and 25.0% (5/20) had keratosis pilaris. None of the participants in the control group had clinical xerosis, palmar hyperlinearity or a history of fissures on hands and feet. The median values of DTI, filaggrin and NMF in the patient population and in the healthy controls according to study population characteristics are listed in Table.

Dermal Texture Index (DTI)

Overall, the patient population had significantly higher DTI values compared to the control group (median DTI; $25^{th}/75^{th}$ percentile, 75; 59/98 vs. 44; 31/78, P=0.015) (Figure 2). In the control group, DTI was significantly higher in the oldest age group (>50 years) compared to the youngest age group (<30 years) (P<0.0001) and those between 30-50 years of age (P<0.0001) (Figure 3). When adjusting

for age and sex, the oldest age group had 74% higher DTI values compared to the youngest age group (P<0.0001). In the patient population, no association between DTI and age was found. No significant differences in DTI were found for the other population characteristics in the patient population or the control group in the unadjusted analyses or after adjustment for age and sex.

Epidermal monomeric filaggrin

Overall, the patient population had significantly lower levels of monomeric filaggrin compared to the control group (AU/mg epidermis; 25th/75th percentile, 58.7;16.5/180.5 vs. 195.2; 108.1/486.3, P=0.001) (Figure 2). In the patient population, participants with *FLG* mutations had significantly lower levels of monomeric filaggrin in the unadjusted analyses (P<0.0001) (Table 1), as previously shown by Bandier et al., [6] and this was also evident after adjustment for age and sex (P<0.0001). When stratified for the number of mutations, heterozygous mutation carriers had 42% lower levels and the homozygous mutation carriers had 90% lower levels of monomeric filaggrin compared to wild types in the age and sex adjusted analyses (P=0.009 and P<0.0001, respectively). Monomeric filaggrin levels were significantly lower in participants with xerosis and palmar hyperlinearity compared to those without such clinical characteristics in the unadjusted analyses (Table 1). Analyses adjusted for age and sex showed that xerosis was associated with a 65% reduction (P=0.001) and palmar hyperlinearity with a 59% reduction (P=0.002) of filaggrin levels. No significant differences in the levels of filaggrin were found with regards to age or sex in the patient population or in the control group.

Filaggrin degradation products (NMF)

Overall, the levels of NMF were significantly lower in the patient population compared to the control group (mmol/g protein; 25th/75th percentile, 0.66; 0.47/0.96 vs. 1.01; 0.78/1.17, P=0.005) (Figure 1). In the patient population, the NMF levels were significantly lower in participants with a history of AD compared to participants without AD (P=0.027), and when adjusted for age, sex and *FLG* mutations, a history of AD was associated with an 18% reduction in NMF (P=0.045). In the unadjusted analyses, no difference was seen for the presence of any *FLG* mutation, but when stratified for the number of mutations, homozygous carriers had significantly lower NMF levels compared to heterozygous (P=0.004) and wild type cases (P=0.020). When adjusted for age and sex, homozygous carriers had a 24% reduction in NMF levels compared to wild type cases (P=0.010). A history of asthma or hay fever was associated with significantly lower levels of NMF in the unadjusted analyses (P=0.020) and after adjustment for age and sex (17% reduction, P=0.015). Furthermore, xerosis was associated with lower levels of NMF when adjusted for age and sex (16% reduction, P=0.025). With regards to sex, no significant differences in the NMF levels were found. The oldest age group (>50 years) had higher NMF

levels compared to the youngest age group (<30 years) in the unadjusted analyses (P=0.017), and when adjusted for sex, a history of AD and *FLG* mutations, the oldest age group had 28% higher NMF levels compared to the youngest age group (P=0.023). In the control group, male participants had significantly lower levels of NMF compared to female participants (P=0.029) (Table 2). Finally, participants with high TEWL values (≥ 12 (g/m^2)/h), defined as higher than the median value of all participants, had significantly lower levels of NMF compared to those with lower TEWL values (P=0.033).

Discussion

Main findings

The 47 patients with dermatitis under control had significantly higher levels of DTI and significantly lower levels of monomeric filaggrin and NMF compared to the 20 healthy controls. In these patients, reduced levels of monomeric filaggrin and NMF seemed to correlate with the presence of *FLG* mutations and clinical phenotypes such as xerosis, palmar hyperlinearity and history of AD. Among healthy controls, DTI was significantly higher in the oldest age group compared to the two younger age groups.

Interpretation

The increased DTI observed in patients with dermatitis is in line with previous studies that investigated the relationship between changes in corneocyte morphology and the presence of dermatitis. In 18 healthy newborns followed from birth and up to 6 months, study subjects (n=5) who developed dermatitis had a tendency of higher number of 'villus-like projections' compared to those who did not develop AD.[2] Furthermore, elevated levels of DTI have been found in both lesional (529 ± 277 counts) and non-lesional (116 ± 53 counts) pediatric AD skin when compared to healthy controls (24 ± 21 counts).[3] In our study, the observed difference between the patient population and the healthy controls was not as pronounced, which could be explained by the absence of active dermatitis and furthermore that the individuals were adults.

To our knowledge, we are the first to describe significantly increasing levels of DTI with age in healthy skin. A small study including 21 healthy individuals aged 1-77 years, found no correlation between DTI and age.[3] Nevertheless, morphological changes of aged corneocytes have been described previously. Thus, aged corneocytes compared to young corneocytes have significantly increased cell size, a rougher cell surface, and possibly corneocyte protrusions, described by Gorzelanny et al. as 'humps'.[14] The authors could not explain the nature of these nano-scale alterations, but hypothesized that they

were due to decreased production of lipids and epidermal proteins involved in the keratinization process. Previous studies have shown that aged skin has decreased levels of lipids and down-regulation of important epidermal proteins such as filaggrin and loricrin compared to young skin, supporting this assumption.[15-17] In the current study, we did not observe a significant decrease in levels of filaggrin monomers with age. This suggests that other factors than monomeric filaggrin deficiency are responsible for the high DTI observed in aged healthy skin. For example, we did not measure amount or distribution of lipids around the cells which could account for the difference with age. No association between age and DTI was found in the patient population, however, we observed significantly higher levels of NMF in older patients compared to the two younger age groups (P=0.023), perhaps due to a compensatory breakdown of filaggrin to keep the skin hydrated.

The nature of the altered topography of the corneocyte surface is still unresolved, but several hypotheses have been proposed. While a strong inverse correlation between DTI and NMF was shown in children with acute and convalescent AD,[4] it cannot be out ruled that skin inflammation associated with AD affected this relationship. However, the DTI level was more closely related to NMF than the SCORAD (SCOring Atopic Dermatitis) value, suggesting that the absence of filaggrin or NMF could be more important for altered corneocyte morphology than inflammation *per se*. This notion was supported by the fact that the DTI was not affected by a change in SCORAD.[4] Moreover, the level of skin hydration might be important for DTI levels, as suggested in a study by a Matsumoto et al.[18] Here, experimentally induced xerosis and inflammation by exposure to the potent contact allergen 2,4,6-trinitrochlorobenzene led to corneocyte protrusions (villi), which disappeared after treatment with moisturizers In the current study, we were unable to detect a significant difference in DTI levels according to a history of AD, the presence of *FLG* mutations, xerosis or elevated TEWL in the patient population. This finding could be due to the heterogeneity of the patient population, use of emollients or the presence of sub-clinical inflammation.

The reduced levels of epidermal monomeric filaggrin and NMF in the patient population compared to the healthy controls are in accordance with previous research. Several inflammatory mediators, such as IL-4, IL-13, IL-17A, IL-22, IL-25, IL-33, TSLP and TNF-α have been shown to downregulate filaggrin expression,[5, 19, 20] even in non-lesional atopic skin.[21] Our study subjects had no or mild dermatitis which was under control, hereby suggesting that even sub-clinical inflammation could negatively affect the skin barrier and in turn reduce filaggrin and NMF levels. As expected,[22, 23] the levels of NMF were lower in participants with a history of AD and in carriers of common *FLG* mutations.

Strengths and limitations

To our knowledge, this is the first study to investigate DTI in a relatively large population of adults with and without dermatitis, and also the first study to examine *FLG* mutations, monomeric filaggrin levels and NMF in the same group. There are some limitations with regards to the study design. The biopsy for monomeric filaggrin quantification and the TEWL measurements were taken on the inner surface of the upper arm, while the tape strips used to determine DTI and NMF were collected from the volar aspect of the forearm close to the elbow, possibly leading to bias regarding anatomical variation. Also, the analytical variability of the DTI measure has not yet been established. It is possible that absolute levels of DTI and NMF were reduced since tape strips had been stored since 2011, but we do not expect the relative levels to have been affected. No information on current AD was collected and no clinical scoring of eczema severity was performed. No correction for multiple comparisons was performed despite a high number of association tests. Significant results should therefore be interpreted with caution as they may be based on chance. Finally, we had no information about emollient use, sun exposure or bathing habits within weeks before sampling.

Conclusion

In this human adult study, a significant difference in DTI, epidermal monomeric filaggrin and NMF was found between patients with a history of dermatitis and healthy controls, indicating that mild dermatitis, or even subclinical inflammation, is sufficient to produce alterations in important skin barrier properties. DTI was significantly increased in aged individuals in the healthy control group, suggesting a steady change in corneocyte morphology with age. Future studies need to identify the nature of DTI-related corneocyte changes including possible elicitors for a better understanding of interplay between cells and skin matrix in the etiopathogenesis of AD and other forms of dermatitis.

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Tables

Table 1. The DTI, level of monomeric filaggrin (AU/mg epidermis) and NMF (mmol/g protein) in dermatitis patients and healthy controls, stratified by study population characteristics.

			DTI* median (25th/75th percentile)			Filaggrin (AU/mg epidermis) median (25th/75th percentile)			NMF (mmol/g protein)† median (25th/75th percentile)		
	Patient population % (n/ntotal)	Controls % (n/ntotal)	Patient population	Controls	Patient vs. Controls p-value[1]	Patient population	Controls	Patient vs. Controls p-value[2]	Patient population (n=47)	Controls n=20)	Patient vs. Controls p-value[1]
Overall			75 (59/98)	43 (31/78)	***0.015***	58.7 (16.5/180.5)	195.2 (108.1/486.3)	**0.001**	0.66 (0.47/0.96)	1.01 (0.78/1.17)	***0.005***
Sex											
Male	38.3 (18/47)	50.0 (10/20)	85 (60/150)	43 (32/109)	*0.090*	64.2 (11.3/193.8)	171.8 (93.9/527.3)	*0.061*	0.64 (0.47/0.89)	0.77 (0.45/1.05)	*0.476*
Female	61.7 (29/47)	50.0 (10/20)	70 (47/98)	44 (30/59)	***0.049***	44.1 (19.8/153.0)	207.5 (102.9/388.3)	***0.015***	0.67 (0.43/0.99)	1.05 (0.89/1.80)	***0.001***
p-value			*0.197*	*0.508*		*0.793*	*0.775*		*0.905*	***0.029***	
Age group											
<30 years	34.0 (16/47)	55.0 (11/20)	67 (51/82)	33 (29/44)	***0.025***	49.5 (12.6/227.1)	211.6 (102.7/523.9)	***0.026***	0.59 (0.46/0.69)	0.99 (0.82/1.11)	***0.002***
30-50 years	48.9 (23/47)	20.0 (4/20)	86 (55/114)	45 (25/63)	***0.023***	44.1 (13.7/180.5)	460.7 (40.2/900.0)	*0.116*	0.72 (0.40/1.01)	1.09 (0.87/1.60)	*0.052*
>50 years	17.0 (8/47)	25.0 (5/20)	67 (59/87)	114 (93/139)	***0.025***	100.2 (27.6/172.2)	156.6 (128.1/280.4)	*0.118*	0.99 (0.83/1.28)	0.77 (0.39/-)	*0.564*
p-value			*0.553*	***<0.0001***		*0.812*	*0.980*		***0.023***	*0.679*	
History of asthma or hay fever											
No	38.3 (18/47)	85.0 (17/20)	75 (62/92)	47 (33/100)	*0.392*	51.7 (23.1/127.5)	187.0 (113.6/448.8)	**<0.0001**	0.81 (0.58/1.10)	1.04 (0.77/1.47)	*0.246*
Yes	61.7 (29/47)	15.0 (3/20)	73 (48/134)	30 (20/-)	***0.012***	58.7 (9.0/209.1)	211.6 (36.8/-)	*0.258*	0.64 (0.39/0.78)	0.99 (0.86/-)	*0.062*
p-value			*0.191*	*0.107*		*0.526*	*0.956*		***0.020***	*0.924*	
TEWL (g/m^2)/h)											
Low (<12)	42.6 (20/47)	65.0 (13/20)	79 (41/118)	40 (29/88)	*0.055*	51.7 (18.1/183.1)	131.6 (81.4/409.6)	***0.040***	0.72 (0.61/1.02)	1.05 (0.90/1.78)	***0.009***
High (≥12)	57.4 (27/47)	35.0 (7/20)	69 (59/90)	47 (37/87)	*0.219*	58.7 (13.7/150.5)	211.6 (187.0/523.9)	***0.008***	0.63 (0.43/0.92)	0.81 (0.44/1.07)	*0.413*
p-value			*0.700*	*0.701*		*0.914*	*0.347*		*0.327*	***0.033***	
Keratosis pilaris											
No	34.0 (16/47)	75.0 (15/20)	86 (63/105)	45 (31/109)	***0.030***	31.8 (10.3/175.6)	211.6 (124.6/537.7)	***0.002***	0.67 (0.47/0.99)	1.05 (0.81/1.76)	***0.025***
Yes	66.0 (31/47)	25.0 (5/20)	67 (47/93)	44 (29/47)	*0.074*	69.7 (23.1/180.5)	125.0 (69.7/363.6)	*0.224*	0.65 (0.41/0.95)	0.92 (0.61/1.08)	*0.285*
p-value			*0.310*	*0.271*		*0.559*	*0.392*		*0.836*	*0.312*	
History of AD											
No	21.3 (10/47)	-	67 (60/87)	-	-	100.2 (23.8/172.2)	-	-	0.94 (0.66/1.19)	-	-
Yes	78.7 (37/47)	-	78 (51/108)	-	-	44.1 (9.0/187.8)	-	-	0.63 (0.41/0.80)	-	-
p-value			*0.786*			*0.659*			***0.027***		
***FLG* mutations**											
No	40.4 (19/47)	-	83 (62/108)	-	-	150.5 (41.1/351.7)	-	-	0.73 (0.47/0.95)	-	-
Yes	59.6 (28/47)	-	67 (49/98)	-	-	23.6 (2.5/116.1)	-	-	0.64 (0.46/1.00)	-	-
p-value			*0.556*			***<0.0001***			*0.872*		
***FLG* mutations**											
Wild type	40.4 (19/47)	-	83 (62/108)	-	-	150.5 (41.1/351.7)	-	-	0.73 (0.47/0.95)	-	-
Heterozygote	42.6 (20/47)	-	71 (57/138)	-	-	64.5 (23.1/134.7)	-	-	0.72 (0.59/1.01)	-	-
Homozygote	17.0 (8/47)	-	67 (39/97)	-	-	0.4 (0.3/1.7)	-	-	0.32 (0.25/0.44)	-	-
p-value			*0.170*			***<0.0001***			***0.006***		
Clinical xerosis											
No	70.2 (33/47)	-	77 (59/98)	-	-	69.7 (26.4/195.8)	-	-	0.70 (0.55/0.99)	-	-
Yes	29.8 (14/47)	-	67 (43/120)	-	-	8.8 (0.4/134.3)	-	-	0.46 (0.28/0.72)	-	-
p-value			*0.519*			***0.008***			*0.100*		
Palmar hyperlinearity											
No	36.2 (17/47)	-	74 (60/90)	-	-	127.4 (40.0/370.9)	-	-	0.75 (0.51/0.97)	-	-
Yes	63.8 (30/47)	-	75 (51/108)	-	-	26.3 (3.5/130.9)	-	-	0.64 (0.43/0.94)	-	-
p-value			*0.979*			***0.004***			*0.709*		
History of fissures on hands and feet											
No	55.3 (26/47)	-	82 (63/110)	-	-	65.9 (23.1/206.6)	-	-	0.72 (0.46/1.05)	-	-
Yes	44.7 (21/47)	-	64 (48/97)	-	-	39.1 (1.3/159.9)	-	-	0.63 (0.47/0.83)	-	-
p-value			*0.336*			*0.140*			*0.414*		

AD, atopic dermatitis according to the UK Working Party's diagnostic criteria; AU, arbitrary units; DTI, Dermal Texture Index; *FLG*, filaggrin gene mutation; NMF, natural moisturizing factors; TEWL, transepidermal water loss
Low and high TEWL values were defined as values below or above the median value of all participants
Wild type: Carriers with 2 functional filaggrin gene copies
Heterozygote: Carriers with one functional filaggrin gene copy
Homozygote: Carriers with double-allele null mutations either compound heterozygous or homozygous
The filaggrin gene mutations analyzed are: R501X, 2282del4, R2447X
†NMF values from 17 participants are missing
*DTI values from 5 participants are missing
[1]Independent samples t-test with log-transformed values of NMF and DTI
[2]Mann-Whitney U test
Highlighted in bold are the significant results (P<0.05)

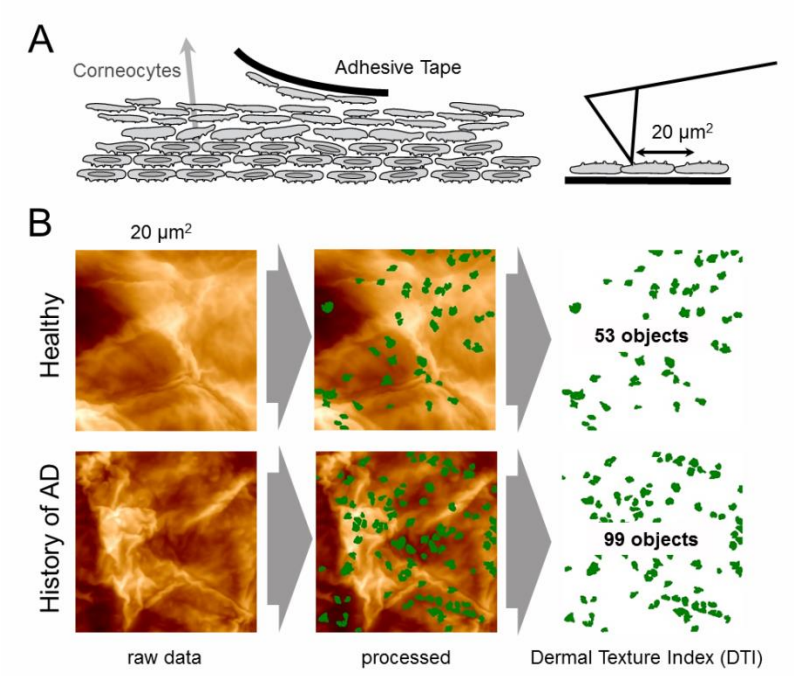

Figure 1. Nanostructure counting. A) Superficial corneocytes are obtained by tape stripping. The basal (bottom) face of corneocytes adhering to the tape is imaged by Atomic Force Microscopy (AFM). B) Arbitrarily addressed areas of 20 μm^2 are scanned and analyzed by computer vision to count circular structures (green spots). The number of objects per area yields the Dermal Texture Index (DTI). DTI is defined as mean value of 10 images. AD, atopic dermatitis

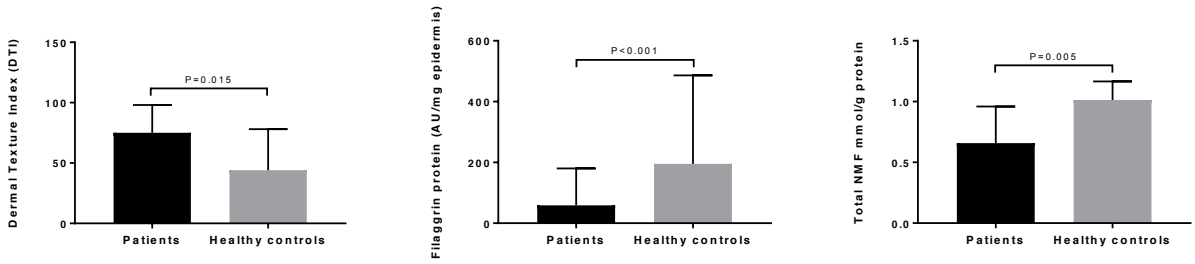

Figure 2. The DTI, levels of monomeric filaggrin (AU/mg epidermis) and NMF (mmol/g protein) in dermatitis patients and healthy controls expressed as median values with interquartile range. For DTI and NMF, independent samples t-test with log transformed values were used to test the statistical difference between the two groups, while for monomeric filaggrin the Mann-Whitney U test was used. P-values <0.05 were considered significant. AU, arbitrary units; DTI, Dermal Texture Index; NMF, natural moisturizing factors

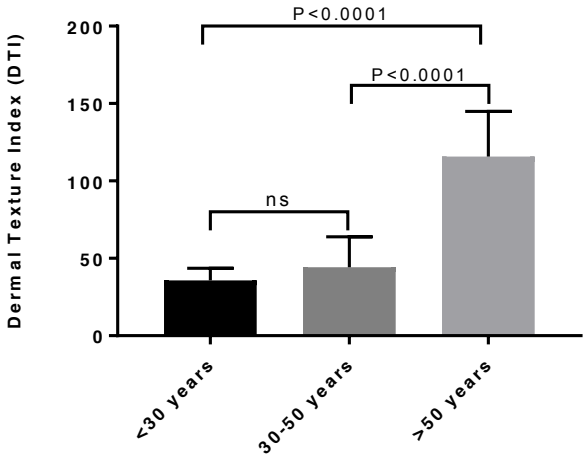

Figure 3. The DTI in healthy participants stratified by age group, expressed as mean (± SD). One-way ANOVA followed by Tukey post hoc test was used to test the statistical difference between the groups. P-values <0.05 were considered significant. DTI, Dermal Texture Index

2.3 Manuscript III

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Effect of daily-life skin stressors on natural moisturizing factors and cytokines in healthy adult skin

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What is already known about the topic?

- Epidermal deficiency of filaggrin and the derived natural moisturizing factors (NMF) due to filaggrin gene mutations cause xerosis and increase the risk of atopic dermatitis (AD). Once AD is present, inflammation further downregulate filaggrin expression.
- Skin stressors such as hard water, bacterial colonization, house dust mite and winter climate have been associated with AD, however limited data is available on their effect on NMF and skin cytokines.

What does this study add?

- In normal adult skin, experimental exposure to water and house dust mite led to decreased levels of NMF, whereas water, bacteria toxin and house dust mite increased secretion of cytokines.

What is the translational message?

- We have identified mechanistic details about common environmental factors that can negatively affect the skin barrier, and suggest that these may be involved AD etiopathogenesis.

Abstract

Background: Epidermal deficiency of filaggrin, and the derived natural moisturizing factors (NMF), is associated with xerosis and increased risk of atopic dermatitis (AD). While loss-of-function mutations in filaggrin gene represent a well-established cause of filaggrin deficiency, there is limited insight in causative environmental factors.

Objective: To explore the effect of selected exogenous skin stressors on NMF and skin cytokines levels in healthy adult skin.

Material and methods: 40 healthy volunteers aged 18-49 years were exposed to hard, soft, and chlorinated water, 0.5% SLS, house dust mite, cat allergen, staphylococcal enterotoxin B (SEB), cooling and histamine. Participants were tape-stripped and biophysiological measurements were performed. NMF was determined after 24 and 48 hours, while skin cytokines were measured after 24 hours for selected exposures.

Results: Exposure to all water types led to a significant decrease in NMF after 24 and 48 hours. Hard, but not soft water led to increased skin levels of IL-4, IFN-ɣ and IL-10. House dust mite exposure led to a significant decrease in NMF and an increase of IFN-ɣ, IL-2, IL-4 and IL-10 after 24 hours. Exposure to SEB led to a significant increase in IL-1α after 24 hours, but no decrease in NMF.

Conclusion: Based on experimental exposure to selected daily-life skin stressors such as different water types, allergens and SEB, we conclude that NMF levels are decreased along with secretion of various skin cytokines in healthy individuals. Our data highlight environmental factors that might play a role in AD pathophysiology, but needs confirmation in AD patients.

Introduction

Filaggrin proteins are essential for normal epidermal architecture and skin barrier function.[1] After alignment of keratin filaments, filaggrin is degraded into hygroscopic amino acids, part of the skin's 'natural moisturizing factors' (NMF), that additionally provide protection against ultraviolet (UV) irradiation and colonization with *Staphylococcus aureus*.[2-5]

Common filaggrin gene (*FLG*) mutations result in reduced epidermal levels of filaggrin and NMF,[6,7] and increase the risk of developing atopic dermatitis (AD) 3-5 fold.[8] Notably, homozygous mutation carriers, who express no filaggrin, have a dramatically elevated risk of AD characterized by early onset and a severe course, hereby emphasizing the pivotal role of filaggrin.[9] Once AD is present, cytokines such as interleukin (IL)-4, IL-13, IL-17A, IL-22, IL-25, IL-31 and tumour necrosis factor (TNF)-alpha may further down-regulate epidermal filaggrin expression,[10] likely in both lesional and non-lesional skin.[11] In a similar way, long-term use of topical corticosteroids may result in decreased epidermal levels of filaggrin and its degradation products.[12-14]

While genetic, inflammatory and drug aetiologies of epidermal filaggrin deficiency are well-established, there is little insight into environmental skin stressors that may reduce filaggrin and NMF levels.[2] Such information is important to reduce culprit exposures in patients with AD and those at risk. In this experimental study, we explored the effect of selected exogenous skin stressors on the levels of NMF and skin cytokines in healthy volunteers.

Material and methods

Study participants

40 healthy volunteers aged 18-49 years were recruited by online advertisement and divided into two groups. Exclusion criteria were: *FLG* mutations, xerosis, history of/current AD, inflammatory diseases (e.g. psoriasis, rheumatoid arthritis and inflammatory bowel disease), asthma or rhinitis in adulthood, pregnancy/lactation, or intense exposure to UV irradiation within 4 weeks. Participants in the second exposure group were excluded if they had house dust mite or cat allergy. First exposures were conducted April-June 2015 and included 7 males and 13 females, median age 24 years (range 19-49). Second exposures were conducted October 2015-January 2016 and included 9 males and 11 females, median age 24 years (range 18-45 years). All participants gave informed and written consent. The study was conducted in accordance with the Declaration of Helsinki principals. The protocol was approved by the regional ethics committee (H-6-2014-100) and the Danish Data Protection Agency.

Genotyping

Participants were genotyped for three prevalent *FLG* mutations in the Northern European population (R501X, 2282del4 and R2447X). Genomic DNA was extracted from buccal swabs (Isohelix, Harrietsham, UK) and analyzed by a multiplex polymorphism analysis.[15]

Biophysiological measurements

Biophysiological measurements were performed in a tempered room (room temperature 21°C-24°C and humidity levels of 35% to 60%). Participants were acclimatized for at least 30 minutes before measurements. Three consecutive transepidermal water loss (TEWL) and skin hydration measurements were done with a multi probe adaptor system (Tewameter® TM 300 and Corneometer® CM 825, Courage and Khazaka electronic GmbH, Cologne, Germany). The mean value was calculated and used in statistical analyses. Participants were instructed not to use emollients on their volar forearms five days prior to study start, or to take a shower/bath one day prior to the study start and throughout the study period.

Exposures and study set-up

All exposures were performed on the volar aspect of both forearms. 20 participants were exposed to different types of water (hard water, soft water and chlorinated water) and 0.5% SLS in extra-large Finn Chambers®. 20 participants were exposed to house dust mite, SEB, cat allergen, histamine by skin prick and cooling for 10 minutes (Table S1). An empty Finn Chamber was applied to evaluate the effect of occlusion alone. All exposures were performed in duplicates allowing us to measure skin changes 24 and 48 hours after application. The order of exposures was randomized to minimize the influence of anatomical variation. Participants were seen over three consecutive days. The first day, the different exposures were either performed or applied onto the skin. After 24 hours, chambers were removed. Excessive moisture and residues were cleaned off, and participants were acclimatized for 30 minutes before measurements. Hereafter, tape stripping was performed at one of two exposure sites and a control site. After 48 hours, the skin measurements and tape stripping were performed at the second exposure sites and a control site.

Determination of filaggrin degradation products (NMF) in the stratum corneum (SC)

The level of filaggrin degradation products (histidine, PCA and UCA (trans- and cis-isomer)) was determined in tape strips by the use of HPLC.[16] Round, adhesive tapes (3.8 cm^2, D-Squame, CuDerm, Dallas, TX, USA) were attached to skin on the volar forearm and pressed on for 5s with a standardized force (D500–D-Squame Pressure Instrument, CuDerm, Dallas TX, USA), gently removed with tweezers

and stored in a closed vial (-20°C). The fourth consecutive tape was used for the analysis. A description of the analysis can be found in Supplementary Materials.

Cytokine analyses

We measured the presence and levels of selected cytokines (IFN-ɣ, TNF-α, IL-1α, IL-1β, IL-2, IL-4, IL-10, IL-13 and IL-18) in the SC at the control site (non-occluded) and after selected exposures (hard water, soft water, 0.5% SLS, house dust mite and SEB). The seventh consecutive tape from tape stripping was chosen for the analysis where the cytokine levels are expected to be stable.[17] The cytokine analyses were performed by using a multiplex assay with human antibodies (MESO QuikPlex SQ 120 assay, MSD, Rockville, Md., USA). A detailed description can be found in Supplementary Materials. If the cytokine values were below the fit curve range, they were assigned with ½ of the lowest value below the detection range. Cytokines where more than 50% of the measured values were below the fit curve range or below the detection range after exposure were excluded from further analyses.[18] Values under the detection range included in the analyses were left as they were to keep the ranking order.

Statistics

Statistical calculations were performed using IBM SPSS Statistics 22 (IBM, Armonk, NY, USA) and Graphpad Prism 7 (GraphPad Software, Inc, San Diego, Calif). To check the distribution of data, the Shapiro-Wilk test was used. In case of normal distribution, a paired sample t-test was used to test the statistical difference between the non-occluded and occluded control spot and the exposed areas. In case of deviation from normal distribution, we used the nonparametric two-sided Wilcoxon signed-rank test. Significance level was set P-values <0.05 for analyses regarding NMF, TEWL and skin hydration. In the cytokine analyses, we chose a more conservative approach and set the significance level to $P<0.01$. Due to the explorative design of the study, no correction for multiple comparisons was performed.

Results

In total, 5% of the study participants (2/40) were *FLG* mutation carriers, one participant in part 1 and one participant in part 2 (both heterozygous for the mutation 2282Del4), and thus excluded.

Part 1: Exposure to different types of water

Table S2 shows the mean values (± standard deviation (SD)) of NMF, TEWL and skin hydration 24 hours and 48 hours after start of exposure. All exposures, including occlusion, led to a significant

decrease in NMF at 24 hours compared to the control site (Figure 1). A significant increase in TEWL was seen for all exposures compared to the control site at 24 hours. There was also a significant decrease in skin hydration for all exposures, except for occlusion alone, which led to an increase in skin hydration compared to the control site at 24 hours. No difference in NMF levels were found between hard and softer water (data not shown).

At 48 hours, the NMF levels were still significantly reduced for all water types and 0.5% SLS compared to the control site measured at 48 hours (Figure 1). No significant difference was found for TEWL, except for 0.5% SLS, where a large significant increase was seen compared to the control site (32.0 g/m^2 h ±14.1 vs. 8.9 g/m^2 h ± 2.3, respectively) ($P<0.0001$). Skin hydration was significantly decreased for all water types, while for 0.5% SLS no significant change was seen (Figure 1).

To evaluate the effect of occlusion, we compared exposure with occlusion only to the other exposures (hard water, soft water, chlorinated water and 0.5% SLS) (Figure 1). At 24 hours, the NMF values were significantly lower for hard water, soft water and 0.5% SLS compared to occlusion alone. TEWL values were only significantly higher for 0.5% SLS at 24 hours, while skin hydration was significantly lower for all water types compared to occlusion alone. After 48 hours, exposure to all water types and 0.5% SLS led to significantly lower levels of NMF, higher TEWL values and decreased skin hydration compared to occlusion alone.

Part 2: Exposure to house dust mite, SEB, cat allergen, cooling and histamine

Table S3 shows the mean values (± SD) of NMF, TEWL and skin hydration at 24 hours and 48 hours after start of exposure. At 24 hours, the only significant decrease in NMF was observed for house dust mite when compared to the control site (0.77 mmol/g protein ± 0.28 vs. 0.87 mmol/g protein ± 0.26) ($P= 0.0459$) (Figure 2). Exposure to occlusion and SEB led to a significant increase in TEWL, while exposure to cat allergen and cooling led to a significant decrease in TEWL compared to the control site. Skin hydration was significantly increased after exposure to occlusion, house dust mite, cat allergen and histamine and decreased after exposure to SEB compared to the control site.

At 48 hours, there was a significant increase in NMF for house dust mite, SEB and cat allergen compared to the control site (Figure 2). TEWL was significantly decreased after exposure to occlusion, cooling and histamine compared to the control site. Finally, hydration was significantly increased for house dust mite and cat allergen compared to the control site.

To evaluate the effect of occlusion, we compared exposure with occlusion only to exposure with house dust mite, SEB and cat allergen. At 24 hours, the levels of NMF were significantly lower for house dust mite, SEB and cat allergen compared to occlusion alone (Figure 2). TEWL was significantly lower for house dust mite and cat allergen, while there was no difference for SEB compared to occlusion alone. Skin hydration was only significantly lower for SEB. At 48 hours, NMF levels were significantly higher for house dust mite and SEB, but not cat allergen, while TEWL was increased for all exposures compared to occlusion alone. Finally, skin hydration was significantly higher for house dust mite and cat allergen, but not for SEB.

Cytokine analyses after 24 hours

The mean (± SD) of log-transformed cytokine values 24 hour after selected skin exposures can be seen in Table S4 and S5. IL-18 was excluded from both parts as more than 90% of the values were below the detection range, or fell below the fit curve range after exposure. Furthermore, TNF-α, IL-2 and IL-13 were excluded from part 1 as more than 50 % of the measured cytokine values were below the detection range, while TNF-α was excluded from part 2 (Table S6).

Exposure to hard water led to a significant increase of IL-4, but also IFN-ɣ and IL-10 compared to the control site (non-occluded) (Figure 3). No significant increase of cytokines where found for soft water compared to the control site. Exposure to 0.5% SLS led to a significant increase in IFN-ɣ and IL-10 compared to the control site.

Compared to the control site, a significant increase in IFN-ɣ, IL-2, IL-4 and IL-10 was seen after exposure to house dust mite (Figure 4). Exposure to SEB only led to a significant increase in IL-1α compared to the control site.

Discussion

Main findings

We showed that 24-hour occluded exposure to soft, hard, and chlorinated water led to a significant decrease in NMF after 24 and 48 hours. Hard water increased epidermal levels of the pro-inflammatory cytokine IFN-ɣ, as well as IL-4 and IL-10. House dust mite exposure led to a significant decrease in NMF and an increase of IFN-ɣ, IL-2, IL-4 and IL-10. Occluded exposure to SEB led to a significant increase in IL-1α, but no decrease in NMF. Collectively, these exposures represent a selection of daily-life skin stressors that negatively affect the skin barrier and ultimately may lead to dermatitis.

Interpretation

Our findings on water corroborate with observations from recent epidemiological and experimental studies. Thus, water indeed downregulated *FLG* expression by 50% after 24 hour occluded exposure.[19] So-called hard water appears to have the most negative effect on the skin barrier, which in turn may increase the risk of AD. [20] Accordingly, studies from the UK, Spain and Denmark identified a higher prevalence of AD in children residing in regions with hard compared to soft domestic water.[21-24]A high content of calcium carbonate is normally associated with elevated pH, which may result in premature activation of serine proteases that in turn degrade corneodesmosomes.[25] Also, pH levels regulate lipid lamellae biosynthesis and desquamation.[26] However, in our study, the soft water surprisingly had a higher pH than the hard water (7.8 vs 7.2). These factors could have obscured stronger differences in skin reactivity between soft and hard water. Nonetheless, hard water led to an increase in IL-4, a cytokine which is often up-regulated in AD skin.[27] This finding suggests that hard water could have a distinct influence on the immune system. However, a recent study showed that accumulation of skin irritating SLS residues following a washing regime is associated with use of hard water.[28] Chlorinated water also had a negative effect on the skin barrier, and indirectly supports findings from a recent study, which showed that a washing regime with chlorinated water induced skin irritation.[28] Moreover, children with a high cumulative swimming pool attendance might have increased occurrence of AD.[29]

The breakdown of filaggrin is controlled by the water gradient within the SC.[30] The decreased levels of NMF after prolonged water exposure could, therefore, be explained by reduced degradation of filaggrin. Another explanation could be a 'wash-out' effect, where water extracted NMF from the SC.[31] Next to NMF reduction, we observed an increase in TEWL, reflecting reduced skin barrier function, and possibly alterations in lipid organization responsible for diffusion of water across the SC. Collectively, the increased flux of water from the skin and reduced NMF levels are consistent with a decrease in skin hydration at 24 hours after exposure to the different water types.

House dust mite was the only other exposure that led to a significant decrease in NMF levels after 24 hours. The role of house dust mite in AD pathophysiology is debated.[32] Application of house dust mite on non-lesional AD skin produces eczema, also in patients with negative skin prick test and without specific IgE against house dust mite.[33,34] Moreover, healthy volunteers with and without specific IgE against house dust mite experience inflammatory skin reactions, although less pronounced and frequent than AD patients.[35] Der p3 and Der p9, two of the major allergens in house dust mite, have proteolytic activity, and can induce a non-allergic inflammatory response in human pulmonary epithelial cells.[36] Furthermore, application of house dust mite delays the recovery rate after

experimentally induced skin barrier disruption.[37] In the current study, exposure to house dust mite led to a significant increase in pro-inflammatory cytokines at 24 hours (IFN-ɣ and IL-2) as well as increased TEWL. At 48 hours there was a significant increase in NMF levels compared to the control site, possibly due to a compensatory breakdown in filaggrin and hence increased levels of NMF. Interestingly, a recent study showed increased expression of Th2, Th9 Th17/Th22 polar cytokines in tissue with eczematous reaction after exposure to house dust mite compared with unexposed skin [38].

SEB, a superantigen that may bypass the normal and controlled activation of the immune system, is produced by strains of *Staphylococcus aureus* isolated from atopic skin.[39-41] Experimental application of SEB provokes eczema in both normal and atopic skin.[42] In our study, exposure to SEB did not reduce NMF levels compared to the control site at 24 hours, however, a significant decrease in skin barrier function was found as indicated by increased TEWL. We observed a significant increase in IL-1α after 24 hours. At 48 hours, the levels of NMF were significantly increased compared to the control site, possibly caused by a compensatory breakdown of filaggrin.

A reduction of ambient humidity and temperature has a negative effect on the skin barrier.[43] However, in the current study, 10 min reduction of skin temperature to 10°C did not influence epidermal NMF levels at 24 or 48 hours. The same applied for as intradermal exposure to histamine. The exposures where only performed once and the short time and limited degree of exposure likely explain these results.

Strengths and limitations

All measurements and sample collections were performed by one person minimizing the risk of interpersonal variation and all exposures were randomized. The difference in water hardness should ideally have been larger in the current study (449 mg $CaCO_3$/L vs. 188 mg $CaCO_3$/L), and our soft water was still relatively hard according to national definitions, as it can be as low as 118 mg $CaCO_3$/L.[24] This could have obscured stronger differences in skin reactivity between soft and hard water. All exposures, except histamine and cooling, were performed under occlusion, which does not mimic real-life exposure. Occlusion can affect the skin barrier in a complex way, thus possibly influencing the results in the current study.[44] Since house dust mite was prepared in petrolatum, the observed increase in skin hydration at 24 hours could, at least in theory, be explained by the effect of petrolatum.[45] Due to limited space, exposure to petrolatum alone was not performed. No clinical scoring of post-exposure reactions was done.

Conclusion

Based on experimental exposure to selected daily-life skin stressors such as different water types, allergens and SEB, we conclude that NMF levels are decreased along with secretion of various skin cytokines in healthy individuals. Our data highlight environmental factors that might play a role in AD pathophysiology and serve as a basis for a future similar study in patients with AD.

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Figures

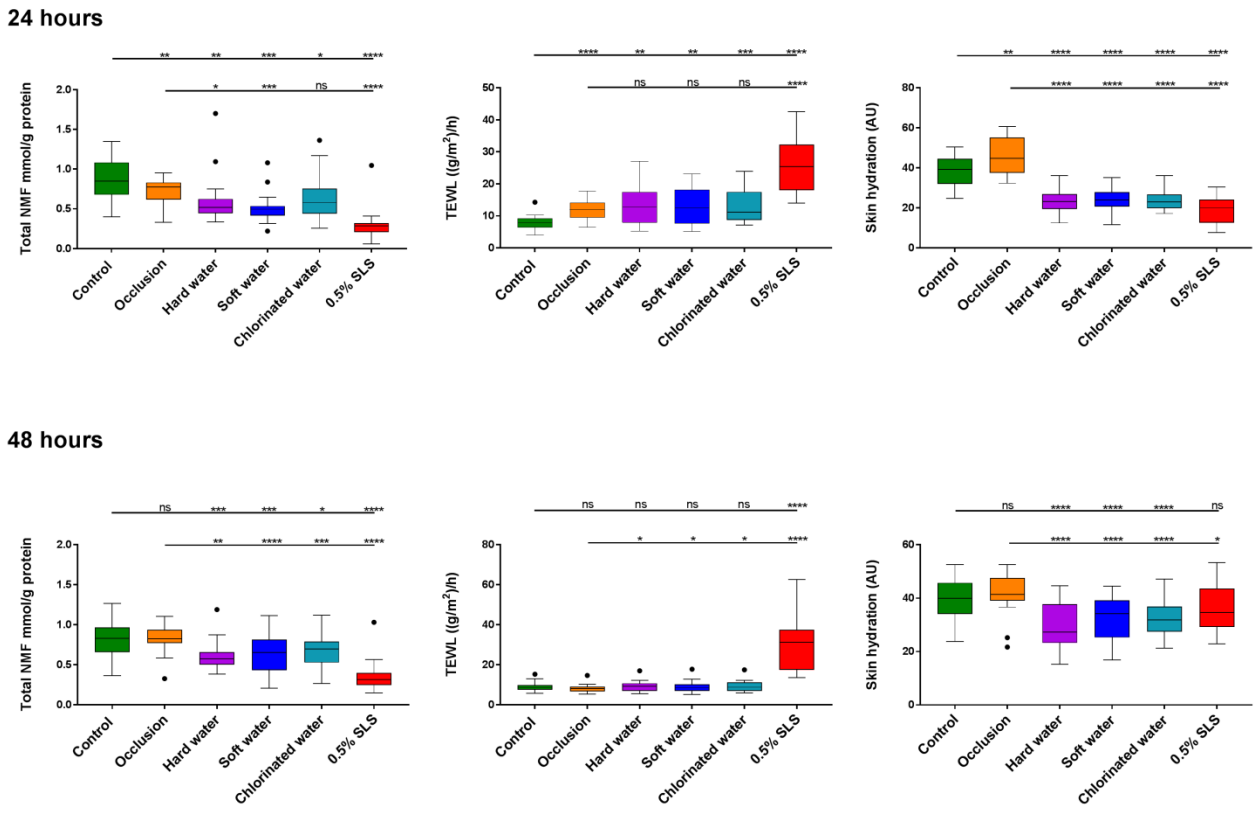

Figure 1: **Total levels of natural moisturizing factors (NMF) (mmol/g protein), TEWL ((g/m²)/h) and skin hydration (AU) at 24 hours and 48 hours in part 1 of the study.** Data are presented as boxplots with Tukey-style whiskers. The statistical difference between respectively the control site values and the occlusion values versus the other exposures were tested. Paired samples t-test was used in case of normal distribution and Wilcoxon signed-rank test in case of deviation from normal distribution. Statistical significance was set at P<0.05, ns = non-significant, * P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. AU, arbitrary units; NMF, natural moisturizing factors; SLS, sodium lauryl sulfate; TEWL, transepidermal water loss

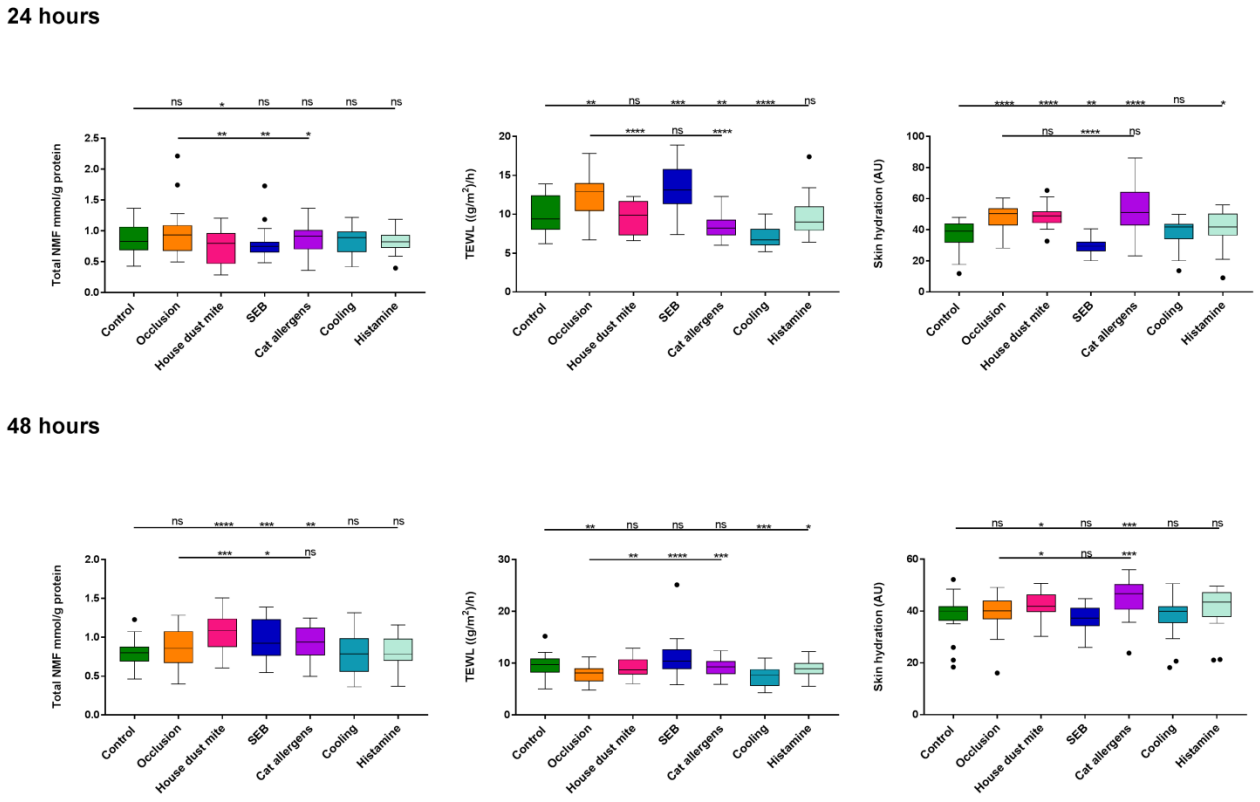

Figure 2: Total levels of natural moisturizing factors (NMF) (mmol/g protein), TEWL ((g/m²)/h) and skin hydration (AU) at 24 hours and 48 hours in part 2 of the study. Data are presented as boxplots with Tukey-style whiskers. The statistical difference between respectively the control site values and the occlusion values versus the other exposures were tested. Paired samples t-test was used in case of normal distribution and Wilcoxon signed-rank test in case of deviation from normal distribution. Statistical significance was set at P<0.05, ns = non-significant, * P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. AU, arbitrary units; NMF, natural moisturizing factors; SEB, Staphylococcal enterotoxin B; TEWL, transepidermal water loss

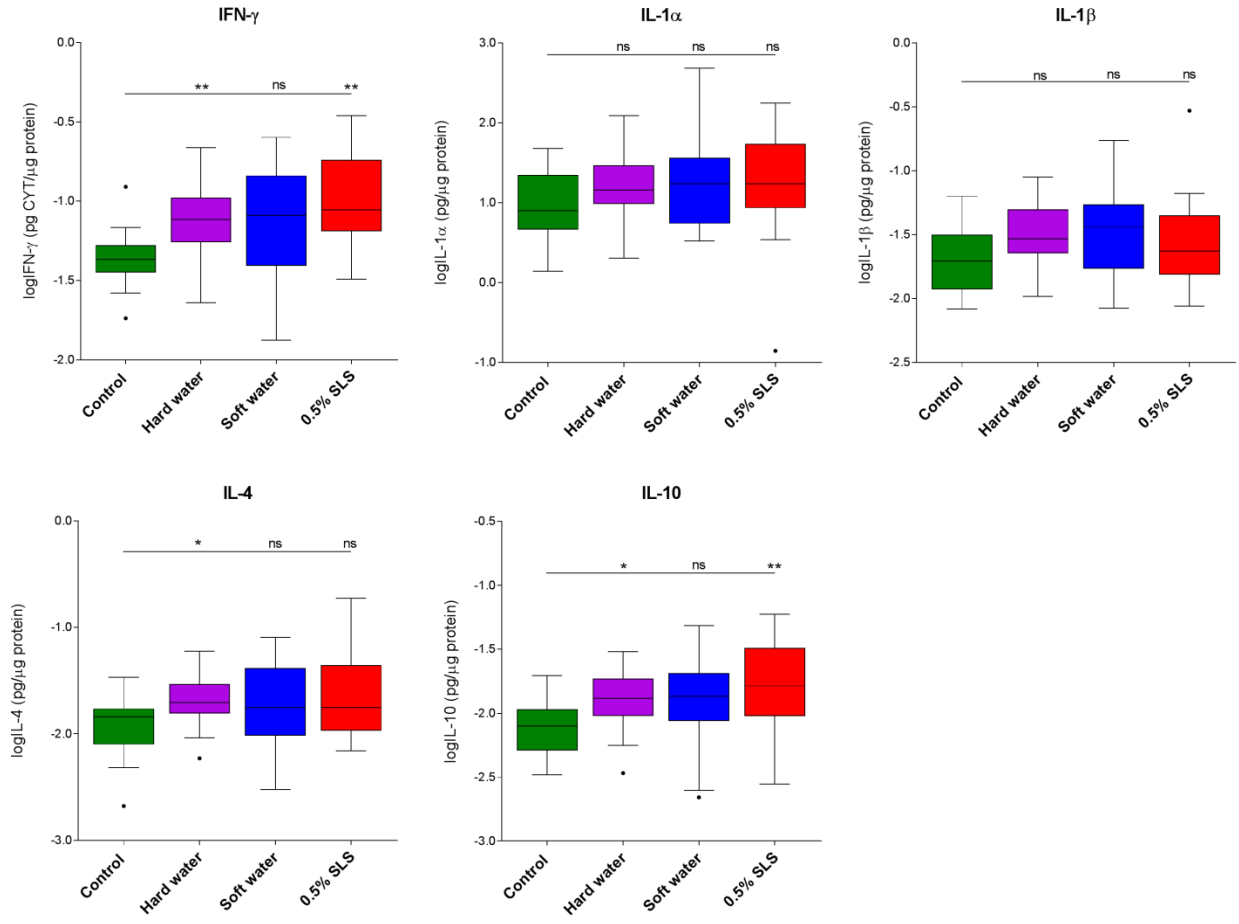

Figure 3: Log transformed cytokine levels (pg/µg protein) at 24 hours measured at the control site and after exposure to hard water, soft water and 0.5% SLS. Data are presented as boxplots with Tukey-style whiskers. The statistical difference in cytokine levels at the control site and after exposure to the different water types was tested with the paired samples t-test in case of normality and the Wilcoxon signed-rank test in case of deviation from normality. The statistical significance level was set at P<0.01, ns = non-significant, * P<0.01, ** P<0.001. SLS, sodium lauryl sulfate

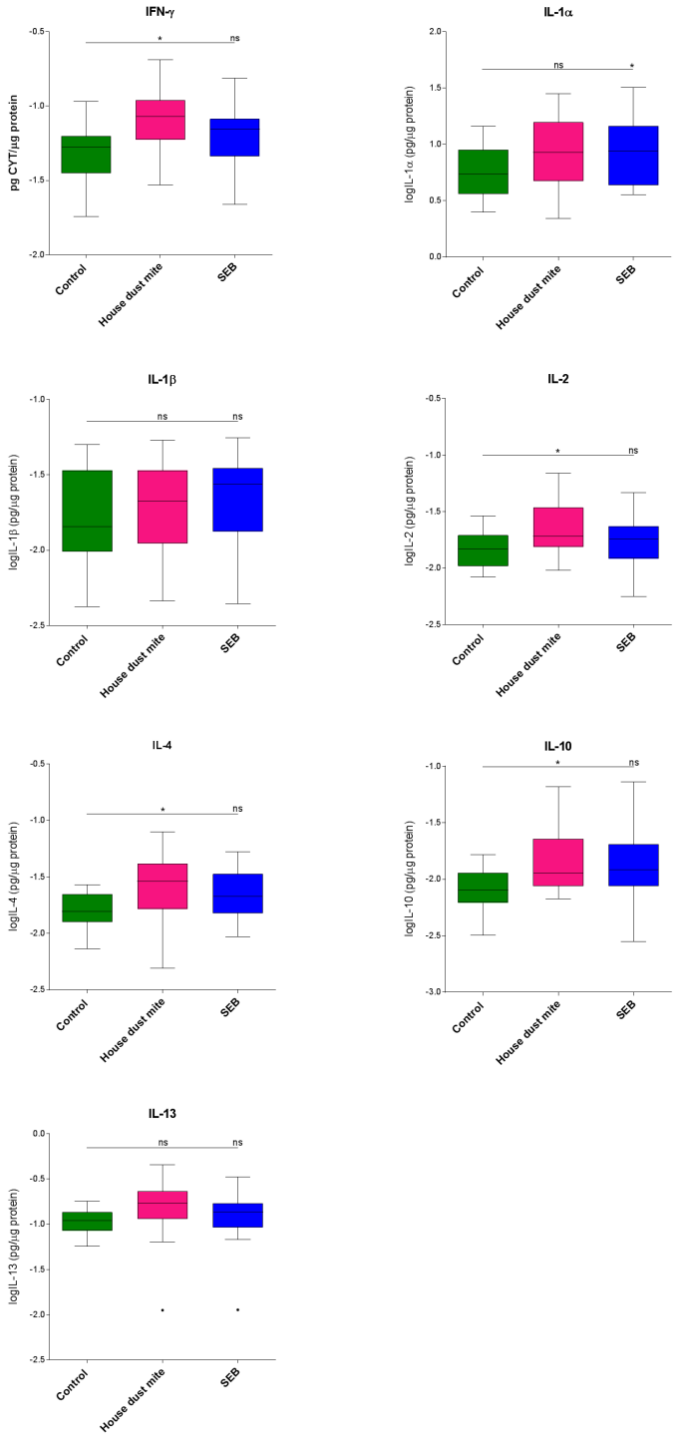

Figure 4: Log transformed cytokine levels (pg/µg protein) at 24 hours measured at the control site and after exposure to house dust mite and SEB. Data are presented as boxplots with Tukey-style whiskers. The statistical difference between the cytokine levels at the control site and after exposure to house dust mite and SEB was tested with the paired samples t-test in case of normality and the Wilcoxon signed-rank test in case of deviation from normality. The statistical significance level was set at P<0.01, ns = non-significant, * P<0.01. SEB, Staphylococcal enterotoxin B

Supplementary Materials

Determination of filaggrin degradation products (NMF) in the stratum corneum (SC)

The fourth consecutive tape was used for the analysis. 500 µL 25% (w/w) ammonia solution was added to each vial to extract the NMF components histidine (His), 2-pyrrolidone-5-carboxylic acid (PCA) and urocanic acid (UCA) (trans- and cis isomer). After 2 hours of shaking (IKA-Vibrax Model 2200, IKA-works Inc, Wilmington, NC, USA), the extracts were transferred to a new vial and evaporated to dryness at 60°C (Eppendorf Concentrator 5301, Eppendorf AG, Hamburg, Germany). The residues were dissolved in 500µl of Millipore water and an aliquot of the sample was analyzed by HPLC. To adjust for the various amount of SC protein on each tape strip, the proteins were first extracted by ammonia and then by 0.1M KOH. The Pierce Micro BCA protein assay kit (Thermo Fischer Scientific, Rockford, IL, USA) was used to determine the protein levels. Finally, they protein measurements were added together, and the levels of NMF expressed as mmol/g protein.

Cytokine analyses

To extract the cytokines from the tape strips, 600µl of phosphate buffered saline containing 0.005% Tween 20 was added to each vial. After 15 minutes on ice, the samples were sonicated for 15 minutes and briefly vortexed before the extracts were distributed in vials and stored at -80°C. The cytokine analyses were performed using the MESO QuikPlex SQ 120 assay (MSD, Rockville, Md., USA). The assays used human antibodies, and an aliquot of 50µl from each tape extraction was incubated overnight together with a calibrator (provided by MSD) in a sealed plate at 2-8°C. The plates were washed with phosphate buffered saline with Tween 20, detection buffer was added, and the plates were read on the MSD Sector Imager 2400 plate reader. The cytokine concentration was corrected by the total protein content which was determined by the Pierce Micro BCA assay kit (Thermo Fischer Scientific, Rockford, IL, USA).[1]

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Supplementary tables

Table S1. Description of exposures used in the study

Type of exposure	Origin of exposure	Mode of exposure	Study part
Hard water - Ca^{2+} 140 mg/L - Mg^{2+} 24 mg/L - Cl^- 63 mg/L - pH 7.2 Analysis performed by GEUS (Geological survey of Denmark and Greenland) in February 2015.	Katrinebjerg water work, - Located in Taastrup, in the eastern part of Zealand, Denmark.	Extra large Finn Chambers® (18-mm diameter, SmartPractice, Phoenix, AZ, USA) - Filter discs were soaked in 200µl of hard water, placed in the Finn Chamber and attached to the skin by the use of tape - Removed after 24 hours of exposure	1
Soft water - Ca^{2+} 67 mg/L - Mg^{2+} 5 mg/L - Cl^- 36 mg/L - pH 7.8 Analysis performed by GEUS (Geological survey of Denmark and Greenland) in October 2014.	Asserbo water work - Located in the northern part of Zealand, Denmark.	Extra large Finn Chambers® (18-mm diameter, SmartPractice, Phoenix, AZ, USA) - Filter discs were soaked in 200µl of soft water, placed in the Finn Chamber and attached to the skin by the use of tape - Removed after 24 hours of exposure	1
Chlorinated water - Free chlorine: 1.37 mg/L - Combined chlorine: 0.33 mg/L - Total chlorine: 1.70 mg/L - pH 7.4.	Kildeskovshallen, public swimming pool - Located close to Herlev and Gentofte Hospital, Hellerup, Denmark - The water was collected from the closed system and not directly from the swimming pool	Extra large Finn Chambers® (18-mm diameter, SmartPractice, Phoenix, AZ, USA) - Filter discs were soaked in 200µl of chlorinated water, placed in the Finn Chamber and attached to the skin by the use of tape - Removed after 24 hours of exposure	1
Detergent - 0.5% SLS (Sodium lauryl sulfate) in distilled water	Sigma-Aldrich, Steinheim, Germany (≥99.0% purity)	Extra large Finn Chambers® (18-mm diameter, SmartPractice, Phoenix, AZ, USA) - Filter discs were soaked in 200µl of 0.5% SLS solution, placed in the Finn Chamber and attached to the skin by the use of tape - Removed after 24 hours of exposure	1
House dust mite - Dermatophagoides mix (Pteronyssinus/Pharinae 50/50) - Concentration (%w/w): 30% pet - 40 mg/cm^2	Chemotechnique, Vellinge, Sweden	Extra large Finn Chambers® (18-mm diameter, SmartPractice, Phoenix, AZ, USA) - 100 mg of house dust mite in petrolatum were placed in on the Finn Chamber and attached to the skin by the use of tape - Removed after 24 hours exposure	2
Bacteria toxin - Staphylococcal enterotoxin B (SEB) from *S. aureus* - SEB was diluted in Phosphate-buffered saline - 10µg/cm^2	Sigma-Aldrich, Steinheim, Germany	Extra large Finn Chambers® (18-mm diameter, SmartPractice, Phoenix, AZ, USA) - Filter discs were soaked in 180µl of 0.139 µg/µl SEB, placed in the Finn Chamber and attached to the skin by the use of tape - Removed after 24 hours of exposure	2
Cat allergen - ALK-555, cat allergen used for skin prick test - Contains a standardized Soluprick® SQ solution with 10 HEP (Histamine Equivalence in skin Prick testing).	ALK-Abello A/S, Hørsholm, Denmark	Extra large Finn Chambers® (18-mm diameter, SmartPractice, Phoenix, AZ, USA) - Filter discs were soaked in 200µl of cat allergen, placed in the Finn Chamber and attached to the skin by the use of tape - Removed after 24 hours of exposure	2
Histamine - ALK Soluprick® Positive control - Histamine 10 mg/ml	ALK-Abello A/S, Hørsholm, Denmark	Three drops of histamine solution were placed on the skin and a lancet was used to prick through the drops. After 1 minute the histamine drops were wiped off with a paper towel.	2
Cooling of the skin - TSA-II NeuroSensory Analyzer was used to cool the skin down	Medoc Advanced Medical Systems, Durham, NC, USA	A thermode (30x30 mm) was attached to the skin and 10 cycles of 60 s cooling (10°C) and 30 s break was performed.	2

Table S2. Total levels of natural moisturizing factors (NMF) (mmol/g protein), TEWL and skin hydration at 24 hours and 48 hours in part 1 of the study.

Measurement Mean (± SD)		Control	Occlusion	Hard water	Soft water	Chlorinated water	0.5% SLS
NMF (mmol/g protein)							
	24 hours	0.87 (± 0.28)	0.71 (± 0.18)	0.61 (± 0.32)	0.51 (± 0.19)	0.64 (± 0.28)	0.31 (± 0.20)
	48 hours	0.80 (± 0.21)	0.82 (± 0.17	0.61 (± 0.19)	0.64 (± 0.22)	0.69 (± 0.21)	0.36 (± 0.20)
TEWL ((g/m^2)/h)							
	24 hours	8.2 (± 2.3)	11.8 (± 3.3)	13.1 (± 6.1)	13.0 (± 6.2)	13.3 (± 5.4)	25.9 (± 8.3)
	48 hours	8.9 (± 2.3)	8.2 (± 2.2)	9.2 (± 2.8)	9.1 (± 3.0)	9.3 (± 2.9)	32.0 (± 14.1)
Skin hydration (AU)							
	24 hours	39.0 (± 7.4)	46.3 (± 9.5)	23.2 (± 5.7)	23.8 (± 6.0)	24.1 (± 5.4)	19.2 (± 6.4)
	48 hours	39.1 (± 7.9)	41.6 (± 8.1)	30.5 (± 8.5)	32.3 (± 8.3)	32.7 (± 7.5)	36.3 (± 9.5)

AU, arbitrary units; NMF, natural moisturizing factors; TEWL, transepidermal water loss; SD, standard deviation; SLS, sodium lauryl sulfate

Table S3. Total levels of natural moisturizing factors (NMF) (mmol/g protein), TEWL and skin hydration at 24 hours and 48 hours in part 2 of the study.

Measurement Mean (± SD)		Control	Occlusion	House dust mite	SEB	Cat allergen	Cooling	Histamine
NMF (mmol/g protein)								
	24 hours	0.87 (± 0.26)	1.00 (± 0.42)	0.77 (± 0.28)	0.80 (± 0.28)	0.89 (± 0.24)	0.86 (± 0.21)	0.83 (± 0.20)
	48 hours	0.80 (± 0.20)	0.85 (± 0.25)	1.06 (± 0.23)	0.97 (± 0.28)	0.92 (± 0.22)	0.81 (± 0.27)	0.80 (± 0.24)
TEWL ((g/m^2)/h)								
	24 hours	10.1 (± 2.4)	12.2 (± 2.7)	9.7 (± 2.1)	13.2 (± 3.0)	8.4 (± 1.7)	7.0 (± 1.5)	9.6 (± 2.6)
	48 hours	9.6 (± 2.2)	7.8 (± 1.8)	9.0 (± 1.9)	11.3 (± 4.2)	9.2 (± 1.9)	7.4 (± 2.0)	8.9 (± 1.8)
Skin hydration (AU)								
	24 hours	37.5 (± 9.5)	48.7 (± 8.3)	49.1 (± 7.4)	29.6 (± 4.9)	54.4 (± 16.3)	38.6 (± 9.3)	41.3 (± 11.7)
	48 hours	38.2 (± 8.4)	39.1 (± 7.1)	42.2 (± 5.4)	37.3 (± 5.0)	45.0 (± 7.4)	38.3 (± 8.6)	40.7 (± 8.0)

AU, arbitrary units; NMF, natural moisturizing factors; TEWL, transepidermal water loss; SD, standard deviation; SEB, Staphylococcal enterotoxin B

Table S4. Log transformed cytokine levels (pg/µg protein) at 24 hours, measured at the control site and after exposure to hard water, soft water and 0.5% SLS

Cytokine mean (± SD)	Control log (pg/µg protein)	Hard water log (pg/µg protein)	Control vs hard water	Soft water log (pg/µg protein)	Control vs soft water	0.5% SLS log (pg/µg protein)	Control vs 0.5% SLS
IFN-γ	-1.362 (0.176)	-1.116 (0.220)	<0.001	-1.139 (0.359)	ns	-0.997 (0.289)	<0.001
IL-1α	0.986 (0.410)	1.227 (0.429)	ns	1.274 (0.561)	ns	1.234 (0.721)	ns
IL-1β	-1.688 (0.272)	-1.525 (0.264)	ns	-1.490 (0.356)	ns	-1.561 (0.371)	ns
IL-4	-1.947 (0.286)	-1.691 (0.248)	<0.01	-1.734 (0.426)	ns	-1.657 (0.376)	ns
IL-10	-2.125 (0.218)	-1.895 (0.235)	<0.01	-1.918 (0.358)	ns	-1.792 (0.339)	<0.001

The statistical difference in log transformed cytokine levels at the control site and after exposure to hard water, soft water and 0.5% SLS was tested by paired sample t-test in case of normality and the Wilcoxon signed-rank test in case of deviation from normality. The statistical significance level was set at $P<0.01$. SLS, sodium lauryl sulfate

Table S5. Log transformed cytokine levels (pg/µg protein) at 24 hours, measured at the control site and after exposure to house dust mite and SEB

Cytokine	Control	House dust mite	Control vs house dust mite	SEB	Control vs SEB
mean (± SD)	log (pg/µg protein)	log (pg/µg protein)	P-value	log (pg/µg protein)	P-value
IFN-γ	-1.323 (± 0.207)	-1.100 (± 0.211)	<0.01	-1.189 (± 0.208)	ns
IL-1α	0.773 (± 0.237)	0.915 (± 0.310)	ns	0.934 (± 0.294)	<0.01
IL-1β	-1.786 (± 0.323)	-1.751 (± 0.306)	ns	-1.670 (± 0.302)	ns
IL-2	-1.835 (± 0.154)	-1.661 (± 0.225)	<0.01	-1.755 (± 0.219)	ns
IL-4	-1.808 (± 0.154)	-1.578 (± 0.276)	<0.01	-1.660 (± 0.219)	ns
IL-10	-2.094 (± 0.206)	-1.857 (± 0.256)	<0.01	-1.863 (± 0.316)	ns
IL-13	-0.976 (± 0.129)	-0.843 (± 0.346)	ns	-0.906 (± 0.312	ns

The statistical difference in log transformed cytokine levels at the control site and after exposure to house dust mite and SEB was tested by paired sample t-test in case of normality and the Wilcoxon signed-rank test in case of deviation from normality. The statistical significance level was set at P<0.01. SEB, Staphylococcal enterotoxin

Table S6. Number of cytokine values under range of detection or under fit curve after selected exposures for 24 hours

Cytokine	Part 1				Part 2		
	Hard water	Soft water	0.5% SLS	% of total	House dust mite	SEB	% of total
Total (n)	**19**	**18**	**19**	**56**	**20**	**20**	**40**
IFN-γ	1	0	0	2%	1	0	3%
TNF-α	9	14	10	**59%**	9	12	**53%**
IL-1α	0	0	1	2%	0	0	0%
IL-1β	0	0	0	0%	8	5	33%
IL-2	15	14	16	**80%**	0	0	0%
IL-4	3	5	10	32%	1	0	3%
IL-10	1	3	2	11%	10	7	43%
IL-13	17	19	19	**98%**	5	6	28%
IL-18	15	18	17	**89%**	19	19	**95%**

Marked in bold are the cytokines excluded from further analyses. SEB, Staphylococcus enterotoxin B; SLS, Sodium lauryl sulfate

3. Considerations and comments on methodology

In the following section the strengths and weaknesses of the methods used in Manuscript I, II and II will be discussed and critically reviewed.

3.1 General considerations

3.1.1 Filaggrin genotyping

In all three studies, the participants were genotyped for three of the most prevalent *FLG* loss-of-function mutations in the Northern European population (R501X, 2282del4 and R2447X). Since the discovery of *FLG* mutations in 2006, more than 60 different mutations have been identified, whereof many are region or race specific [16]. The three mutations tested for account for 83% of known *FLG* mutations in the Northern European population [13], and it is therefore a possibility that we were not able to correctly identify all mutation carriers. In Manuscript I and III, a small number of participants was of Middle-Eastern and Pakistani decent (4 participants in Manuscript I and 2 participants in Manuscript III), and thus not tested for the most relevant *FLG* mutations corresponding to non-European populations [112]. In Manuscript III, dry skin was an exclusion criterion, limiting the risk of misclassification with regards to *FLG* mutations. Furthermore, in Manuscript I and III, we investigated the difference in skin barrier measurements within in each participant (winter vs. summer and unexposed vs. exposed), and we do not believe that a misclassification of the non-European participants with regards to *FLG* mutation status would have a substantial effect on our results.

3.1.2 Biophysiological measurements

In Manuscript II and III, TEWL (g/m^2)/h) was measured by using to different protocols. In Manuscript II, the principal investigator (J.B.) measured TEWL for 30 seconds, and the average of the last 10 seconds of steady state was calculated and used in the statistical analyses. In Manuscript III, TEWL was measured until steady state was reached and the average of three consecutive measurements was used in the statistical analyses. There is a possibility that this may have resulted in different TEWL values, but as the same investigator used the same method within each manuscript, we believe that the potential difference is minor.

3.1.3 Interpretation of data on NMF

In Manuscript I, II and III, we measured the level of histidine (His), 2-pyrrolidone-5-carboxylic acid (PCA) and UCA (trans- and cis-isomer) in tape strips. They are all degradation products of filaggrin and represent the main NMF components in the SC [113]. At the time we performed the studies, it was not possible to measure profilaggrin expression or filaggrin proteins by the use of tape strips. This information would have required invasive skin biopsies, as performed in Manuscript II where epidermal filaggrin levels were measured. The lack of data on profilaggrin and filaggrin expression is a limitation with regards to interpretation of the NMF results in Manuscript I and III, as increased levels of NMF could either be due to increased expression of profilaggrin and filaggrin in general, or due to degradation of filaggrin into NMF as a response to inflammation or external factors (climatic factors, irritants, allergens and bacteria toxins).

3.2 Manuscript I

Manuscript I is based on an experimental study where 80 participants were tape stripped on the cheek and dorsal aspect of the hand during the winter and summer. The aim was to evaluate the effect of season on filaggrin degradation products and corneocyte surface texture in healthy adult skin.

The main strength of the study was the high number of participants and the paired design with a high participation rate in the follow-up sampling (90%). Due to the knowledge that filaggrin degradation might be affected by external factors [87, 107, 108, 114], we asked the participants whether they had used cream or taken a shower/bath on the day of the sampling. In the winter, we additionally recorded the lowest temperature at the day of sampling, and in the summer, we asked about recent UV-exposure and use of sunscreen (within the last week).

Several of the potential confounders were self-reported, possibly influencing the accuracy of the adjusted analyses. In the statistical analyses, we did not distinguish between different types of cream (lipid content, perfumed or non-perfumed products) or the time between application of cream and tape stripping. Furthermore, we did not ask about the number of hand washes or use of disinfectants, which possibly could also influence the NMF levels and corneocyte surface texture on the dorsal aspect of the hand [107]. Frequent hand washing may lead to skin irritation, and it has been shown to denaturate skin proteins and reduce skin lipids and skin hydration [115]. Moreover, a recent study

concluded that frequent hand washing was more prevalent among women [116], possibly introducing a bias with regards to behavioral differences between men and women in the study. In line with gender differences, we recorded the use of make-up among our female participants. The make-up data were, however, not included in the final manuscript, as we did not differentiate what type of make-up the participants had used. In the winter there was no difference in NMF or DTI according to use of make-up (P=0.145 and P=0.988, respectively). In the summer, female participants who had used make-up had significantly lower NMF levels compared to those who had not used make-up (P<0.0001), while no significant difference was observed for DTI (P=0.382). The same reduction in NMF levels in cheek skin during the summer was observed for participants who had applied cream to the face on the day of the sampling. Since many make-up products are cream based, it is possible that they contribute to skin hydration and thus reduce the need for filaggrin degradation. However, no sex-related differences were found for NMF levels in cheek skin during the winter or summer, so we do not believe that use of make-up had any substantial influence on the overall results.

We observed that a high self-reported UV-exposure led to a significant increase in DTI in cheek skin. Since we did not have a non-exposed control spot, we cannot rule out that the observed results were due to a systemic effect of UV-irradiation. However, if the effect of UV-exposure was systemic, we would have also expected an effect on DTI in hand skin and not only in cheek skin.

With regards to the analytical methods used in manuscript I, the DTI was determined by the use of AFM and the nAnostic™-method applying custom-built, proprietary algorithms (Serend-ip, GmbH, Münster, Germany) [110]. Since the method is rather new, limited data on healthy volunteers is available. A future study addressing the natural variance among healthy individuals could thus be of interest. It could also be relevant to analyze several tape strips from the same individual to assess intra-individual variability. Furthermore, the exact cause of these nanoscale alterations on corneocytes is not clear, and we cannot rule out that there are different etiologies behind the nanostructures that are used to calculate the DTI.

3.3 Manuscript II

In Manuscript II, we investigated the level of filaggrin protein, NMF and DTI in dermatitis patients and healthy controls. This is the first study to investigate all three components in the same study population, and to date it is the largest study on DTI.

Most of the data used in Manuscript II was collected in conjunction with a previous study performed by Bandier et al. [106], where the epidermal level of filaggrin before and after exposure to SLS in dermatitis patient and healthy controls was investigated. The tape strips used to measure NMF and DTI in Manuscript II were collected in 2011 and stored until analysis in 2016. This might have affected the total levels of NMF and DTI. However, since the samples were stored under the same conditions, we expect that this bias would be systematic and not affect the relative levels of NMF and DTI. Furthermore, the tapes strips used to determine DTI and NMF were taken from the volar aspect of the forearm, while the TEWL measurements and biopsy for epidermal filaggrin quantification were taken from the inner surface of the upper arm. This might have led to a bias regarding anatomical variation.

Another possible limitation of the study is the heterogenic population of dermatitis patients. Recruitment was done by revision of medical charts at the Department of Dermatology and Allergy at Herlev and Gentofte Hospital. The patients were eligible for inclusion if the medical doctor treating them had found it relevant to test the patient for *FLG* mutations. The dermatitis patients were therefore a mixture of patients with different forms of dermatitis such as AD, allergic contact dermatitis, irritant contact dermatitis and other forms of unclassified dermatitis.

A history AD was diagnosed by the U.K. Working Party's Diagnostic Criteria [117]. In a systematic review, the U.K. criteria have been shown to have a sensitivity and specificity ranging from 10-100% and 77.6-93.8%, respectively [118]. The validation studies have mainly been performed in children, and a recent publication has highlighted the challenges when applying these criteria on an adult patient population [119]. This might have affected the accuracy of the AD diagnosis in the current study population. Unfortunately, we have no information regarding the presence of AD or other forms of dermatitis at the time of the measurements and sampling, as no clinical scoring was performed. This information would have been valuable as it would allow us to stratify the patient population into "present dermatitis" and "history of dermatitis" and investigate the effect of present inflammation on DTI and levels of epidermal filaggrin protein and NMF.

The participants were instructed not to use emollients on the day of the measurements and sampling, but we had no information on recent sun exposure or bathing habits. However, as shown in Manuscript I, a high self-reported UV-exposure was not shown to affect NMF or DTI on the dorsal aspect of the hand during the summer. Since the samples were collected during the winter (October to March) and from the volar forearm and inner surface of the upper arm, we do not believe that UV-exposure might have influenced the results. Finally, shower or bath the same day of the sampling did not influence NMF or DTI in Manuscript I.

3.4 Manuscript III

In Manuscript III, we exposed 40 healthy volunteers to several daily-life skin stressors and measured skin barrier function and NMF after 24 and 48 hours. Furthermore, the level of various skin cytokines was measured after 24 hours for selected exposures (soft water, hard water, 0.5% SLS, house dust mite and SEB). All exposures were compared to a non-occluded control spot measured at the same time point (24 or 48 hours). Except from cooling of the skin and histamine prick, all exposures were performed using Large Finn Chambers® measuring 18 mm in diameter to best match the surface area of the tape strips used for measuring NMF and skin cytokines (22 mm in diameter). The volume (200µl) used to soak the filter discs with the different types of water, SLS and cat allergen was chosen according to a previous publication [105]. For SEB, we chose to use 180 µl to avoid spreading of the bacteria toxin to adjacent skin.

Due to the number of exposures, we divided the study into two parts. In both parts the exposures were randomized to minimize the risk of bias with regards to anatomical variation. An experimental study has shown that baseline TEWL values did not differ significantly between 3 test areas on the volar forearm (proximal, mid and distal), but after exposure to 1% SLS for 24 hours, TEWL values were significantly higher at the proximal test area [120]. In part 1, the right and left volar forearm were exposed to the same exposures in the same randomized order, while the control measurements after 24 and 48 hours were performed on the mid area from both arms (Fig. 3).

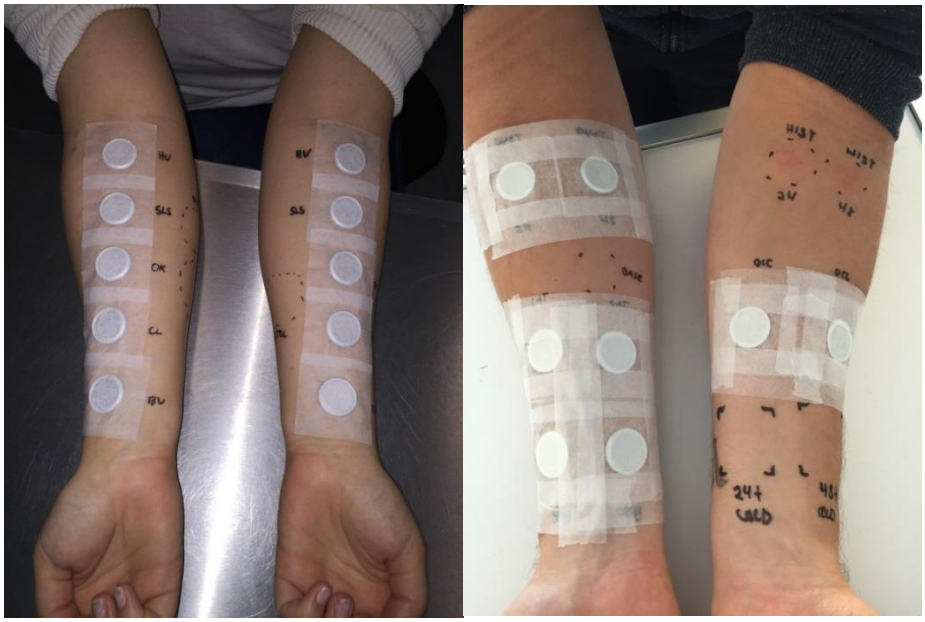

Figure 3: Exposures in part 1 (left) and 2 (right) of Manuscript III.

In part 2, we also randomized the control spot. Furthermore, three exposures and a baseline area were fixed to be randomized on the right arm (house dust mite, cat allergen and SEB) and three exposures and a control spot were fixed to be randomized on the left arm (histamine prick, occlusion and cooling of the skin). Two identical exposures were performed next to each other (Fig. 3), allowing us to perform measurements and tape stripping after 24 and 48 hours. We did not randomize which of the two exposure sites that was used for measurements at 24 hours and 48 hours. Retrospectively, this would have been more ideal, as it is possible that the skin barrier and response to exposure differ between the medial and lateral part of the volar forearm.

Furthermore, it is also possible that the different exposures were placed too close and thus influenced each other. This would especially be the case for 0.5% SLS, house dust mite and SEB where we observed the largest inflammatory response.

With regards to the different exposures, there are also some limitations that need to be considered. In part 1 we exposed the participants to different types of water (soft water, hard water and chlorinated water) and 0.5% SLS. Ideally, the difference in water hardness should have been larger (188 mg $CaCO_3$/L vs. 449 mg $CaCO^3$/L) and the soft water was quite hard according to both national and international definitions [50, 121]. Usually, a high content of calcium carbonate is associated to a higher pH, but surprisingly the soft water had a higher pH (7.8) than the hard water (7.2) in the current study. Taken together these factors might have obscured a possible difference in skin reactivity between the two water types, and if we were to perform a similar study in the future we would make sure to use the softest and hardest water available in Denmark. Furthermore, this observation may also limit the generalization of the results.

In part 2, we observed that exposure to cat allergen resulted in a significant decrease in TEWL and a significant increase in skin hydration at 24 and 48 hours. Furthermore, a significant increase in NMF levels was observed at 48 hours. This suggests that cat allergen positively affects skin barrier function and epidermal NMF levels. However, since the skin prick test fluid contained 25% glycerol, known to have a beneficial effect on skin barrier function [122], the results could be misleading and should be interpreted with caution. Furthermore, house dust mite was prepared with petrolatum which is also known to have a positive effect on skin barrier function [123]. The observed increase in skin hydration at 24 hours after exposure to house dust mite could therefore, at least in theory, be explained by the effect of petrolatum, rather than exposure to house dust mite. Ideally, we would have included occlusion with petrolatum alone, but this was not possible due to limited space on the volar forearms.

Absence of allergy to house dust mite and cat was only assessed by questioning the participants and not confirmed by skin prick test or specific IgE measurements.

Exposure to the different water types, 0.5% SLS, house dust mite, SEB and cat allergen were all performed under occlusion. Occlusion is known to affect the skin barrier in a complex way [124]. Thus, we chose to compare these exposures with occlusion alone for NMF levels, TEWL and skin hydration, and the results are described in detail in Manuscript III. It might be discussed whether the increase in TEWL after exposure to soft, hard and chlorinated water at 24 hours was due to evaporation. However, there was a clear difference in skin hydration for occlusion alone and the different water types. Occlusion led to a significant increase in skin hydration, while the different water types led to a significant decrease. This supports our hypothesis, that the increased TEWL after water exposure was due to skin barrier impairment. Taken together, our results suggest that occlusion alone had had no major effect on NMF levels; however, an increase in TEWL and skin hydration was observed after 24 hours.

Another challenge we faced in Manuscript III was how to handle the cytokine values that were below the detection range or fell below the fit curve range. The use of tape strips to measure the cytokine levels in the SC is a non-invasive alternative to other available techniques such as punch biopsies, suction blister fluid and skin derived lymph [125]. However, the technique can only measure cytokines that have diffused into the SC, and hence represent most likely a sample of cytokines that can be found in the dermis and lower levels of the epidermis. Thus, it is unknown whether the concentrations and relative distribution truly reflect the cytokines in the epidermis. In the current study, the cytokine analyses were performed with the MESO QuikPlex SQ 120 assay (MSD, Rockville, Md., USA) using human antibodies. The Mesoscale Discovery (MSD) electrochemiluminescence detection system was developed to enable measurements of a broad range of cytokines in small sample volumes with a very high sensitivity [126, 127]. The method uses plates where the wells contain up to 10 carbon electrodes coated with different capture-antibodies. When the cytokine of interest is captured, a secondary cytokine specific ruthenium-conjugated antibody attaches to the cytokine. Electrochemical stimulation makes the ruthenium emit light at the surface of the electrode, and the intensity of the light is used to calculate the concentration of the specific cytokine [126]. Although the method has been extensively used on a range of biological samples, the use of multiplex assays on tape strips is a rather newly introduced method. In a recent study by Koppes et al., they measured a wide range of cytokines and other inflammatory mediators in tape strips from healthy volunteers and AD patients before and after therapy with an emollient [114]. Out of 38 inflammatory mediators, 24 could be quantitatively determined in the majority of samples.

In the current study, we followed the same method as Koppes et al., and excluded cytokines where more than 50% of the measured values were below detection range or fell below the fit curve range. Values below the detection range included in the analyses were left as they were to keep the ranking order. Due to the low concentration and values below detection range, we chose a more conservative approach and set the significance level at $P<0.01$. Of the 9 cytokines analyzed (IFN-ɣ, TNF-α, IL-1α, IL-1β, IL-2, IL-4, IL-10, IL-13 and IL-18), IL-18 was excluded from both parts, while TNF-α, IL-2 and IL-13 were excluded from part 1 and TNF-α were excluded from part 2. Although we had to exclude some of the cytokines due to low concentrations, we believe that our choice of method was the most optimal, as the multiplex assay gave us the opportunity to evaluate a range of cytokines in a large group of participants in a non-invasive manner.

4. Discussion

4.1 The effect of season on important skin barrier measures

In Manuscript I, we investigated the effect of season on filaggrin degradation products and corneocyte surface texture on the cheek and dorsal aspect of the hand. Previous studies have suggested that climatic factors such as low temperature, low humidity and deprivation of UVB irradiation may negatively affect the skin barrier function and ultimately lead to inflammation and dermatitis [45, 51, 128]. Due to clothing, cheek and hand skin are more frequently exposed to weather compared to other anatomical locations. Interestingly, AD often has its debut on the cheek, chin and forehead skin [129], and the dorsal aspect of the hands are often involved in both pediatric and adult AD patients [130].

We observed seasonal effects on both corneocyte surface texture (DTI) and the levels of filaggrin degradation products (NMF). In cheek skin, NMF levels were lower during the winter compared to the summer, while DTI was increased. In hand skin, NMF levels were higher during the winter, while DTI was not significantly different between the two seasons.

We cannot explain why we observed a different response in NMF levels for the cheek and hand skin. One possible explanation could be that cheek and hand skin are morphologically different. Cheek skin has smaller and less matured corneocytes compared to other body regions, suggesting a higher epidermal turnover and possibly a less sufficient proteolysis of filaggrin [131]. Furthermore, facial skin has a higher content of lipids compared to the dorsal aspect of the hand [44], which could decrease the need for filaggrin degradation to maintain a well hydrated SC. We did not account for other day-to-day factors such as use of gloves, hand wash and soap usage, which might also have affected the NMF levels [107].

The higher NMF levels on the hands during the winter could be explained by increased degradation of filaggrin due to low humidity [60]. A recent study on human skin cultured in dry (RH 30-50%) or humid (RH >90%) conditions for 10 days showed that low humidity led to increased *FLG* expression, decreased levels of filaggrin monomers and increased amount of filaggrin degradation products, supporting our assumption [132].

The number of corneocyte surface protrusions (DTI) was higher in the winter compared to the summer in cheek skin. The same tendency was observed for the hand, but the difference was not

statistically significant (P=0.154). The nature and exact cause of these protrusions is unknown. Previous studies have hypothesized that the alterations could be due to disturbances in the cytoskeleton or the cornified envelops, or that they are related to corneodesmosomes [111, 133-135]. Filaggrin plays an essential role in the intracellular architecture, as it aggregates keratin filaments and contributes to the development of the cornified envelope [7, 8]. Thus, it is plausible that filaggrin deficiency might be involved. A higher DTI has been found in both non-lesional and lesional AD skin and in individuals with *FLG* mutations compared to healthy controls [110, 111], suggesting an association to impaired skin barrier function. While we did not measure TEWL in the current study, a positive correlation between DTI and TEWL has previously been shown, supporting this assumption [111]. Furthermore, a high self-reported UV-exposure within the last week was associated to higher DTI, suggesting that UV-irradiation alters the corneocyte surface texture. Previous studies have shown that high doses of UV-irradiation may negatively affect the skin barrier function and decrease skin hydration [136-138], and the increased DTI found in the current study could possibly be explained by UV-induced xerosis and skin barrier impairment.

Our results demonstrate that, independent of AD and *FLG* mutations, climatic factors influence important skin barrier measures on both a biochemical and ultrastructural level. In a previous study, we have shown that Danish children born during the fall and winter have a higher prevalence of AD compared to children born during the spring and summer [50]. We hypothesized that the first months spent in a dry and cold climate could be a possible explanation for this observation. While the current study was performed on adult skin, we believe that the results might also apply to children. Filaggrin deficient skin is dry and more prone to inflammation and epidermal penetration of allergens [139-141] which may all contribute to the development of AD. In a clinical perspective, primary prevention in form of moisturizers could be an easy and feasible way to restore skin barrier function in children born during the fall and winter, and thus reduce the risk of AD. In a recent study on neonates with a high risk of developing AD, daily application of moisturizers reduced the relative risk of AD development by 50% within the first 6 months of life compared to the control group [142], supporting this assumption. Furthermore, neonates with a high TEWL on day 2 and 2 months after birth have been shown to have an increased risk of AD at 1 year of age, again stressing the importance of an intact skin barrier to reduce the risk of AD development [143].

Our findings are also relevant for other forms of dermatitis with impaired barrier function where climate has been shown to have an effect. Winter season, low temperatures and low ambient humidity have all been associated to an increased risk of irritant skin changes and dermatitis in both hairdresser apprentices and healthcare workers in previous studies [144, 145]. Furthermore, the prevalence of

hand dermatitis has been found to be higher in the northern parts of Norway compared to a similar survey in the southern parts of Sweden where the climate is milder [146]. Finally, the response to skin irritants such as SLS and sodium hydroxide (NaOH) is stronger during the winter [147, 148], and the number of positive patch test reactions is higher [149]. Use of barrier restoring emollients during the winter season would thus be an applicable preventive measure, also for other forms of dermatitis.

4.2 A history of dermatitis influence the epidermal level of filaggrin, its degradation products and corneocyte surface texture

In Manuscript II, we investigated the level of epidermal filaggrin protein, NMF and DTI in dermatitis patients and in healthy controls. For all measures, a significant difference was observed between the 47 dermatitis patients and 20 healthy controls, where the level of filaggrin protein and NMF was lower and DTI higher. The patient population was recruited from the Department of Dermatology and Allergy at Herlev and Gentofte Hospital by reviewing medical records. Exclusion criteria included widespread and active dermatitis and the use of systemic immunosuppressants, and accordingly none of the patients had severe dermatitis.

In the patient population, we stratified the participants according to history of AD, the presence of *FLG* mutations, history of asthma and hay fever and clinical features such as xerosis, keratosis pilaris, palmar hyperlinearity and history of fissures on the hands and feet. TEWL was measured to evaluate skin barrier function. No significant differences in DTI were found with regards to the different population characteristics within the patient population. We were a bit surprised by this finding, as previous studies have shown a clear increase of DTI in patients with AD and *FLG* mutations [110, 111]. The lack of difference could be due to the heterogeneity of the patient population, and that none of the patients had severe or active AD. Furthermore, in the study by Riethmüller et al., the median age of the AD patients was 8.25 years (range 2.3-57.3 years) while in our patient population it was 41 years (range 18-67 years). It is possible that this difference might have affected our results if corneocyte morphology and the inflammatory response are different in pediatric and adult AD skin. Recently, it was shown that early pediatric AD and adult AD share a common increased Th2 and Th22 response, however, in pediatric AD a significantly higher activation of Th17- and Th9-related cytokines was found [150].

As expected, the level of epidermal filaggrin protein and NMF was significantly lower for dermatitis patients with *FLG* mutations [20, 106]. Patients with a history of AD had significantly lower NMF levels compared to those without a history of AD, while no difference in filaggrin levels was found. The same applied for a history of asthma or hay fever. To further investigate the relationship between filaggrin and NMF, we performed extra correlation analyses, stratified by the presence of *FLG* mutations and AD. This showed a strong positive correlation between filaggrin and NMF, however only in patients with *FLG* mutations (Spearman's rho 0.667, P=0.002) or a history of AD (Spearman's rho 0.433, P=0.027). No significant correlation was found in patients without *FLG* mutations (P=0.820) or a history of AD (P=0.352). These data suggest that the relationship between epidermal filaggrin protein and NMF differs in healthy skin and skin with filaggrin deficiency due to genetic mutations or inflammation.

Taken together, our results suggest that mild dermatitis and even subclinical inflammation is sufficient to produce alterations in important skin barrier properties.

4.3 Exposure to common daily-life skin stressors affect the level of filaggrin degradation products, skin barrier function and cytokines

In Manuscript III, we investigated the effect of common daily-life skin stressors such as different types of water (hard, soft, chlorinated), 0,5% SLS, house dust mite, bacteria toxin (SEB), cat allergen, cooling and histamine on NMF, skin barrier function (TEWL and skin hydration) and selected cytokines.

4.3.1 Exposure to different water types

Exposure to all water types and 0.5% SLS led to a significant decrease in NMF levels and skin hydration, while TEWL was significantly increased after 24 hours compared to the unexposed control spot. After 48 hours, NMF levels and skin hydration were still significantly decreased for all water types, while TEWL was only significantly higher for 0.5% SLS. The decrease in NMF after exposure to water could be due to decreased degradation of filaggrin, as the breakdown of filaggrin is controlled by the water gradient within the SC [60]. Alternatively, the results could be due to a 'wash-out effect' where the excess water extracted NMF from the SC [86].

Due to the previously shown association between hard domestic water and increased prevalence of AD, we hypothesized that hard water would lead to a more pronounced decrease in NMF and skin barrier function than exposure to softer water. No significant difference between hard and soft water was found in the current study. However, hard, but not soft water led to a significant increase in INF-γ, IL-4 and IL-10 after 24 hours, suggesting a direct effect. As discussed previously, the soft water used in the current study was quite hard (188 mg $CaCO_3$/L) and had a higher pH than expected (7.8), possibly contributing to the lack of difference between the two water types. A high content of calcium carbonate results in a higher pH that may lead to premature activation of skin serine proteases and initiate skin inflammation [11]. Furthermore, calcium is an important regulator of protein synthesis in the epidermis, and normal epidermis has a steep calcium gradient [2]. The concentration is low in basal and spinous layers, high in the granular layers, and low again in the SC [151]. Interestingly, the dephosphorylation of profilaggrin is regulated by calcium concentration and initiated when the calcium concentration is high [152]. Although speculative, a possible direct effect of hard water could thus be due to a disturbance of the epidermal calcium gradient.

There are several possible explanations of the association between hard domestic water and dermatitis. When water is hard, more soap and detergent are needed to produce lather. The increased use of soap and detergent might irritate the skin in itself [98], but it may also lead to deposition of irritant soap salt residues on the skin or on the clothes that are not easily rinsed off [99]. It was recently shown in an experimental study that washing regimes using hard water led to an increased skin deposition of SLS compared to washing regimes using soft water [100]. Increased TEWL and skin irritation were also found, especially in AD patients with *FLG* mutations. A summary of the possible and observed effects of hard domestic water on the skin can be seen in Figure 4.

Another possible explanation of the increased prevalence of AD in regions with hard domestic water could be a systemic effect of hard water. In an experimental study on mice genetically programmed to develop type-1 diabetes, it was shown that the pH of drinking water influenced the gut microbiome and promoted a Th1 immunologic response that was associated with rapid disease progression [153]. The pH of drinking water is closely related to domestic water hardness, and although speculative, it could be interesting to see whether hard or soft drinking water could have the same influence on gut microbiome and inflammatory response in humans and thus influence the development of AD.

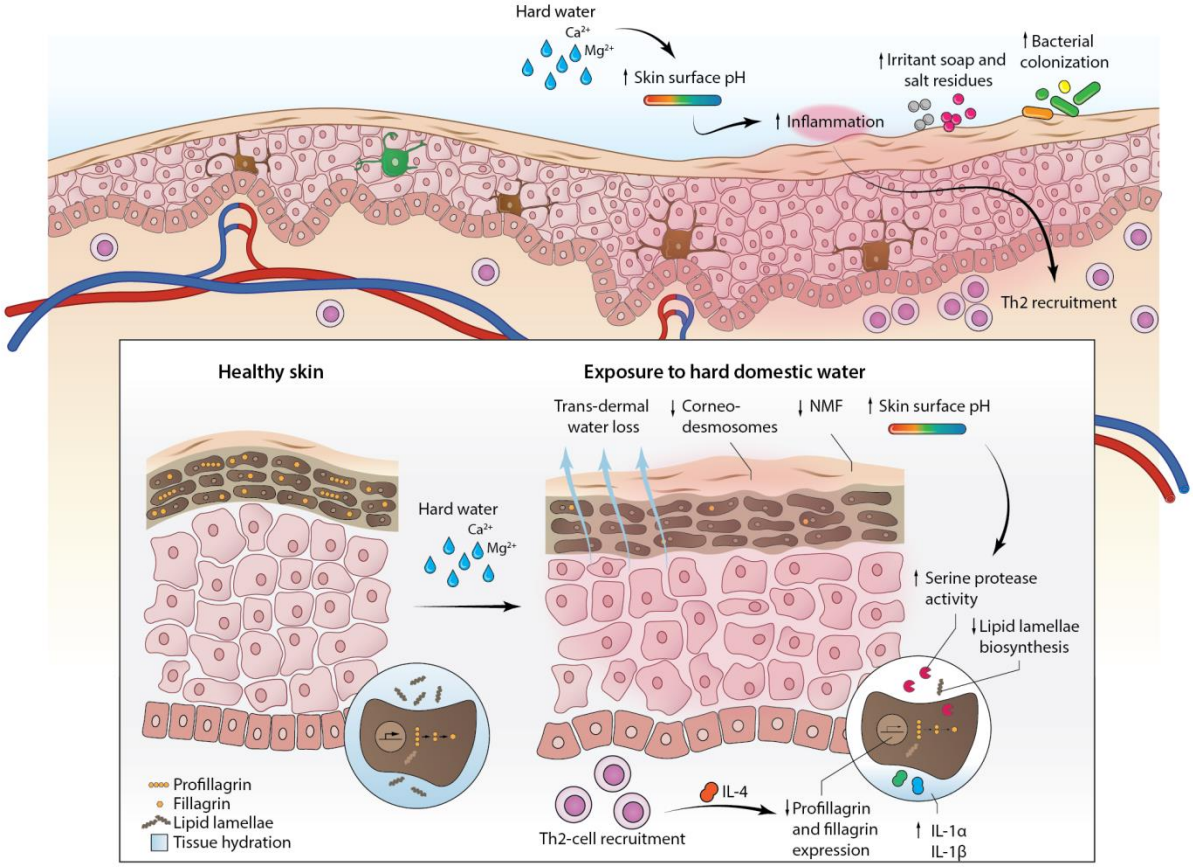

Figure 4. The possible and observed effects of hard domestic water on the skin. Hard domestic water has a high content of calcium and magnesium that may elevate skin surface pH. This may in turn lead to a premature activation of serine proteases, leading to decreased lipid synthesis and premature degradation of corneodesmosomes. An increased skin surface pH may also facilitate bacterial colonization. Furthermore, deposit of irritant soap salt residues on the skin surface may further cause irritation and inflammation. In the current study we observed a significant decrease in NMF after exposure to hard domestic water. TEWL was significantly increased and skin hydration decreased, suggesting impaired skin barrier function. We also observed increased secretion of the Th2-associated cytokine IL-4 that may downregulate profilaggrin and filaggrin expression, further compromising the barrier function. NMF, natural moisturizing factors; TEWL, transepidermal water loss

From a clinical point of view, domestic water softening could be an easy and feasible preventive measure with regards to AD. A previous study from the UK showed that domestic water softening did not have any effect on AD severity in already established moderate to severe AD [154]. However, two studies from Japan have shown that bathing in ultrapure soft water led to an improvement in skin barrier function and a reduction in symptoms in children with mild to moderate eczema and women

with mild eczema [155, 156]. No study has yet investigated whether domestic water softening could have a primary preventive effect on AD development. This is currently being investigated by a research group in the UK, but the study is not yet completed [96].

Besides exposure to hard and soft water, chlorinated water was also shown to have a negative effect on NMF levels and skin barrier function in the current study. The chlorinated water was collected from a closed system in a public swimming pool and had a high content of free chlorine (1.37 mg/L). Chlorine and its degradation products, chloramines, are potent oxidants that may disrupt cellular junctions and the epithelial barrier [157]. Swimming in chlorinated water for 1 hour reduce sebum content on the skin's surface [158], and immersion of one arm in heated, chlorinated water for 10 minutes has been shown to reduce the water holding capacity of the skin [159]. Furthermore, a washing regime with chlorinated water has been shown to produce skin irritation in a recent study from the UK [100]. Workers that are in frequent contact with chlorinated water report a high prevalence of skin symptoms such as pruritus and dermatitis [101], and an epidemiologic study from Spain showed that a high cumulative swimming pool attendance in children seemed to be associated with an increased prevalence of eczema (OR 1.71, 95%CI 1.38-2.12 for >5 years versus 0 years) [160].

Although exposure to chlorinated water may have negative effects on the skin barrier function, its anti-microbial effect might be beneficial in AD treatment. A Malaysian study showed that use of bleach baths (0.005% sodium hypochlorite) in patients with moderate to severe AD reduced the clinical severity (EASI score), itch and colonization with *S. Aureus* [161]. Moreover, children with moderate to severe AD, who used a body wash cleanser containing sodium hypochlorite three times per week in 12 weeks, had a significant reduction in clinical severity (IGA score) and percentage of body surface area affected [162]. No statistical difference in TEWL, skin hydration or skin surface pH was found in another study investigating the effect of 10 minutes bathing in regular water or bleach water in healthy controls and AD patients [163]. Taken together, these results could imply that short exposure to chlorinated water might have a beneficial effect due to its anti-microbial properties, while longer and more excessive exposure affects the skin barrier negatively and increases the risk of dermatitis.

4.3.2 Exposure to house dust mite

The role of house dust mite in AD pathophysiology and its relation to AD exacerbation is debated. A Cochrane review from 2015 concluded that the available literature on house dust mite reduction or

avoidance did not show any clinical beneficial effect on AD [164]. However, its role in the development of AD has, to the best of our knowledge, not been investigated.

Except from exposure to the different water types and 0.5% SLS, house dust mite was the only exposure that led to a significant decrease in NMF levels after 24 hours in the current study. Furthermore, a significant increase in the cytokines IFN-γ, IL-2, IL-4 and IL-10 was found. Previous studies have shown that application of house dust mite induces dermatitis in AD patients and healthy controls without a positive skin prick test and specific IgE towards house dust mite [77-79], suggesting that non-immunological mechanisms might be involved. None of the participants in our study had a known allergy towards house dust mite. Although we did not perform an objective clinical scoring, we observed that most of our healthy participants developed signs of dermatitis after exposure (Fig. 5). This is supported by the significant increase in the pro-inflammatory cytokines IFN-γ and IL-2 [165, 166]. Interestingly, exposure to house dust mite also led to a significant increase in the Th2- associated cytokine IL-4 known to downregulate filaggrin expression [36], and IL-10 which has been shown to have a direct regulatory effect on Th2 cell survival *in vitro* and *in vivo* [167].

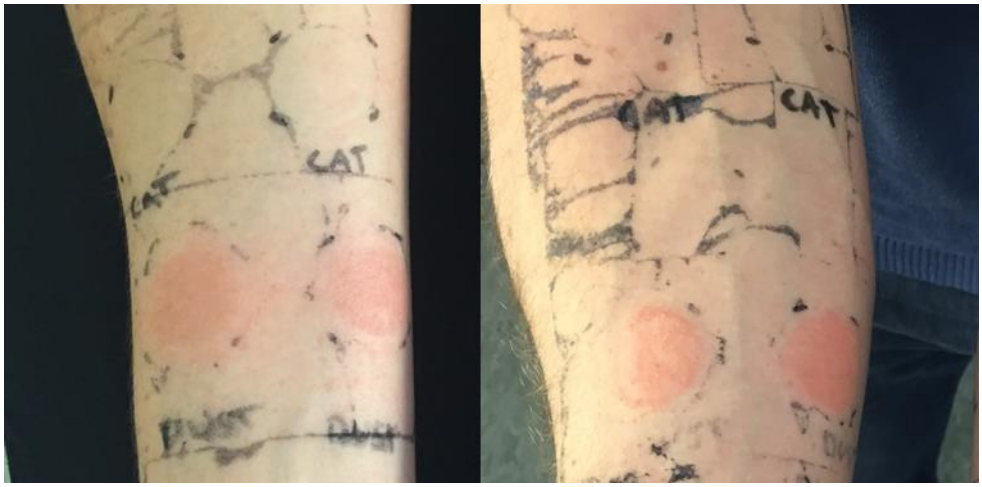

Figure 5. Clinical response in two participants after exposure to house dust mite for 24 hours. The pictures were taken after 48 hours. CAT, cat allergen; DUST, house dust mite

The effect of house dust mite on NMF levels and skin barrier function could be related to the proteolytic activity of house dust mite allergens [168]. In an experimental study, application of house dust mite on human and murine skin delayed the recovery rate after skin barrier disruption, most likely due to an activation of the protease-activated receptor 2 (PAR-2) [169] . PAR-2 is involved in epidermal barrier function, and inflammatory mediators such as TNF-α, IL-1α and lipopolysaccharide (LPS) have been shown to up-regulate PAR-2 expression in endothelial cells [170]. One of the major

allergens in house dust mite, Der p1, increases the permeability of airway epithelium by disruption of tight junction proteins [168]. If the same applies in skin epithelium, the decrease in skin barrier function could lead to an increased TEWL and need for NMF to maintain skin hydration. In the current study, no effect on TEWL or skin hydration was observed after 24 hours. As previously discussed, this could be explained by the fact that house dust mite allergens were prepared with petrolatum [123].

The increase in NMF levels after 48 hours might have several possible explanations. The degradation of filaggrin into free amino acids depends on skin proteases, and Caspase-14 and Bleomycin hydrolase are two of the proteases suggested to be involved in this process [10]. It has been shown that barrier disruption leads to an increased activity of Caspase-14 [171]. Although we did not observe an increase in TEWL after 48 hours, the clinically observed erythema and signs of dermatitis could suggest skin barrier impairment. Although speculative, the increased levels of NMF could thus be due to increased activity of Caspase-14 and degradation of filaggrin. Alternatively, the increased levels could be due to occluded exposure to petrolatum [123], or a compensatory breakdown of filaggrin due to skin inflammation [38].

4.3.3 Exposure to Staphylococcus enterotoxin B

SEB is produced by strains of *S. aureus* isolated from AD skin [70, 71]. Recently, a meta-analysis showed that the pooled prevalence of *S. aureus* colonization in AD patients was 70% in lesional and 39% in non-lesional skin, and that in lesional skin the prevalence of colonization increased with disease severity [172]. The prevalence of enterotoxin producing strains of *S. aureus* in lesional skin varied from 31.5% and 80%, SEB being the most frequent. Experimental exposure to SEB on intact skin of healthy controls and AD patients has previously been shown to produce eczema like reactions [73]. In the same experiment, 50% (3/6) of the patients with AD experienced flair of their disease in the ipsilateral, non-exposed elbow flexure, suggesting a systemic effect of SEB.

In the current study, no significant decrease in NMF levels was observed after exposure to SEB for 24 hours. However, we saw a significant increase in TEWL and decrease in skin hydration, suggesting skin barrier impairment. After 48 hours there was a significant increase in NMF levels. A possible explanation could be the that the decrease in skin hydration initiated a compensatory breakdown of filaggrin [60]. As for house dust mite, we observed that most of the participants developed inflammation on the exposed sites (Fig. 6), and a significant increase in the pro-inflammatory cytokine IL-1α was found after 24 hours. Intact epidermis and SC contain high levels of preformed IL-1α which

may be released in response to mechanical stress or injury and serve as a bridge between the innate and acquired immune system [173]. In a study by Kezic et al., filaggrin deficient skin contained increased levels of IL-α compared to healthy skin, and the level of IL-1α correlated inversely with NMF levels [141]. The activity of serine proteases responsible for activating pro-forms of IL-1α is calcium dependent, and the authors hypothesized that decrease in skin hydration and the subsequent increase in calcium concentration could be responsible for increased levels of IL-1α.

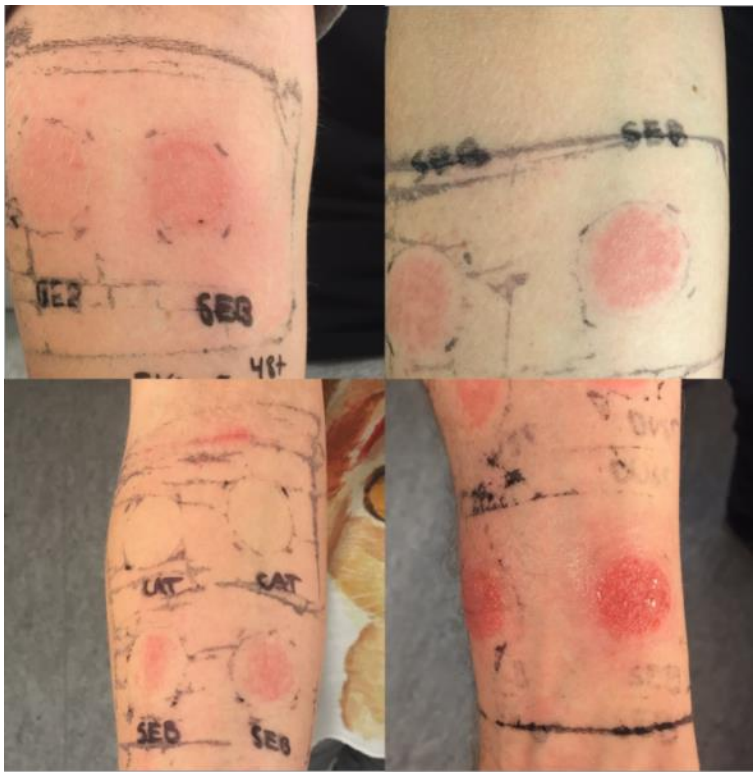

Figure 6. Clinical response in four participants after exposure to Staphylococcal enterotoxin B (SEB) for 24 hours. The pictures were taken after 48 hours.

4.3.4 Exposure to cat allergen

Neonatal exposure to cat has been found to increase the risk of early-life AD in children with *FLG* mutations in two independent birth cohorts in Denmark [81]. Furthermore, a worldwide, multicenter study found that early-life exposure to cats was a risk factor for eczema in children aged 6-7 years [174]. The reason for the increased risk is not known, but it has been hypothesized that it could be due to endotoxin exposure or helminth infections caused by close contact to cats [174]. In the current study we wanted to investigate whether cat allergen could have a direct effect on NMF and skin barrier function. As described previously, exposure to cat allergen seemed to have a positive effect on the skin

barrier function and NMF levels in the skin. However, we choose to interpret these results with caution as the cat allergen used in the study was prepared with 25% glycerol. Exposure to pure cat allergen in form of cat dander would have been preferred, but this was not available in a standardized form at the time of the study.

4.3.5 Exposure to histamine

Histamine has previously been shown to downregulate profilaggrin expression in keratinocytes [83]. In the current study, exposure to histamine prick did not lead to a significant change in NMF levels compared to the control spot at 24 or 48 hours. The exposure was only performed once and the limited degree of exposure could possibly explain this finding. Nonetheless, histamine prick led to a significant increase in skin hydration after 24 hours and decrease in TEWL after 48 hours suggesting a positive effect on the skin barrier. This finding could be a coincidence; however, exposure to histamine has been shown to upregulate genes associated to skin barrier function [83]. Although speculative, the observed increase in skin hydration and decrease in TEWL in the current study could be related to this.

4.3.6 Exposure to low temperature

Low temperatures have previously been shown to negatively affect the skin barrier, amongst other by reducing lipid production and delay skin barrier recovery after disruption [62, 63]. No significant effect on NMF levels was observed after cooling of the skin in the current study. The cooling was performed using a thermode holding a temperature of 10°C. A total of 10 cycles with 60 seconds of cooling was performed, with a break of 30 seconds between each cycle. The short duration of exposure could explain the lack of effect on NMF levels. Alternatively, exposure to cold alone has no direct effect on NMF levels in the skin. We did, however, observe a significant decrease in TEWL after 24 and 48 hours compared to the control spot, suggesting a positive effect on the skin barrier. We were surprised by this finding as previous studies have shown that TEWL might be reduced immediately after exposure to low temperatures, but that it increases significantly when the skin temperature returns to normal [68].

4.3.7 Exposure to sodium lauryl sulfate

Experimental exposure to SLS is known to reduce epidermal filaggrin protein and NMF levels in the skin [106-108]. In the current study, exposure to 0.5% SLS led to a significant decrease in NMF after 24 and 48 hours and an increase in INF-γ and IL-10 compared to the control site. Furthermore, a large significant increase in TEWL and decrease in skin hydration was observed, conforming previous findings [105, 175]. IL-10 has a potent anti-inflammatory effect, and the increased level was likely a counteractive response of the inflammation caused by SLS exposure [176, 177]. Although experimental exposure to SLS does not mimic real-life exposure, our findings confirm that excessive use of soap and detergents may have a negative effect on skin barrier function and increase the risk of xerosis and dermatitis, also in healthy skin.

4.3.8 Alternative exposures

The exposures performed in the current study were chosen due to their possible association to AD pathophysiology or exacerbation, as discussed previously. Several other exposures would have been of interest, but due to limited time this was not feasible. When preparing and planning the study, we attempted to build an installation that could expose the participants for dry and cold air to mimic winter climate in Denmark. The installation was inspired by a previous publication [65], but due to technical difficulties we were not able to complete the project. Exposure to heat and sweating has also been associated to AD flares, and exposure to artificial sweat and heating of the skin could thus be two other possible exposures [40, 41]. Furthermore, it would be interesting to evaluate the effect of stress, as this has been shown to have a negative effect on several important skin barrier properties, including filaggrin expression in an experimental mouse model [178]. However, this would be challenging to perform in an experimental setting, as it would require a standardized and ethically acceptable form of experimentally induced stress.

4.4 The effect of age and sex on filaggrin degradation products and corneocyte surface texture

We investigated the effect of age on NMF levels and DTI in Manuscript I and II. In Manuscript I, we found that old participants (≥ 70 years) had increased NMF levels on the cheek compared to young participants (18 ≤ 40 years) in the winter and summer. However, after adjustment for possible

confounders (shower/bath, use of cream, temperature/sun exposure) the difference was only evident in the winter. No significant differences with regards to age were found for NMF levels on the hand. For DTI, no age-related differences were found for the cheek or hand, neither during the winter, nor the summer. In Manuscript II we saw an effect of age on NMF levels in the patient population, where NMF levels were significantly higher in the oldest age group (>50 years) compared to youngest age group (<30 years) in both the unadjusted and adjusted analyses. No significant age-related difference was seen in the healthy control group for NMF, but DTI showed a significant increase with age.
Aged corneocytes have larger cell surface [135, 179], and the SC transition time has been found to be longer than in young skin [180]. It has been suggested that the slower SC turnover rate could mean more time for filaggrin to be degraded into NMF and hence lead to increased NMF levels [44]. Moreover, aged skin has decreased levels of all major lipids [181]. This is known to negatively affect skin hydration [182], and the increased levels of NMF could thus also be due to a compensatory breakdown of filaggrin to restore skin hydration. In the study by Rinnerthaler et al., aged skin was also shown to have decreased levels of calcium in *stratum granulosum* were the *FLG* is transcribed to produce profilaggrin. As the transcription of profilaggrin is thought to be calcium dependent, a decrease in calcium levels could affect the expression of profilaggrin and hence the levels of filaggrin and NMF [42].

The reason why we only saw a difference in NMF levels in the healthy participants in Manuscript I, but not in Manuscript II, could be due to the age composition of the study population and the difference in anatomical localization. The aged, healthy participants in Manuscript I were older (all ≥ 70 years) than the healthy participants in Manuscript II (oldest participant was 68 years old). Furthermore, in Manuscript II, the participants were tape-stripped on the volar forearm and not on the cheek as in Manuscript I.

We are the first to report that DTI increases significantly with age in Manuscript II. However, morphological changes of aged corneocytes have previously been described in a small study by Gorzelanny et al. In two male individuals aged 39 and 70 years, it was shown that the corneocytes from the old, but not the young participant had small protrusions described as “humps” [135]. As mentioned previously, an increased DTI has been associated to skin barrier impairment and decreased skin hydration. In the current study, we were not able to see a significant difference in DTI according to high or low TEWL values, suggesting that there might be a different mechanism responsible for the altered corneocyte surface texture in aged individuals, such as decreased levels of lipids or other structural proteins [42].

With regards to sex, female participants had higher levels of NMF and lower levels of DTI on the hands during the summer compared to the male participants in Manuscript I. No sex-related differences were found for cheek skin. In Manuscript II, there was no significant difference in the patient population, however, in the healthy controls, female participants had higher NMF levels on the volar forearm. No sex-related differences were seen for DTI. Several studies have investigated gender related differences in skin physiology, but the results are conflicting. TEWL has been found to be higher in men in one study [183], while in another large study on 300 participants, male participants had lower TEWL values compared to female participants [184]. The observed difference in our study could be due to hormonal differences between men and women. Testosterone upregulates sebum production, while estrogen has the opposite effect [185]. However, estrogen has also been shown to have positive effect on skin barrier function and hydration [186]. This suggests that other mechanisms may also contribute to observed difference between the male and female participants in Manuscript I and II. This could be behavioral differences (i.e. clothing habits, frequency of hand washing, use of emollients, sun exposure, bathing/showering etc.) that we did not account for in the manuscripts.

5. Conclusions

This PhD-thesis has investigated how important skin barrier measures such as filaggrin and its degradation products, corneocyte surface texture and cytokines are affected by both internal (age, sex and inflammation) and external (climatic factors, different water types, allergens, irritants and bacteria toxin) factors to further elucidate their role in AD pathophysiology, but also in seasonal and age-aggravated dermatoses. The main findings from the three included manuscripts are listed below:

- Climatic factors have a significant influence on filaggrin degradation products and corneocyte surface texture on weather exposed areas such as the cheek and dorsal aspect of the hand

- High self-reported UV-exposure was associated with an increased number of corneocyte surface protrusions in cheek skin

- Experimental exposure to different types of water (soft, hard and chlorinated) all led to a significant decrease in filaggrin degradation products, skin barrier function and skin hydration

- Hard, but not soft water, led to a significant increase in the AD associated cytokine IL-4, suggesting a direct effect of hard water on the skin

- Exposure to house dust mite led to a significant decrease in filaggrin degradation products and an increase in the pro-inflammatory cytokines IFN-ɣ and IL-2

- Exposure to SEB led to a significant decrease in skin barrier function and skin hydration, but no decrease in filaggrin degradation products

- Patients with dermatitis had significantly lower levels of epidermal filaggrin protein and filaggrin degradation products and an increased number of corneocyte surface protrusions compared to healthy controls

- Old age was associated with higher levels of filaggrin degradation products and an increased number of corneocyte surface protrusions compared to young age

- Female participants had higher levels of filaggrin degradation products and a decreased number of corneocyte surface protrusions on the hands compared to male participants

6. Future research

Over the last decades it has become increasingly evident that an impaired skin barrier increases the risk of dermatitis. In the current thesis, I have shown that important skin barrier properties are significantly affected by both internal and external factors, but also that the interplay between them is complicated. To further elucidate their role in the development of dermatitis I here propose some future research perspectives.

First, I experienced that it was a challenge to interpret the level of filaggrin degradation products without having concomitant data on profilaggrin expression and epidermal level of filaggrin protein. To date, this information requires invasive skin biopsies, and a future perspective could be to determine whether there is a correlation between the three, and if it differs between healthy skin and skin with inflammation or filaggrin deficiency. In a pilot study, I attempted to investigate this in 20 healthy male participants, but due to technical difficulties the study did not succeed. Information on other important skin barrier features such as SC lipids and level of proteases involved in filaggrin processing would also be a great asset in the interpretation of data on filaggrin degradation products.

Furthermore, I observed that experimental exposure to daily-life skin stressors had a significant effect on skin barrier function, filaggrin degradation products and level of skin cytokines in healthy volunteers. The study was originally meant to serve as a basis for a larger study on AD patients with and without *FLG* mutations to investigate the difference in response between healthy skin and skin with inflammation and filaggrin deficiency. Due to limited time, I was unable to proceed with the patient population, but this is currently being planned. Moreover, I have also considered studies with less experimental and more real-life exposures such as washing with hard and soft water and exposure to cold and dry weather in the winter (e.g. biking with and without gloves).

Interestingly, I observed that corneocyte surface texture seemed to be affected by high self-reported UV-exposure. I am the first to report this, and to further elucidate the effect of UV-irradiation on corneocyte surface texture, an experimental study with controlled doses of UV-irradiation and measurements at several time points following the irradiation would be of great interest. Moreover, in future studies, it would be of value to include other important skin barrier features such as TEWL, skin hydration, filaggrin, NMF and SC lipids and correlate these to DTI to further investigate the cause of these changes in corneocyte surface texture.

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