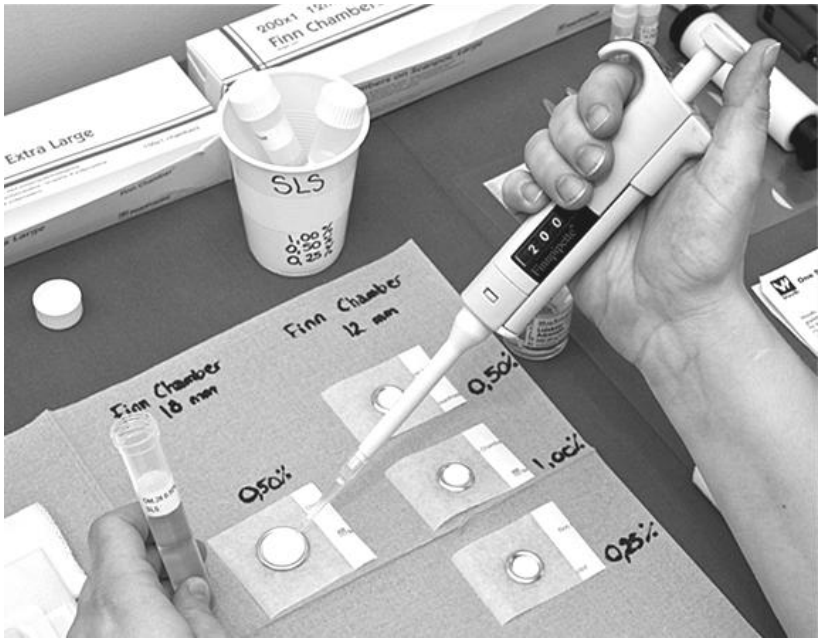
FACULTY OF HEALTH AND MEDICAL SCIENCES
UNIVERSITY OF COPENHAGEN

PhD thesis

Filaggrin and skin reactivity to irritants – Epidemiological and Experimental studies



Josefine Bandier, MD
National Allergy Research Centre
Department of Dermato-Allergology
Copenhagen University Hospital Gentofte
Denmark

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NATIONAL ALLERGY RESEARCH CENTRE
STATENS SERUM INSTITUT
REGION H
Gentofte Hospital

Filaggrin and skin reactivity to irritants

– Epidemiological and Experimental studies

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I. Bandier J, Ross-Hansen K, Carlsen BC, Menné T, Linneberg A, Stender S, Szecsi PB, Meldgaard M, Thyssen J, Johansen JD. *Carriers of filaggrin gene (FLG) mutations avoid professional exposure to irritants in adulthood*. Contact dermatitis. 2013; Dec; 69 (6): 355-62.
II. Bandier J, Carlsen BC, Jelstrup Petersen L, Johansen JD. *Skin reaction and regeneration after single SLS exposure stratified by filaggrin genotype and atopic dermatitis phenotype*. British Journal of Dermatology. 2015 Jan 10. doi:10.1111/bjd. 136512014
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PhD supervisors

Jeanne Duus Johansen, Professor, MD, DMSc
National Allergy Research Centre
Department of Dermato-Allergology
University of Copenhagen
Gentofte Hospital, Denmark

Berit Christina Carlsen, PhD, MD
National Allergy Research Centre
Department of Dermato-Allergology
Gentofte Hospital, Denmark

Niels H. Heegaard, MD, DSc, DMSc
Department of Autoimmunology & Biomarkers
Statens Serum Institut, Denmark

Assessment committee

Asger Dirksen, Professor, MD, DMSc (Chair)
Department of Clinical Medicine
University of Copenhagen, Denmark

Charlotte Gotthard Mørtz, Associate Professor, MD, Ph.D.
Department of Dermatology
Odense University Hospital, Denmark

Sanja Kezic, Senior researcher, Principal Investigator, Ph.D.
Coronel Institute of Occupational Health
Academic Medical Center, AMC, Holland

Preface

This dissertation is based on scientific work carried out from August 2010 to March 2015 at the National Allergy Research Centre, Copenhagen University Hospital Gentofte and at the Department of Autoimmunology & Biomarkers, Statens Serum Institute.

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The greatest gratitude is, however, reserved for my loving husband and children for being ever supporting in this process in both its ups and downs – words cannot express my love for you guys.

Gentofte, Marts 2015

Josefine Bandier

Abbreviations

The abbreviations are listed alphabetically.

AD – Atopic Dermatitis

ELISA – Enzyme-Linked Immunosorbent Assay

FLG – The filaggrin gene

FLGnull – Filaggrin null mutations

FLGwt – Filaggrin wild type

Het – Heterozygotes

Hom – Homozygotes

LDF – Laser Doppler Flowmetry

PCA – Pyrrolidone carboxylic acid

SLS – Sodium Lauryl Sulfate

TEWL – Transepidermal Water loss

UCA – Urocanic acid

TABLE OF CONTENTS

1 ABSTRACT

Background: Contact dermatitis is commonly induced by irritants, which can affect the skin barrier after a single skin contact or repetitive contacts. Filaggrin mutations are the most important explanatory genetic factor to date for the development of atopic dermatitis (AD) and may carry an increased risk of occupational irritant contact dermatitis with a poor prognosis. However, the interactions between genes, skin irritants and behaviour are still not clear and the effect on filaggrin content in the skin in response to irritant exposure has not been investigated.

Objectives: Firstly, the aim was to assess individual behaviour according to occupational irritant exposure in relation to filaggrin mutations. Secondly, to evaluate the skin response to an irritant in a population stratified for AD and filaggrin mutations. Lastly, to estimate the effect of irritant exposure on the filaggrin protein level in the epidermis.

Material and Methods: The first part of the study was conducted on an epidemiological cohort of 3471 Danish individuals. The population was genotyped for FLG polymorphisms and answered a questionnaire on occupational exposures. The second part was a clinical study comprising 67 individuals stratified for AD and filaggrin mutational status. A known irritant, sodium lauryl sulfate (SLS), was applied to the skin in three different doses and assessed at four different time points by non-invasive assessments. The third part of the study was a biochemical study based on biopsies taken from the skin site exposed to 0.50% SLS and adjacent unexposed skin and evaluated at different time points. Epidermis was isolated and epidermal filaggrin protein was extracted and quantified after developing an enzyme-linked immunosorbent assay.

Results: The epidemiological study showed that individuals with early onset of hand eczema and filaggrin mutations avoided occupational irritant exposure and that this avoidance was not driven by the absence or presence of AD. The clinical study revealed a marked difference in skin reactivity to SLS between the healthy control group and individuals with either AD or filaggrin mutations or both, by univariate analysis. Pattern analysis assessment showed that the reaction pattern of individuals with AD with and without filaggrin mutation could be significantly separated from each other due to individuals with filaggrin mutations having a rapid inflammatory response, primarily driven by increased inflammatory alertness and a higher clinical response, separating them from the other study groups. The amount of epidermal filaggrin protein decreased with the number of functional filaggrin alleles. Additionally, a non-significant decrease was associated with AD presence. In response to SLS, a sizable increase was seen, which could be due to increased granular filaggrin release or decreased degradation. This was followed by a substantial decrease in filaggrin to levels lower than at baseline even 145 hours after removal of the Finn chamber, which could be due to an increased turnover of filaggrin to repair the SLS induced barrier disruption.

Conclusion: The filaggrin levels in the epidermis depend on genotype but also seem to be influenced by the phenotype of atopic dermatitis in non-lesional skin. We showed that SLS exposure induces a substantial and long lasting decrease in filaggrin levels, an exposure which is relevant to many occupations. Filaggrin mutations, and thus indirectly filaggrin levels, are associated with higher inflammatory alertness and an early inflammatory response, especially among individuals with concomitant AD, which offers an explanation of the increased severity and worse prognosis associated with filaggrin mutations as described in the literature. It may also partly explain why individuals with filaggrin mutations avoid occupational irritant exposures.

2 INTRODUCTION

The skin is the largest organ in the human organism and its surface area accounts for approximately 2m^2. The skin is a dynamic organ with a complex structure. It functions both as the first line of bodily defence and protects the inner milieu from exogenous stressors, while still being able to excrete water and waste products. The skin is able to adapt to its surroundings and in general the skin is capable of resisting multiple stimuli; however, some individuals are more susceptible to skin challenges than others. Exposure to exogenous substances, such as irritants, can cause eczema, which is a common disease often associated with functional impairment and psychological distress, especially in relation to hand eczema. An intact skin barrier and consideration concerning exogenous skin hazards is thus important in the prevention of eczema.

2.1 The skin barrier

The structure of the skin can be divided into 3 sections: 1) the outer skin compartment, the epidermis; 2) the underlying dermis including connective tissue and cells involved in the immune system, pressure sensitive cells and many more[1]; 3) the subcutis, which lies beneath these structures, containing layers of subcutaneous fat. The many layers of the skin are structurally very different from each other.

Epidermis: The most prominent function of the epidermis is to provide a barrier between the internal bodily milieu and the external environment. This requires a continuous renewal of the epidermis effected by differentiation of keratinocytes, which comprise 95% of the resident cells[1]. The inner cell layer of the epidermis (the stratum basale) functions as stem cells. From the stratum basale, the cells travel outwards, perpendicular to the plane of the cell sheet while differentiating and undergoing transformation from the stratum basale to stratum spinosum, stratum granulosum, stratum lucidum and lastly ending up as dead cells reduced to squames in the stratum corneum[2] (Figure 1). This turnover with the shedding of old cells and the production of new cells is in a constant balance[3]. The flattening of the keratinocytes depends on a filament aggregating protein, filaggrin, which together with keratin comprises 80–90% of the epidermal mass[1].
In the outer cell layers, the keratinocytes have lost their nuclei and organelles, and are now called corneocytes. This outer visible skin layer thus consists of dead skin cells and functions as the first line of defence and is known as the stratum corneum.

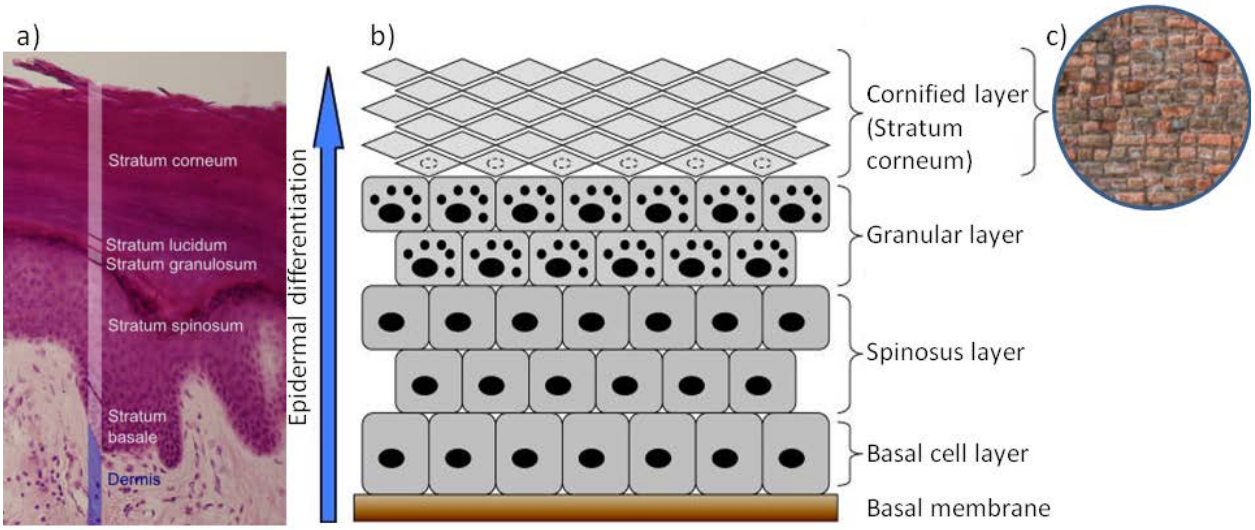

Figure 1. Epidermis. a) Epidermis illustrated histologically by Haematoxylin-Eosin staining[4]. b) Graphic illustration of keratinocyte differentiation through the epidermis, modified from[5]. c) Illustration of bricks and mortar, where the bricks imitate the terminally differentiated keratinocyte (the corneocyte) and the mortar the lipids (membrane bilayers) by courtesy of Colourbox.

Bricks and mortar: The stratum corneum is often compared to a structure similar to that of bricks and mortar (Figure 1c), where the corneocytes are pictured as the bricks while the mortar mimics the inter-corneocyte lipids embedding the corneocytes[6]. Both the bricks and the mortar contribute to the integrity of the skin barrier[6]. The lipid bilayer mainly consists of three large groups: ceramides, cholesterol and free fatty acids, varying in composition according to the epidermal differentiation stage[7].

Humectants: In addition to the lipid bilayer, another important system protecting the skin is the natural moisturising factor (NMF). NMF primarily consists of free amino acids and/or their derivatives such as pyrrolidone carboxylic acid (PCA), urocanic acid (UCA), lactic acid, urea, citrate and sugars, which are effective humectants[8]. In the 1980s it was found that the aminoacid parts of NMF originate from the degradation of the protein filaggrin[9;10].

PH: In normal, intact skin the pH range is 4–6[11], i.e., slightly acidic. The pH of the stratum corneum is crucial to uphold a proper antimicrobial defence, permeability, skin immune defence, formation of the lipid bilayer and skin cohesion[12]. The proton sources to uphold an acidic pH are thought to come from filaggrin degradation products, α-hydroxy acids and from acidic lipids[11].

The structure of the stratum, with both the corneocytes and the lipid bilayer, as well as the humectants and pH outline important parameters that secure a proper skin barrier. Disturbances in these parameters can result in a defective skin barrier.
One protein, filaggrin, is known to influence all the mentioned parameters.

2.2 Filaggrin

In 1977 filaggrin was purified and recognised as “stratum corneum basic protein”[13]. The name filaggrin was introduced in 1981 by Peter Steinert[14] when researchers started to identify filaggrin as a protein associated with intermediate filaments and when defective synthesis of filaggrin was found to correlate with the absence of keratin filaments by light and electron microscopy[15].

Filaggrin is a protein derived from the Filaggrin gene (FLG), which is located on the largest chromosome in the genome in the epidermal differentiation complex on chromosome 1q21[16;17]. The entire FLG gene consists of 3 exons and 2 introns[18;19], where profilaggrin is encoded by the third exon (Figure 2).

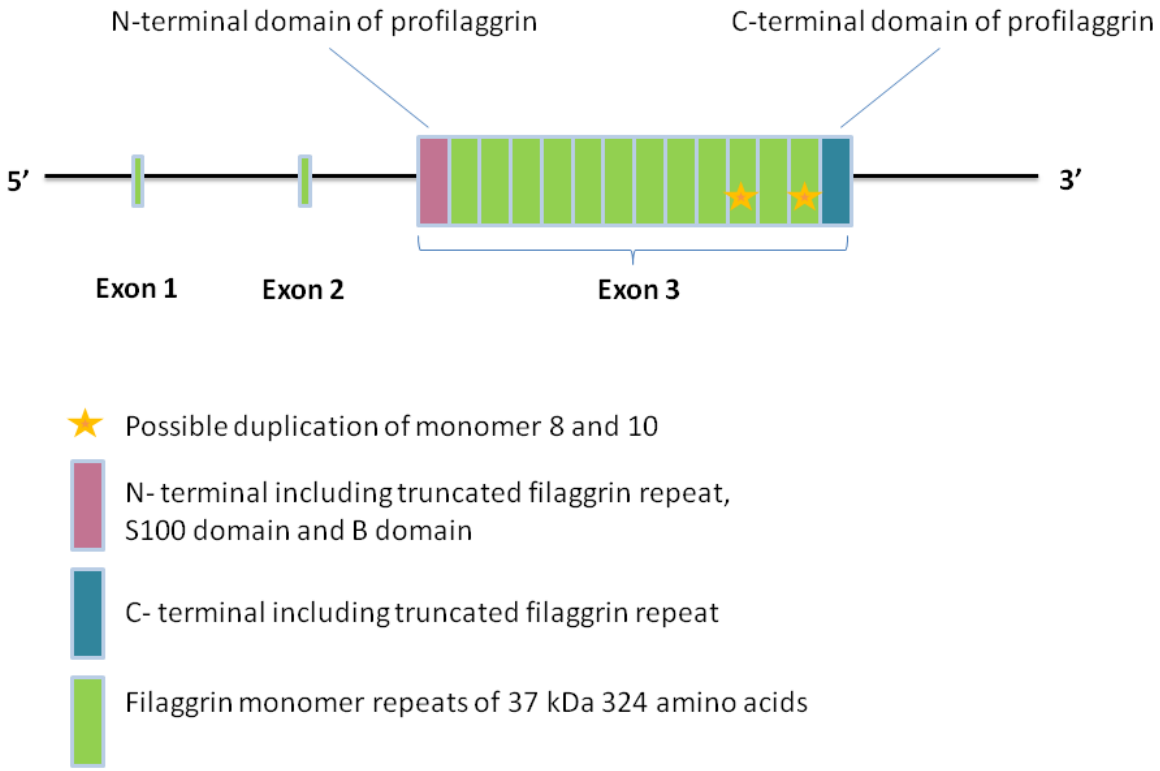

Figure 2. Schematic overview of the FLG structure. The FLG gene is located on the epidermal differentiation complex on chromosome 1q21. Exon 1 is non-coding and within exon 2 protein translation is initiated. Exon 3 encodes profilaggrin which is dephosphorylated and cleaved to yield 10–12 filaggrin monomer repeats depending on duplication of monomer 8 and/or 10.

Profilaggrin is the large precursor protein from which filaggrin derives (Figure 2). Profilaggrin is a highly phosphorylated and insoluble protein residing in granules in the stratum corneum. During the later state of terminal epidermal differentiation, profilaggrin is dephosphorylated and cleaved

by proteases to filaggrin. Each profilaggrin molecule generates 10–12 filaggrin monomers[19;20]. The monomers are found to be almost identical in animal studies, with > 90% similarity in amino sequence[5]. The repeats are of approximately 37kDa each[5] and function to aggregate intermediate keratin filaments into macrofilaments, hence the name filament aggregation protein[21]. This alignment into larger bundles promotes the collapse of the cytoskeleton of the corneocyte causing a change of cellular shape to a more flattened form, thereby enabling a more densely packed barrier[17]. Filaggrin thus functions to strengthen the skin barrier.
During cell differentiation, filaggrin undergoes internal reorganisation and becomes susceptible to proteases hydrolysing filaggrin into free amino acids, which constitute approximately 50% of the NMF in the stratum corneum[5;8;22;23]. The turnover of filaggrin into NMF is sensitive both towards changes in external humidity and also to the turnover time of the stratum corneum[8]. Apart from the central role of filaggrin breakdown products in stratum corneum hydration, the breakdown into the chromophore UCA is also important for the absorption of ultraviolet radiation[24]. UCA and PCA further contribute to maintaining the stratum corneum acidity[25].

When individuals have filaggrin mutations they can either be heterozygous with only one functional allele or homozygous with no functional alleles. Filaggrin mutation carriers have been described as having a specific phenotype with keratosis pilaris, and skin xerosis, and when focusing on the hands, filaggrin mutation carriers have been characterised with presenting dorsal hyperkeratosis, palmar hyperlinearity and fissuring/chapping[26-33]. The lack of filaggrin can be caused by genetic mutations[34] but can also be caused by functional filaggrin deficiency, where inflammatory and proinflammatory mediators modulate the expression or processing of filaggrin[35-39].

It is known that having filaggrin mutations increases the risk of developing AD[34] and that AD increases the risk of developing irritant contact dermatitis[40]. However, whether filaggrin mutations alone increase the risk of developing irritant contact dermatitis is still not clarified.

2.3 Atopic dermatitis

AD is a common disease affecting up to 20% of individuals in Europe[41]. It has shown increasing prevalence[42-44]. AD usually manifests in early childhood[45] and its cardinal features are dry skin, lichenisation and flexural dermatitis as well as personal or family disposition to atopy. AD is characterised as an itching, non-contagious inflammatory skin disorder. The anatomical predilection is age dependent and changes distinctively over time. AD can be a transient disease with flares; however, it can also develop into a chronic or intermittent, relapsing disease even despite treatment[46]. Even though it is possible to outgrow AD, the risk of persistence into adulthood exists[47]. In adulthood, AD manifestations are commonly seen as hand eczema, which is known to have substantial socioeconomic implications[48;49].

In twin studies a higher concordance of atopic dermatitis has been found among monozygotic twins (0.75) in comparison with dizygotic twins (0.25), suggesting that genetic susceptibility is an element in the development of AD[50].
Individual susceptibility has been studied for decades with the aim of explaining why some people are more prone than others to developing AD. This has led to identification of disease genes for AD[51-53].

Genetic barrier defect: In 2006, filaggrin mutations were found to be a cause of ichtyosis vulgaris[54], a disease clinically characterised by xerosis, keratosis pilaris, scaling, hyperliniarity both palmar and plantar and a prominent association with AD[21]. Palmer et al. established that filaggrin mutations strongly predispose to having AD[34].
Filaggrin mutations are frequent, affecting 8–10% of adults from the general population[34;55], but among individuals with AD, the frequency is higher. An Irish study reported that 55.8% (26/52) of the children with AD had filaggrin mutations, compared with 8.6% (16/186) of controls[34]. In a Danish birth cohort the frequency of filaggrin mutations among individuals with AD was 17.6% (25/142) compared with 7.9% (15/190) among individuals without AD[34]. Having a mutation in the gene encoding filaggrin does not necessarily mean that an individual has AD since a penetrance of only 60% is seen among heterozygous and 90% among homozygous individuals for developing ichtyosis vulgaris[21]. This has been further supported by an epidemiological study and a cohort study showing that not all individuals with filaggrin mutations develop dermatitis[56-58]. However, many studies have reported that concomitant AD and filaggrin mutations are associated with early onset of disease, disease severity and persistency[59-63].
Apart from mutations in the filaggrin gene, numerous potential susceptibility loci have been identified by genome-wide association studies (GWAS) to explain AD[52;64-66]. Some of these loci are related to immunological features, whereas others are related to skin barrier deficiencies[52]. Even though the increased susceptibility among individuals with AD can be caused by numerous factors, mutations in the gene encoding filaggrin are the most prominent explanatory factors to date for the pathogenesis of AD[52;53].

Antimicrobial barrier deficiency: The barrier deficiency in AD is also associated with a compromised antimicrobial barrier, with 90% of AD individuals being colonised with *Staphylococcus aureus*, compared with only 5–30% of healthy individuals[67], causing impetigo[68]. Even on non-lesional skin, individuals with AD have an increased colonisation in comparison with healthy controls[69]. Additionally, frequent viral infections, such as herpes simplex, mainly occur in individuals with AD[70]. The susceptibility to skin infections is also linked to filaggrin mutations; a study has shown that among AD patients with eczema herpeticum there is an increased frequency of the 501X filaggrin mutation compared with wild type individuals[71]. For staphylococcus aureus infections, a study has shown reduced bacterial growth and protein expression in the presence of UCA and PCA, the degradation products of filaggrin[72].

Lipid disturbances: In individuals with AD, a disturbance in the lipid composition has been found[73], and it has been suggested that there might be a genetic link to filaggrin mutations[74;75]. This finding is, however, contradictory[76-79].

Immunological disturbances: The cytokine levels in stratum corneum have been shown not to differ according to AD[80], whereas the keratinocyte release of inflammatory cytokines is found to be greater among individuals with AD than in controls in response to stimulation[81]. This could also be a factor in the pathophysiology of AD. When the skin barrier is in contact with skin stressors, a complex immunological process is initiated. The mechanisms of acute irritant contact dermatitis and acute allergic contact dermatitis are nearly similar; however, whereas specific T cells are activated in the response to an allergen, no prior sensitisation or memory is required in the irritant skin response[82].

Disruptions of the skin homeostasis and/or structure of the stratum corneum can result in an impaired skin barrier, which may facilitate the penetration of exogenous substances. AD is therefore a predisposing factor for developing irritant contact dermatitis[83]. In an occupational setting, individuals with AD tend to have increased recurrence of their hand eczema and a poorer prognosis than do non-atopic individuals[45;84;85].

2.4 Irritants

The skin is an interface in daily contact with multiple substances. Some of these substances can induce an irritant skin reaction, which can occur after only a single encounter or after repetitive stimulation. Many types of exposure may cause skin irritation[86;87]. Irritant contact dermatitis is described later (under section 2.5). Some of these irritants are listed in Table 1.

Table 1

Skin irritants		
Organic solvents	Occlusive gloves	Concentrated salt solutions
Water	Hand washing	Oxidising/reducing agents
Oil,	Certain foods	Alcohol, disinfectants
Detergents	Corrosive metals	Plant extracts
Friction	Acids	Wool
Pressure	Alkaline substance	Rockwool fibres

The exposure pattern and the irritant load vary between sexes. Women are more exposed to occupational irritant contact dermatitis due to water and soap exposures and men are exposed to oils in particular[88;89]. The local inflammatory response that may be caused by contact with skin irritants varies according to many different factors: different irritants, irritant potency, occlusion, dose and concentration, frequency and duration of skin exposure, season and climate, anatomical site, atopic status, race and skin barrier properties[90-93] and interindividual susceptibility has been shown[94].

Detergents are surfactants, which are non-sensitising chemicals functioning to lower surface tension between non-mixable agents. They are common ingredients in many of today's personal hygiene products e.g. toothpastes, shampoos and various soaps. They possess both polar and non-polar regions, i.e., detergents are amphiphilic (with both lipophilic and hydrophilic properties). This characteristic enables them to mix hydrophobic compounds with water[95]. Surfactants are categorized into four types according to their polar portions: anionic, cationic, amphoteric and non-ionic[96].

A common anionic detergent is sodium lauryl sulfate (SLS)[93]. SLS has been widely used to mimic irritant contact dermatitis due to its potential for inducing eczema. To date, it is probably one of the best tried irritants in research on irritant contact dermatitis[93;95;97;98]. Figure 3 illustrates the chemical and structural formula of SLS.

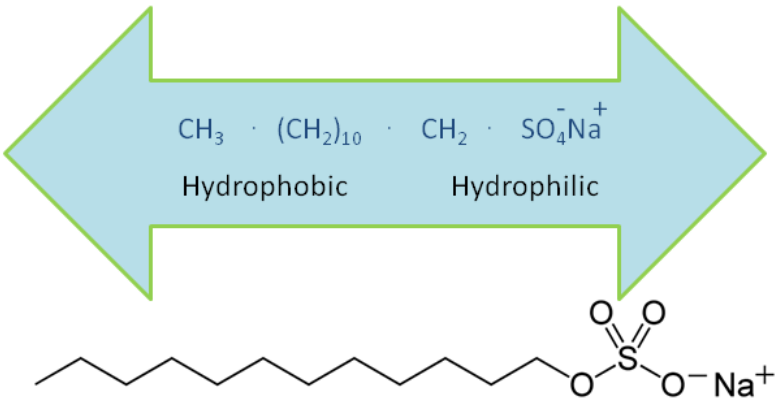

Figure 3. The chemical formula and structure of SLS, depicting its amphiphilicity. Modified from Tupker et al[93].

In 1980 different criteria for experimental topical irritants were suggested. These criteria include: reactions should occur in > 90% upon application, reactions should be reproducible, the irritant should be chemically well-defined and be a non-volatile liquid or solid with no extreme pH value, and be non allergenic, non-allergenic, non-carcinogenic, and have no systemic toxicity, leading to no scarring or staining and give an easily readable skin reaction[99]. SLS fulfils these criteria.

2.5 Irritant contact dermatitis

Irritant contact dermatitis occurs when the irritation load exceeds the defence and the repairing capacity of the skin. The clinical definition of irritant contact dermatitis has been posited as follows: "Irritant contact dermatitis may be defined as a non-allergic inflammatory reaction of the skin to an external agent"[100]. Hence, the diagnosis is based on the exclusion of allergic contact dermatitis. This is a sub-optimal approach but the only feasible method to date. For certain types of irritant contact dermatitis, the prognosis is good[40;87]; however, development into a chronic disease is a risk despite the elimination of the irritant stimulus[101]. Irritant contact dermatitis can occur after a single contact, often with a stronger irritant, causing an acute reaction, or by repetitive exposures of a weaker irritant, causing stepwise deterioration of the skin barrier, leading to a more chronic disease[102].

Recently, studies on both acute and chronic irritant contact dermatitis and skin regeneration after SLS barrier disruption and the role of filaggrin mutations have been published[74;103-111].

Acute Irritant contact dermatitis: There are few studies concerning acute irritant contact dermatitis and the role of filaggrin mutations. Studies comparing wild type mice with filaggrin deficient mice (flaky tail mice) have showed a reduced inflammatory threshold towards irritants when applied in moderate concentrations and an increased irritant reaction in the filaggrin deficient mice[108;109]. Experimental studies with SLS irritation in individuals stratified by AD and filaggrin mutational status have not been able to confirm this increased risk of acute irritant contact dermatitis due to filaggrin mutations[74;103]. Thus more studies are warranted in this field of research.

Chronic irritant contact dermatitis: In a German study filaggrin mutations were found to be risk factors of chronic hand eczema in patients with combined allergic and irritant contact dermatitis, but not in patients diagnosed with irritant contact dermatitis alone[104]. A case-control study has shown an increased risk of developing chronic irritant contact dermatitis among individuals with AD and carriers of filaggrin mutations[105]. In another study both AD and filaggrin mutations were found to be independent risk factors of irritant contact dermatitis, but with the highest risk in those with concomitant AD and filaggrin mutations[106]. However, a follow up study could not confirm that filaggrin was an independent risk factor[110]. It has been posited that filaggrin mutations modify the severity of the clinical course of irritant contact dermatitis in individuals with both AD and filaggrin mutations[107].

Skin regeneration: Only a few studies have addressed skin regeneration after SLS skin irritation taking filaggrin mutations into account. A Swedish study comprising healthy individuals has shown that profilaggrin expression was reduced 6 hours after removal of a chamber with 1% SLS, followed by increased expression on Day 1, 4 and 7 upon chamber removal[111]. In a clinical experimental study investigating 37 filaggrin genotyped AD patients and 20 healthy controls, 1% SLS was applied for 24 hours and the response evaluated at 3, 24, 48 and 72 hours after removal of the chamber. Throughout the 72 hours, no significant difference in TEWL or chromametry a* values was seen between groups[74]. Thus, no difference in regeneration has so far been shown to explain the higher risk of chronicity in the presence of both filaggrin mutations and AD. In the German study, individuals without AD but with filaggrin mutations were not included, and therefore the regeneration associated with carrying filaggrin mutations in the absence of AD could not be assessed[74].

2.6 Methods for non-invasive skin barrier assessments

Different methods can be utilised to evaluate the skin barrier integrity, and below is a short description in alphabetical order of the methods applied in this thesis.

- *Clinical Scoring*: A simple scoring system is available for assessing the clinical reaction followed by SLS irritation. In the ESCD (European Society of Contact Dermatitis) guidelines of acute SLS reactions from 1997[93], six possible scores are given depending on severity of the skin reactions. The clinical scoring guidelines of acute SLS irritant reactions according to ESCD are depicted in Table 2[93].

Table 2

Clinical score	Quality of irritation	Description of skin reactions
0	Negative	No visible reaction
½	Doubtful	Very weak erythema or minute scaling
1	Weak	Weak erythema, slight oedema, slight scaling and/or roughness
2	Moderate	Moderate degree of erythema, oedema, scaling and/or roughness or minute degree of erosions, vesicles, crusting and/or fissuring
3	Strong	Marked degree of erythema, oedema, scaling, roughness, erosions, vesicles, bullae, crusting and/or fissuring
4	Very strong/caustic	As in clinical score 3, with necrotic areas

- *Laser Doppler Flowmetry, LDF:* a blood flow monitor measurement at a wavelength of 785nm +/- 10 nm and a flux range of 0–1000 PU. The probe is a combined large optic and temperature probe, with a probe tip diameter of 8 mm and 90° right-angled probe. The LDF functions by laser light reflecting a moving object (blood cells) and undergoing a Doppler frequency shift depending on the speed of the moving object—the higher speed, the higher the frequency shift and the higher flux[112]. The output is therefore the flux of blood cells, which is defined by the number of blood cells passing times their velocity. An increase in blood flow can indicate a higher inflammatory reactivity.

- *TransEpidermal Water Loss, TEWL:* The measurement of the Tewameter is based on Fick's law of diffusion, where the passive diffusion of water transport from the skin surface is measured in $g/m^2/h$. The probe is an open chamber probe with sensors inside the cylinder measuring the density gradient of water evaporation from the skin indirectly by two sensors measuring temperature and relative humidity. TEWL thereby indicates the ability of the skin barrier to prevent water from evaporating from the skin surface[113]. When the skin barrier is compromised, more water will evaporate, and the output of the measurement will be an increased $g/m^2/h$ as a proxy of the compromised barrier integrity.

3 OBJECTIVES

This thesis consists of an epidemiological, a clinical-experimental and a biochemical study. The aims of this thesis were:

- To explore potential behavioural changes concerning occupational exposures to irritants depending on filaggrin mutations and hand eczema.

- To elucidate whether skin reactivity and regeneration to a detergent (SLS) were affected by the presence of filaggrin mutations and/or atopic phenotype under experimental conditions.

- To develop a method to quantify filaggrin at the protein level in the epidermis from 4 mm skin biopsies and study the level of filaggrin in different patient groups.

- To study whether exposure to an irritant (SLS) induced an effect on the level of filaggrin in epidermis over time.

3.1 Manuscript I

Bandier J, Ross-Hansen K, Carlsen BC, Menné T, Linneberg A, Stender S, Szecsi PB, Meldgaard M, Thyssen J, Johansen JD. *Carriers of filaggrin gene (FLG) mutations avoid professional exposure to irritants in adulthood*. Contact dermatitis. 2013; Dec; 69 (6): 355-62.

Carriers of filaggrin gene (*FLG*) mutations avoid professional exposure to irritants in adulthood

Josefine Bandier[1,*], Katrine Ross-Hansen[1,*], Berit C. Carlsen[1], Torkil Menné[1], Allan Linneberg[2], Steen Stender[3], Pal B. Szecsi[3], Michael Meldgaard[3], Jacob P. Thyssen[1] and Jeanne D. Johansen[1]

[1]*Department of Dermato-Allergology, National Allergy Research Centre, Copenhagen University Hospital Gentofte, Hellerup 2900, Denmark,* [2]*Research Centre for Prevention and Health, Copenhagen University Hospital Glostrup, Glostrup, Denmark, and* [3]*Department of Clinical Biochemistry, Copenhagen University Hospital Gentofte, Hellerup 2900, Denmark*

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Summary

Background. Loss-of-function mutations in the filaggrin gene (*FLG*) are associated with xerosis, atopic dermatitis, and early onset of hand eczema. Irritant exposure is a risk factor for occupational hand eczema, and *FLG* mutations increase the risk of occupational irritant contact dermatitis on the hands in hospital cohorts. It is unknown whether *FLG* mutations affect the level of irritant exposure.

Objectives. To evaluate whether exposure to occupational irritants was dependent on *FLG* mutations, atopic dermatitis, and age at hand eczema onset.

Methods. Randomly chosen Danish adults completed a questionnaire on general health and occupational exposures. Genotyping for *FLG* mutations (R501X, 2282del4, and R2447X) and patch testing were performed.

Results. Overall, 38.7% of subjects reported present or previous occupational exposure to irritants. Among individuals who reported hand eczema onset before entering their work life, 50.6% (45/89) of *FLG* non-mutation carriers became exposed to irritants, as compared with 28.6% (4/14) of heterozygous and 0% (0/6) of homozygous mutation carriers ($p = 0.006$). Avoidance was conspicuous among mutation carriers reporting childhood hand eczema and atopic dermatitis (odds ratio 0.08, 95% confidence interval 0.01–0.65).

Conclusions. Carriers of *FLG* mutations who have had hand eczema onset in childhood avoid occupational exposure to irritants; the association is most marked with homozygous mutation status combined with atopic dermatitis.

Key words: filaggrin mutations; hand eczema; occupational irritant exposure.

Filaggrin proteins help to align keratin filaments in the stratum corneum (1, 2), and are then degraded to hygroscopic amino acids, 'the natural moisturizing factors' (3, 4). When the skin is inflamed, or when ambient humidity drops below 80%, degradation of filaggrin proteins is accelerated to maintain skin hydration (5). Loss-of-function mutations in the filaggrin gene (*FLG*) are observed in ~10% of Caucasian individuals (6), and cause xerosis, scaly skin, and palmar hyperlinearity (7, 8). Pertinently, *FLG* mutations increase the skin pH, reduce hydration, and result in increased transepidermal water loss.

FLG mutations are associated with atopic dermatitis, and early onset and persistence of hand eczema, with a predilection for the dorsal aspects of the hands (9, 10). An association between *FLG* mutations and

Correspondence: Katrine Ross-Hansen, Department of Dermato-Allergology, National Allergy Research Centre, Copenhagen University Hospital Gentofte, Niels Andersens Vej 5, 2900 Hellerup, Denmark. Tel: +45 39777310; Fax: +45 39777118. E-mail: katrine.ross-hansen@regionh.dk

Equal responsibilities as first authors.

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irritant contact dermatitis, as well as combined irritant and allergic contact dermatitis on the hands, has been shown in dermatitis patients (11, 12). Additionally, irritant contact dermatitis caused more sick-leave and a three-fold increased risk of job loss in patients with both *FLG* mutations and atopic dermatitis from a tertiary clinic as compared with controls (13), and was independently associated with *FLG* mutations (14). The above findings indicate that *FLG* mutation carriers have particularly sensitive skin, which increases the risk of hand eczema in early childhood, and which, in occupational patients from tertiary hospitals, increases the risk of irritant contact dermatitis and leads to a poor prognosis. Moreover, *FLG* mutations are associated with skin fissures on the hands and fingers, emphasizing the reduced skin hydration (15).

Whereas atopic dermatitis is the single most important risk factor for hand eczema, exposure to irritants is the most common cause of occupational hand eczema (16). Exposure to wet work alone was reported by 20% of employees in Sweden in 2009 (17). Hairdressing is associated with intense exposure to allergens and irritants, and it is therefore noteworthy that the prevalence of atopic dermatitis in hairdressing apprentices was significantly lower than in controls (18). This 'healthy-worker effect' could be explained by healthcare provider advice prior to choice of profession or reluctance to pursue a professional career with irritant exposure in individuals who already suffer from skin barrier problems. This observation led us to hypothesize that there exists a hitherto unrecognized subtype of atopic dermatitis combined with *FLG* loss-of-function mutations where particularly sensitive skin and early-onset hand eczema could affect career choice, directing people towards professions with little or no exposure to irritants.

In this study, using cross-sectional general population data, we investigated whether exposure to occupational irritants was dependent on common *FLG* mutations, atopic dermatitis, and early or late onset of hand eczema.

Materials and Methods

Study population

In 2006–2008, a cross-sectional study was performed in the western part of Copenhagen. A general health examination was offered to 7931 randomly selected individuals aged 18–69 years, and 3471 people participated (43.7%). The participants completed a questionnaire on general health and occupational exposures. The characteristics of the population have been described previously (19). In total, 3460 (99.7%) individuals were patch tested, and 3346 (96.4%) were genotyped for the three most common Caucasian *FLG* mutations: R501X, 2282del4, and R2447X. The study was approved by the Ethics Committee of Copenhagen County (KA-20060011), and a written informed consent form was obtained from all of the participants.

Questionnaire

Questions concerning the skin were preceded by a description of eczema: 'Eczema is an itching skin disorder showing redness, dryness and possibly vesicles and exudation. Eczema is present in the same area for some time.' Participants who gave an affirmative answer to the question: 'Have you ever had hand eczema' (yes/no), were also asked 'At what age did you have hand eczema on the first occurrence? (before 6 years of age/age 6–14 years/age 15–18 years/after 18 years of age)'. Occupational exposures were assessed with the question: 'Have you, at your past or present workplace, been exposed to any of the following exposures several times a week' (yes/no). Affirmative answers to conditions concerning wet work (partially defined according to the German Approved Code of Practice no. 401) were included in the analyses as occupational irritant exposures: (i) wet work; (ii) use of protective gloves for more than 2 hr/day; and (iii) hand washing at least 20 times daily (20). Additionally, the following exposures were included: 'Cooking or food handling', 'solvents', 'cleaning agents', 'water vapour or moist environments', and 'wet work' (Table 1). The participants were asked to report their educational level based on the categories: 'Skilled or unskilled blue-collar worker', 'short-cycle higher education (< 3 years)', 'medium higher education (3–4 years)', 'long-cycle higher education (> 4 years)', or 'other education'. Atopic dermatitis was defined according to the UK Working Party's diagnostic criteria, with one major criterion and at least two of four minor criteria (21).

Patch testing

Patch testing was performed with the standardized TRUE Test® (Mekos Laboratories, Hilleroed, Denmark). Readings were performed on D2 only (22). Patch test readings of +1, +2 or +3 were considered to be positive. If a patch did not have direct skin contact upon reading, or if participants had removed individual patches from the test series before application because of known allergy status, data were registered as missing.

Table 1. Self-reported occupational irritant exposures

Exposure type	All, % (n) ($n_{total} = 3038$)	*FLG* mutation carriers*, % (n) ($n_{total} = 270$)	*FLG* non-mutation carriers*, % (n) ($n_{total} = 2768$)	χ^2 statistics (*p*)
Cooking or food handling	11.5 (349)	12.7 (34)	11.4 (315)	0.540
Solvents	10.5 (320)	11.5 (31)	10.5 (289)	0.613
Cleaning agents	14.9 (454)	14.6 (39)	15.1 (415)	0.836
Water vapor or moist environment	6.9 (210)	10.1 (27)	6.7 (183)	0.034
Wet work (unspecified)	7.0 (213)	7.2 (19)	7.1 (194)	0.950
Glove use for at least 2 hr/day	16.4 (499)	19.4 (52)	16.3 (447)	0.193
Hand washing at least 20 times/day	15.4 (467)	15.4 (41)	15.5 (426)	0.966
At least one irritant exposure	38.7 (1176)	38.9 (105)	38.7 (1071)	0.949

*Genotyped for the three most common filaggrin gene (*FLG*) loss-of-function mutations (R501X, 2282del4, and R2447X).

FLG genotyping

Genomic DNA was purified from blood samples, and typed for the *FLG* loss-of-function mutations R501X, 2282del4 and R2447X by means of asymmetric, allele-specific polymerase chain reaction (PCR) and tag-specific microbead detection (23). Briefly, multiplex PCR was performed with an excess of biotinylated reverse primers and tagged forward primers, to create single-stranded, allele-specific PCR products. The PCR products were then hybridized to MagPlex C beads with allele-specific capture probes (Luminex, Austin, TX, USA). Analyses were performed on a Bio-Plex 200 device (Bio-Rad, Hercules, CA, USA).

Statistical analyses

Data were processed in SPSS (SPSS Statistics Inc., Chicago, IL, USA; IBM PASW statistics) for Windows™ (release 15.0 and 18.0). Hardy–Weinberg tests were performed with the Hardy–Weinberg calculator from 'Online Encyclopedia for Genetic Epidemiology Studies' (24). The χ^2-test was used to compare categorical characteristics in different groups. For participants reporting hand eczema before 15 years of age, a logistic regression analysis was performed with occupational irritant exposure (yes/no) as the dependent variable and with *FLG* genotype (mutations/no mutations) combined with atopic dermatitis history (atopic dermatitis/no atopic dermatitis), giving four categories, as the independent variable. In participants who denied having hand eczema onset before 15 years of age, a logistic regression analysis was performed with hand eczema onset after 15 years of age as the dependent variable. The independent variables were: sex (male/female), age (18–35/36–55/56–69 years), educational level (skilled or unskilled blue-collar workers/short-cycle higher education/medium-cycle higher education/long-cycle higher education/other), atopic dermatitis (yes/no), contact allergy (yes/no), occupational irritant exposure (yes/no), and *FLG* mutations (yes/no). Associations were expressed as odds ratios (ORs) with 95% confidence intervals (CIs).

Results

In total, 3348 individuals were genotyped for the *FLG* loss-of-function mutations R501X, 2282del4, and R2447X. The combined carrier frequency, including both heterozygous and homozygous carriers, was 9.0% (302/3348), and 12 (0.4%) were homozygotes. The population distribution of the 2282del4 mutation was in Hardy–Weinberg equilibrium ($p = 0.149$), whereas Hardy–Weinberg testing gave *p*-values of 0.05 and 0.0007 for R501X and R2447X, respectively.

All participants

Similar proportions of *FLG* mutation carriers and non-mutation carriers reported exposure to at least one type of irritant in their past or present occupation (38.9% versus 38.7%). The homozygous *FLG* mutation carriers reported less occupational irritant exposure than heterozygous and non-mutation carriers, but the difference was not statistically significant significant [18.2% (2/11), 39.8% (103/259), and 38.7% (1071/2768), respectively, $p = 0.354$]. In the case of atopic dermatitis, occupational irritant exposure was reported more often in individuals who had atopic dermatitis than in individuals who did not [45.5% (136/299) versus 37.8% (1036/2739), $p = 0.01$]. No difference in the overall number of occupational irritant exposures was observed between mutation carriers and non-mutation carriers (Fig. 1a).

Participants who reported hand eczema at some point

The irritant exposure load was also evaluated among individuals who reported having had hand eczema at

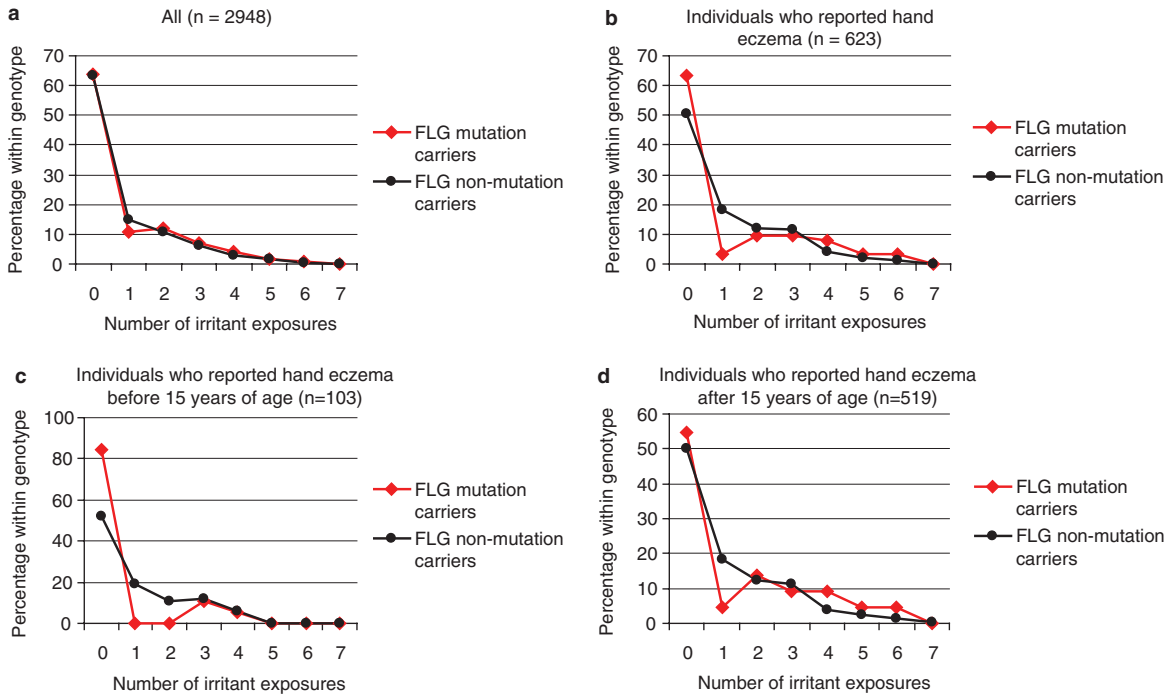

Fig. 1. Number of occupational irritant exposures per individual stratified by filaggrin genotype (*FLG*).

some point in their lives (Fig. 1b). More *FLG* mutation carriers than non-mutation carriers reported absence of occupational irritant exposure [63.5% (40/63) versus 50.5% (283/560), $p = 0.051$], and of the 8 homozygous carriers, none reported occupational irritant exposures. Fewer individuals with *FLG* mutations reported a single irritant exposure [3.2% (2/63) versus 18.4% (103/560), $p = 0.002$) (Fig. 1b)].

Participants who reported having had hand eczema before 15 years of age

We evaluated the irritant exposure pattern among individuals who had their first occurrence of hand eczema before entering their work life and thus exposure to occupational irritants, that is, before 15 years of age in Denmark (Fig. 1c and Table 2). Here, the number of irritant exposures in adulthood differed markedly between *FLG* mutation carriers and non-mutation carriers (Fig. 1c), and avoidance of occupational exposure was associated with *FLG* mutation status in a dose-dependent manner (Table 2). Hence, among individuals who reported childhood hand eczema, 50.6% (45/89) of non-mutation carriers became occupationally exposed to irritants, as compared with 28.6% (4/14) of heterozygous carriers and 0% (0/6) homozygous carriers ($p_{\text{linear-by-linear}} = 0.006$). The four *FLG* mutation carriers with occupational irritant exposure all reported glove use for more than 2 hr/day, whereas only 37.8% (17/45) of the non-mutation carriers did so ($p = 0.028$). To evaluate whether the observation was confounded by a varying prevalence of atopic dermatitis according to genotype status, we analysed the combined effect of *FLG* mutations and atopic dermatitis (Table 3). Although significantly more *FLG* mutation carriers [70.0% (14/20)] than non-mutation carriers [(30.6% (26/85)] ($p = 0.001$) had atopic dermatitis, avoidance of occupational irritant exposure was not explained by having a history of atopic dermatitis. Hence, non-mutation carriers with atopic dermatitis chose occupations with irritant exposure as frequently as individuals without atopic dermatitis, irrespective of *FLG* genotype status (~ 50.0%, Table 3). Avoidance of irritant exposure in adulthood was associated with combined *FLG* mutation and atopic dermatitis status ($OR_{\text{FLG mutations, atopic dermatitis}} = 0.08$, 95% CI 0.01–0.65; Table 3), and 6 of the 6 carriers who were homozygous for *FLG* mutations were found in this group.

Participants who reported hand eczema after 15 years of age

In individuals who reported hand eczema onset after 15 years of age, absence of occupational irritant

Table 2. The implications of filaggrin genotype for occupational irritant exposure in adulthood among individuals who reported hand eczema onset before 15 years of age

		Occupational irritant exposure, % (n/n_{total})	χ^2 statistics
FLG mutation carrier status*	None	50.6 (45/89)	$p_{linear\text{-}by\text{-}linear} = 0.006$
	Heterozygous	28.6 (4/14)	
	Homozygous	0.0 (0/6)	
Atopic dermatitis†	Yes	35 (14/40)	$p = 0.197$
	No	47.8 (32/67)	

FLG, fillagrin gene.
*Genotyped for the three most common loss-of-function mutations (R501X, 2282del4, and R2447X).
†Defined according to the UK Working Party's diagnostic criteria for atopic dermatitis (one major criterion and at least two of four minor criteria).

exposure was independent of *FLG* mutation status (Fig. 1d), whereas a tendency not to be exposed to just one occupational irritant was again found among mutation carriers as compared with non-mutation carriers.

A risk factor analysis on hand eczema onset in adulthood (after 15 years of age) is presented in Table 4, and shows that atopic dermatitis ($OR_{crude} = 3.59$, 95% CI 2.77–4.66; $OR_{adjusted} = 3.35$, 95% CI 2.48–4.54), contact allergy ($OR_{crude} = 1.57$, 95% CI 1.19–2.06; $OR_{adjusted} = 1.39$, 95% CI 1.01–1.91) and occupational irritant exposure ($OR_{crude} = 2.04$, 95% CI 1.69432.46; $OR_{adjusted} = 1.89$, 95% CI 1.53–2.34) were significantly associated factors, whereas *FLG* mutations did not increase the risk of hand eczema with an adult onset. In the adjusted analysis, the variables sex, age and educational level were included, in addition to the above-mentioned variables.

Table 3. Logistic regression analysis on the implications of filaggrin genotype and atopic dermatitis for occupational irritant exposure in adulthood among individuals who reported hand eczema onset before 15 years of age

	Occupational irritant exposure, % (n/n_{total})	OR (95% CI)
No *FLG* mutations† No atopic dermatitis‡	49.2 (29/59)	1.00 (reference)
No *FLG* mutations† Atopic dermatitis‡	50.0 (13/26)	1.03 (0.41–2.60)
FLG mutations† No atopic dermatitis‡	50.0 (3/6)	1.03 (0.19–5.55)
FLG mutations† Atopic dermatitis‡	7.1 (1/14)	0.08 (0.01–0.65)*

CI, confidence interval; *FLG*, fillagrin gene; OR, odds ratio.
*$p < 0.05$.
†At least one of the three most common loss-of-function mutations (R501X, 2282del4, and R2447X).
‡Defined according to the UK Working Party's diagnostic criteria for atopic dermatitis (one major criterion and at least two of four minor criteria).

Discussion

This cross-sectional general population study showed that individuals with and without *FLG* loss-of-function mutations were equally exposed to irritants in their professional lives. However, having *FLG* mutations and hand eczema before the age of 15 years resulted in significantly and markedly reduced occupational exposure to irritants, particularly among the carriers who were homozygous for *FLG* mutations. From the pioneering studies by Rystedt (25), it is clear that individuals with atopic dermatitis and hand eczema in childhood have a particularly high risk of having hand eczema as adults. As we expected that having childhood hand eczema would affect career choice in adulthood, it was decided to perform data analyses taking the age at onset of hand eczema into consideration. This led to the novel finding that only 20% of individuals with childhood hand eczema carrying *FLG* null mutations were occupationally exposed to irritants, as compared with 50% of the non-mutation carriers. Thus, a large group in the population tends to avoid professional exposure to irritants, which can be identified by combining a clinical disease (hand eczema in childhood) and a genotype (*FLG* loss-of-function mutations). Furthermore, it appeared that *FLG* mutation carriers protected their hands by more extensive use of gloves, although large case series are required to confirm this. We also showed that individuals with atopic dermatitis, childhood hand eczema and *FLG* loss-of-function mutations seemed to completely avoid occupational exposure to irritants.

In a recent study on hairdressing apprentices, a significantly lower frequency of atopic dermatitis was found among the apprentices (21.4%) than in a general population control group (29.8%) (18). Earlier studies have shown that there is a group of individuals with atopic dermatitis dominated by females, who develop early-onset occupational hand eczema (26, 27). The lower prevalence of atopic dermatitis in hairdressing apprentices could be explained by successful primary prevention of hand eczema following healthcare provider advice, or by individual avoidance behaviour because of dermatitis. Two recent studies from a German/Dutch group showed that *FLG* mutations alone, and especially

Table 4. Risk analyses of hand eczema onset in adulthood

		All except individuals who reported hand eczema onset before 15 years of age, % (n/n_{total})	Hand eczema onset in adulthood†, % (n/n_{total})	OR for the explanatory variables (95% CI)	
				Crude OR‡	Adjusted OR§
Atopic dermatitis¶	No	91.1 (2807/3082)	16.7 (468/2807)	1.00 (reference)	1.00 (reference)
	Yes	8.9 (275/3082)	41.8 (115/275)	3.59 (2.77–4.66)**	3.35 (2.48–4.54)**
Contact allergy††	No	90.3 (2867/3173)	18.0 (515/2867)	1.00 (reference)	1.00 (reference)
	Yes	9.7 (309/3176)	25.6 (79/309)	1.57 (1.19–2.06)*	1.39 (1.01–1.91)*
Occupational irritant exposure††	No	61.6 (1791/2907)	14.3 (256/1791)	1.00 (reference)	1.00 (reference)
	Yes	38.4 (1116/2907)	25.4 (283/1116)	2.04 (1.69–2.46)**	1.89 (1.53–2.34)**
FLG mutations§§	No	91.4 (2913/3187)	18.6 (543/2913)	1.00 (reference)	1.00 (reference)
	Yes	8.6 (274/3187)	19.0 (52/274)	1.02 (0.75–1.40)	0.95 (0.65–1.38)

CI, confidence interval; *FLR*, fillagrin gene; OR, odds ratio.
*Significant difference in χ^2-tests, $p < 0.001$.
**Difference in χ^2-tests, $p < 0.05$.
†An affirmative answer to the question: 'Have you ever had hand eczema?' and reporting year of onset $\geq$ 15 years of age.
‡Effect of the noted variable on the risk of hand eczema onset in adulthood (each separately).
§Effect of the noted variable on the risk of hand eczema onset in adulthood in a regression analysis including adjustment for sex, age, and educational level.
¶Defined acording to the UK Working Party's diagnostic criteria for atopic dermatitis (one major criterion and at least two of four minor criteria).
††Defined as positive patch test readings (+, ++, +++) according to the International Contact Dermatitis Research Group criteria.
††An affirmative answer regarding past or present occupational exposure to at least one of the following: cooking or food handling, solvents, cleaning agents, water vapour or moist environment, wet work (unspecified), glove use for at least 2 hr per day, and hand washing at least 20 times per day.
§§At least one of the three most common *FLG* loss-of-function mutations (R501X, 2282del4, and R2447X).

in combination with atopic dermatitis, were associated with occupational irritant contact dermatitis on the hands (13, 14). Furthermore, *FLG* mutations and atopy resulted in a poor prognosis (13). Interestingly, we showed that *FLG* mutations did not constitute an independent risk factor for adult-onset hand eczema in a general population cohort. This finding emphasizes that having an inherited barrier abnormality is of less importance than excessive environmental exposure to irritants and contact allergens for the development of hand eczema. Also, atopic dermatitis was, as expected, a strong risk factor for hand eczema, which could be explained by the chronic capacity in an individual to develop skin inflammation when exposed to various stimuli rather than the barrier abnormality. Our findings fit with the recent findings from Germany and The Netherlands, as *FLG* mutations impair the prognosis markedly once hand eczema has developed because of the barrier abnormality.

Among Danish dermatitis patients with occupational hand eczema, irritant contact dermatitis preceded allergic contact dermatitis, as it occurred at a lower age (27). This finding is in line with immunological and epidemiological data, which were recently reviewed (28). Also, a recent general population study showed that *FLG* mutations were strongly associated with contact sensitization in individuals who reported dermatitis (29). One may therefore suggest that the risk of allergic contact dermatitis can ultimately be reduced by limiting the onset and severity of irritant contact dermatitis, especially in those with *FLG* mutations. Future studies are required to investigate this.

The definitions of hand eczema, occupational irritant exposure and atopic dermatitis relied on questionnaire data, which potentially introduce bias and misclassification. However, the questions concerning atopic dermatitis have been validated by the UK Working Party, and have a high specificity and a moderate sensitivity (21). Unfortunately, we did not have detailed information about irritant exposure. Patch test readings were performed only on D2, which may cause both false-negative and false-positive reactions. Nonetheless, studies like this usually convey important information on the burden of disease in a general population. Owing to a lack of the relevant parameters, we were not able to evaluate the course of hand eczema in individuals who reported childhood hand eczema and yet became occupationally exposed to irritants.

In summary, *FLG* mutations combined with childhood hand eczema onset result in reduced occupational

exposure to irritants, which is most markedly associated with homozygote status combined with atopic dermatitis. On the basis of the recent finding that *FLG* mutation carriers have an increased risk of occupational irritant contact dermatitis and a poor prognosis (13, 14), health-care providers should continue to take specific preventive measures for this particular group of individuals.

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3.2 Manuscript II

Bandier J, Carlsen BC, Jelstrup Petersen L, Johansen JD. *Skin reaction and regeneration after single SLS exposure stratified by filaggrin genotype and atopic dermatitis phenotype*. British Journal of Dermatology. 2015 Jan 10. doi:10.1111/bjd. 136512014

Title: Skin reaction and regeneration after single SLS exposure stratified by filaggrin genotype and atopic dermatitis phenotype

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Authors: Bandier J[1], Carlsen BC[1], Rasmussen MA[2], Petersen LJ[3,4] and Johansen JD[1].

Institutions:

[1]National Allergy Research Centre, Department of Dermato-Allergology, Copenhagen University Hospital Gentofte, Hellerup, Denmark. [2]Faculty of Science, University of Copenhagen, Frederiksberg. [3]Department of Nuclear Medicine, Aalborg University Hospital and [4] Department of Clinical Medicine, Aalborg University, Aalborg, Denmark.

Corresponding author:

Josefine Bandier
National Allergy Research Centre,
Department of Dermato-Allergology
Copenhagen University Hospital Gentofte
Niels Andersens Vej 65
2900 Hellerup, Denmark
Email: josefine.Bandier@regionh.dk
Phone: 39 77 73 40
Fax: +45 39 77 71 18

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What's already known about this topic?

Subjects with AD have barrier defects and increased susceptibility to irritants.
Studies have suggested that filaggrin mutations facilitate penetration of irritants, which could explain an increased risk of ICD among patients with AD and filaggrin mutations.

What does this study add?

Skin response and regeneration after SLS barrier disruption is more depended on AD than on filaggrin genotype.
Those with both mutations and AD have a higher inflammatory alertness, with a more rapid inflammatory response.

Abstract

Background: Filaggrin is key for the integrity of the stratum corneum. Mutations in the filaggrin gene (FLGnull) play a prominent role in the atopic dermatitis (AD) pathogenesis. Persons with AD have an increased susceptibility to irritants. However, little is known about the effect of filaggrin genotype and AD phenotype concerning irritant response and skin regeneration.

Objectives: To investigate the role of FLGnull and AD concerning skin reaction and recovery after Sodium Lauryl Sulfate (SLS) irritation.

Methods: Case-control study comprising 67 subjects, including healthy controls and patients with and without FLGnull and AD. Reactivity to different doses of SLS at 24, 48, 72 and 145 hours after SLS application was measured by transepidermal water loss (TEWL) and laser Doppler flowmetry (LDF). Reactivity was assessed univariately and by pattern analysis technique.

Results: All patient groups showed a higher degree of skin barrier disruption and inflammation than did controls in response to SLS. Assessing reactivity by ΔAUC both TEWL and LDF showed significant difference between healthy controls and those with the AD phenotype irrespective of filaggrin mutation. The poorest regeneration was among those with the AD phenotype.
The two AD phenotype groups were separated by multivariate technique, due to earlier inflammatory reactivity among subjects with FLGnull$_{plus}$AD compared to the AD phenotype alone.

Conclusions: Both skin reaction and regeneration was significantly different between the patient population and the healthy controls. Additionally, response severity and regeneration depended more on phenotype (atopic dermatitis) than on filaggrin genotype, whereas the response was more rapid among FLGnull$_{plus}$AD individuals.

Introduction

Filaggrin is a key epidermal protein primarily located in the stratum corneum. Filaggrin has several complex interrelated functions involving properties of mechanical strength, hydration and resistance, and in regulation of the epidermal homeostasis [1-3]. Frameshift or nonsense mutations in the epidermal differentiation complex on chromosome 1q21, result in either a partial or total lack of filaggrin dependent on heterozygote or homozygote status [4-6], which endogenously influences the skin barrier integrity.

Exogenous substances, such as irritants, commonly affect skin barrier integrity. The severity of the clinical manifestation of dermatitis is influenced by: the concentration and potency of the irritant, the skin contact time, anatomical site, seasonal changes and individual susceptibility [7-11].

In 1982 Malten suggested that the greater the response to irritancy, the poorer the skin regeneration[12]. Studies showing increased penetration of water-soluble substances in mice and penetration of dye in an in vitro skin model, suggests that filaggrin deficiency facilitates penetration through the skin barrier [13;14]. Accordingly, subjects with filaggrin-deficient skin barrier may have a more severe primary response and/or a longer skin recovery after induced irritant reactions; concomitantly, both the skin response and recovery to irritancy would also depend on the AD phenotype, as other studies have shown increased response to Sodium Lauryl Sulfate (SLS) due to phenotype alone [15;16]. Only few studies have investigated the skin response and regeneration according to AD phenotype and filaggrin genotype together [17-20]. Moreover, skin regeneration after SLS barrier disruption has only been investigated in a study with the last measurement on Day 3 [18] and in a study on mRNA expression of profilaggrin on Day 7 [21].

Hence, we investigated the response to SLS with non-invasive methods in 4 groups: a healthy control group—without AD and without filaggrin mutations; a group without AD and with filaggrin mutations; a group with AD and without filaggrin mutations; and a group with both AD and with filaggrin mutations. We investigated the reaction severity and the following regeneration after barrier disruption and hypothesized that filaggrin mutations would influence the skin barrier function with increased reactivity and decreased skin recovery.

Material Methods

Participants

This case-control observational study comprised 67 individuals, of whom 47 were patients recruited through review of medical files at the department of Dermato-Allergology at Gentofte Hospital Denmark. We recruited 20 healthy controls through online advertising. The distribution of age and sex was equal between all groups.

The pre-sample size to identify significant higher TEWL among $FLGnull_{plus}AD$ compared to controls were calculated by online calculator (http://www.openepi.com)

with 80% power and p-value= 0.05.
Table 1 shows the eligibility criteria.

AD was assessed by using the UK Working Party´s Diagnostic Criteria [22] and filaggrin mutation status for R501X, 2282del4 and 2447X were determined by multiplex analysis[23]. Filaggrin homozygosity, compound heterozygosity, and heterozygosity were pooled and analysed as one group (FLGnull).

None of the patient had Ichtyosis Vulgaris.

All participants received both oral and written information and consent was obtained from all participants. The study was approved by the Ethics Committee of Copenhagen County (project identification: H-1-2011-011) and by the Danish Data Protection Agency.

Table 1

Inclusion, exclusion and restrictions of the study population during the trial

Eligibility criteria	Hospital population, Ntotal= 47 ($FLGwt_{plus}AD$, $FLGnull_{non}AD$ and $FLGnull_{non}AD$)	Healthy controls, Ntotal = 20
Inclusion	Known atopic status, known filaggrin status and age between 18 and 69 years. Caucasian.	Age 18–69 years. Caucasian.
Exclusion	Disease: Active or widespread eczema (but minor eczema was accepted) or chronic inflammatory diseases besides eczema (rheumatoid arthritis, Crohn's disease, Colitis ulcerosa, Systemic lupus erythromatosis or psoriasis). Treatments: No (UV- treatment of test area within 3 weeks of trial, treatment with topical steroids 2 weeks before trial, application of emollients on the test areas throughout the trial, immune-modulating medical treatment).	No actual or prior eczema. Otherwise both disease and treatment exclusion were as for the hospital population.
Restrictions	No smoking and caffeine consumption within 2 hours before measurements, no consumption of food within 4 hours of measurements, and no analgesics within 24 hours prior to measure.	The same as for the hospital population.

FLGwt are participants without one of the three most common filaggrin mutations (501X, 2282del4, and 2447X). FLGnull are participants with at least one of the three most common filaggrin mutations (501X, 2282del4, and 2447X). nonAD are participants without atopic dermatitis (AD) but with eczema and plusAD are with AD according to the UK Working Party's diagnostic criteria for atopic dermatitis.

Distribution of the 4 different groups according to the phenotype AD/non AD and genotype (FLGnull/FLGwt) constituted 20 $FLGwt_{non}AD$ (healthy controls), 10 $FLGnull_{non}AD$, 19 $FLGwt_{plus}AD$, and 18 $FLGnull_{plus}AD$ as illustrated in Fig. 1.

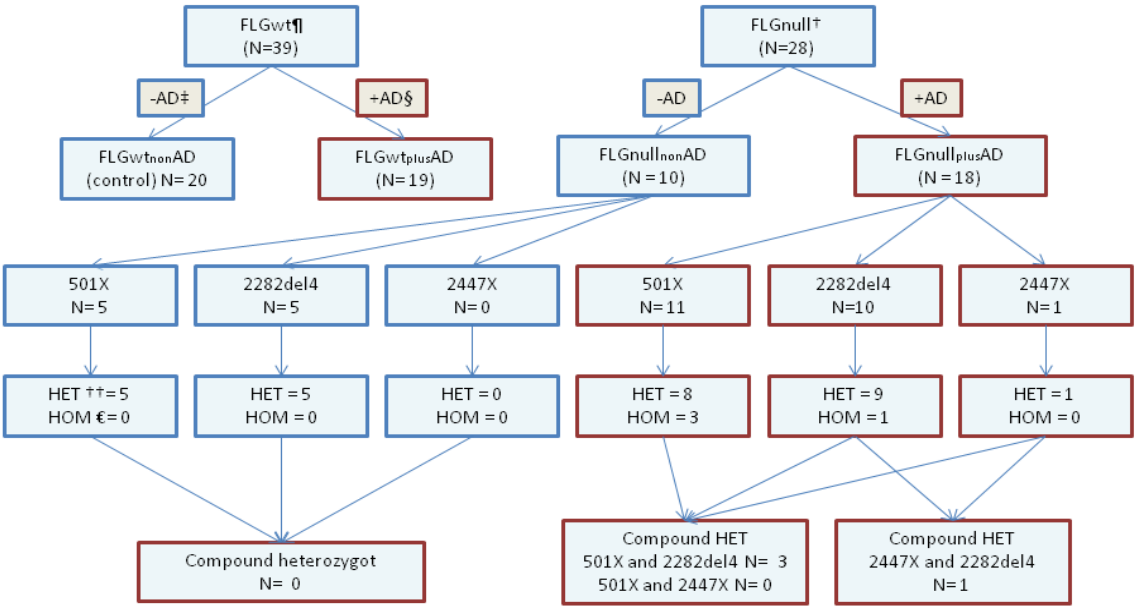
During the study, 1 participant dropped out on Day 2 and one on Day 8. Their data were addressed as missing those days and forward in the trial.

Test parameters and equipment

To investigate response and regeneration of the skin, measurements reflecting skin barrier were performed; respectively transepidermal water loss (TEWL), dermal inflammation by laser Doppler flowmetry (LDF), and skin pH. Clinical assessment of the irritant reactions due to SLS was performed using the simple scoring system by the ESCD guidelines [9]. The TEWL (g/m^2/hour) measurements were performed according to established guidelines [24].

Figure 1

Distribution of the atopic dermatitis phenotype and filaggrin genotype



Categorization of types and distribution of filaggrin mutation and atopic dermatitis (AD). Ntotal = 67 subjects. The red boxes illustrate those with AD. ¶ Subjects without any of the three most common filaggrin mutations (501X, 2282del4, and 2447X).
† Subjects with one of the three most common filaggrin mutations (501X, 2282del4, and 2447X).
‡ Without AD, according to the UK Working Party's diagnostic criteria for atopic dermatitis.
§ With AD, according to the UK Working Party's diagnostic criteria for atopic dermatitis.
†† Heterozygote for a filaggrin mutation, having filaggrin mutation on one allel.
€ Homozygote for a filaggrin mutation, having filaggrin mutations on both allels.

All probes were calibrated according to fabrication guidelines. Information on the non-invasive bioengineering electronic devices is shown in Table 2 and the experimental protocol presented in Table 3.

Data analysis and statistics

The normal distribution of the data was tested by the Shapiro-Wilks test, showing in general skewed data ($p < 0.05$), thus non-parametric tests were used (Kruskal-Wallis and Mann-Whitney test).

Wilcoxon test was performed to analyse repeated measures of 2 conditions. Chi^2 statistics was performed to analyse the distribution of the clinical scores by linear-by-linear associations between individual groups.

AUC, Δreactions and regeneration (R) of the skin was evaluated as described under forms in table 3.

Table 2

Information on the non-invasive bioengineering electronic devices and SLS

Manufacturer	Probe name	Performance of measurement
The MPA5 multiprobe adaptor system from Enviroderm, Courage+ Khazaka electronic GmbH, CK electronic	TM300, a preheated probe to measure TEWL	Duration, 30 sec and an average was created from the last 10sec of steady state.
	PH905, measuring pH.	Triplet measurements with 5 sec between measurements, the mean value was used in the analysis
	Skin thermometer probe	Two consecutive skin measurements were performed creating an average.
	A room condition probe	Measuring temperature and humidity continuously.
The laser Doppler flowmetry was a MoorVMS-LDF1 from Moor Instruments Ltd, UK.	MoorVMS-LDF1 single channel 785nm vascular monitoring system with VP1T/7 probe with integrated temperature probe	A mean LDF value was made from 3 consecutive measurements with a steady state for 15 sec. The skin blood flow was measured in arbitrary units (PU).
SLS from Sigma-Aldrich, Steinheim, Germany, 99% purity.		Dilations in aqueous solution were made, 1%, 0.50% and 0.25% SLS. 50µL SLS was added to a 12mm Finn chamber® on scanpore® tape with filter disc from vitaflo Scandinavia (navamedic AB) [9;47] and left for approximately 24 hours upon removal. Measurements were made 1 hour after removal of the chambers [48] and all subjects were acclimatized for 15 min in a quiet setting before all measurements.

Supplementary to the univariate evaluations of LDF, TEWL, pH, clinical score and the derived delta measures and R, we employed a pattern analysis technique Anova Simultanous Component Analysis (ASCA)[25] for extraction of patterns while sustaining the power of the study design. This was conducted on a matrix with 201 samples (67 by three SLS concentrations) and 26 variables.

Multivariate significance testing was by permutation testing with 10.000 permutations examining the effect of SLS concentration and FLG/AD classes. Data were processed in the Statistical Products and Service Solutions package (SPSS statistics Inc., Chicago, IL, USA; IBM PASW statistics) for Windows, version 19.0 for the univariate analysis, and by Matlab R2014a for MAC (Mathworks® Inc, Natick, MA, USA) for the multivariate analysis.

Table 3

Clarification of the study protocol during the trial

Experimental protocol	
The study period	End October 2011– mid-March 2012.
Randomization of skin sites to SLS	Skin sites on the upper right arm were blindly randomized to an aqueous solution of 0.25%, 0.50% and 1.00% SLS. The first chamber was placed 9 cm from the centre of the axillary fossa and the other chambers in distal extension hereof.
Positioning of the study population	All test subjects were positioned in the same manner with the head of the bed slightly elevated and with the right arm placed behind the head.
Measurement days	The baseline measurements were performed 9 cm distal from the centre of the axillary fossa on non-lesional skin, before irritant application. The measurements were made at baseline before SLS application (Day 1), at 24 hours (Day 2, 1 hour after removal of chamber), 48 hours (Day 3, 25 hours after removal of chamber) and Day 8 after the SLS application (145 hours after removal of Finn chamber).
Forms	- Δreactions: Here we subtracted the baseline measurements from the Day 2, 3 and 8 measurements, to evaluate the pure reaction caused by SLS. - AUC: This was calculated by i.e. ((medianLDF0.25%SLS+median0.50%SLS)/2)·(0.50-0.25) + ((medianLDF0.50%SLS+median1.00%SLS)/2)·(1.00-0.50) - Regeneration (R): This was evaluated as follows: if an increase from baseline to max response was Δ_{max} and the difference between baseline and Day 8 was Δ_8, then R can be expressed in percentage of Δ_{max}: $R= 100 \cdot (\Delta_{max}-\Delta_{78})/ \Delta_{max}$. Thus if R= 100%, the irritated skin will be fully regenerated back to baseline value.
Order of the different measurements and measurement conditions	All visual scorings and instrumental measurements were performed by J. Bandier and in the following order: clinical scoring, skin temperature, TEWL, pH and LDF. Measurements were performed without direct influence of external light sources (blinds drawn and with only soft indirect lighting).

Results

Of the participants, 28 were men and 39 women, median age 38 years (range 18–68 years). During measurements, the room temperature was kept constant at a mean 24.3 ± 0.83 (range 22.0–26.8) ^{o} C and the mean humidity of 36% ± 6.23 (Range 22%-64%).

Assessment of baseline values

Baseline values of TEWL, LD, pH and skin temperature according to phenotype and genotype are illustrated in Fig. 2.

There was no significant difference in overall analysis between groups in either LDF, TEWL, pH or skin temperature between the groups (respectively p= 0.17, p= 0.066, p= 0.75 and p= 0.130). The results on pH are published elsewhere[26]. No further notion will be taken on skin temperature.

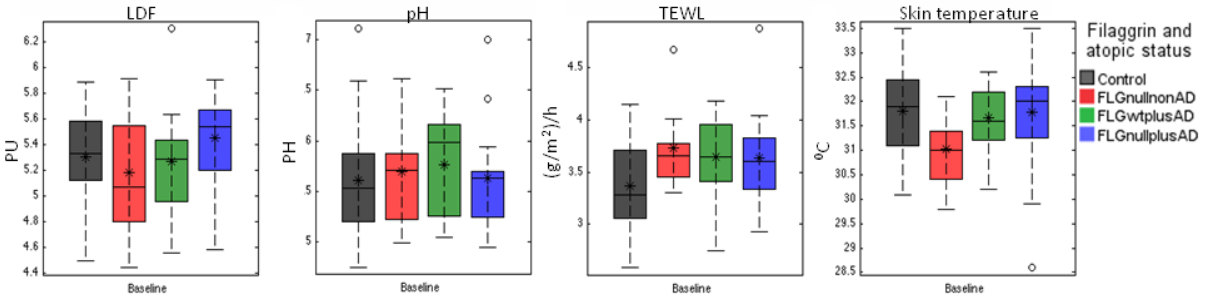
Skin responses to SLS

Assessment of pH

Variation in pH over time is illustrated in Fig. 3.

Figure 2

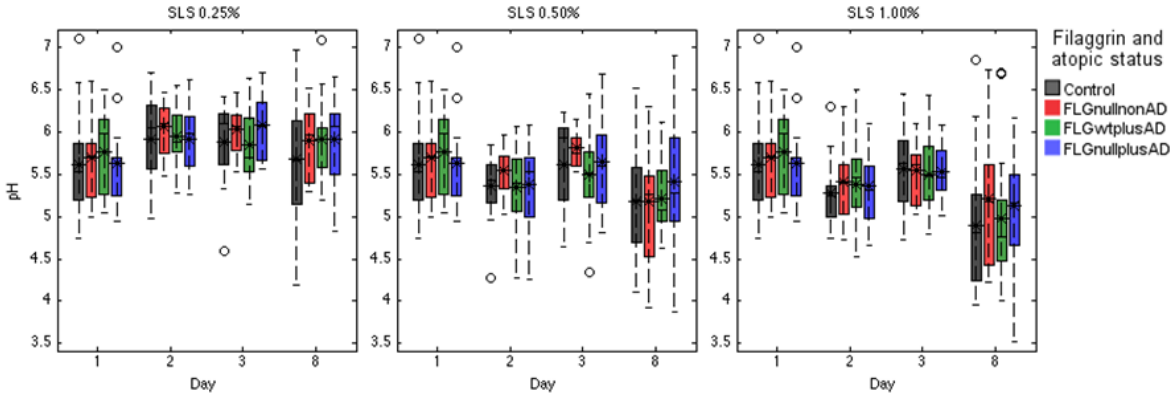
Baseline values (Day 1)



Box Whisker-Plots of baseline laser Doppler flowmetry (LDF), transepidermal water loss (TEWL), pH and skin temperature values, stratified by AD and filaggrin genotype, Ntotal= 67.

Figure 3

Changes in pH over time according to phenotype and genotype



Time variation of pH from baseline to Day 8 according to atopic dermatitis (AD) and filaggrin mutations. $Ntotal_{Day1}$ = 67, $_{Day2}$ = 66, $_{Day3}$= 66 and $_{Day8}$= 65.

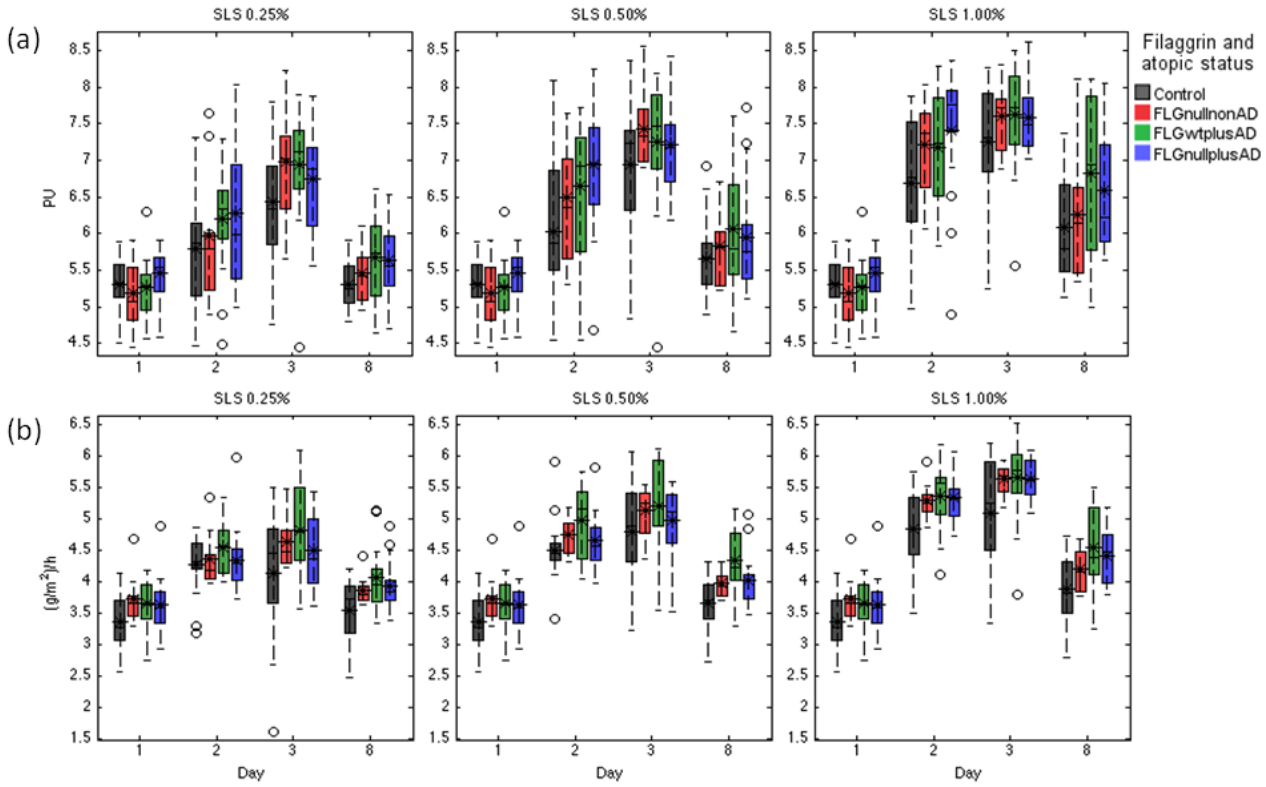
Laser Doppler flowmetry and TEWL

Skin reactions from baseline values to the different doses of SLS are illustrated in Fig. 4. Significant differences in the Δreactions are shown in Table 4.

At the highest concentration, 1.00% SLS, a significant difference in ΔLDF and ΔTEWL was seen in all 4 groups from baseline to Day 2 and Day 3, assessed by the Wilcoxon signed rank test ($p < 0.005$).

Figure 4

Measurement of laser Doppler flowmetry and TEWL according to filaggrin genotype and atopic status



a) LDF box Whisker-Plots of the 4 groups at 0.25%, 0.50% and 1.00% SLS over time, stratified by AD and genotype, $Ntotal_{dDay1} = 67$, $_{Day2} = 66$, $_{Day3} = 66$ and $_{Day8} = 65$.

Table 4

Skin reaction to different doses of SLS Day 2

Comparison of skin reactions	0.25% SLS	0.50% SLS	1.00% SLS
Δreaction LDF	-	Control vs FLGwtplusAD * Control vs FLGnullplusAD ***	Controls vs FLGnullplusAD***
Δreaction TEWL	-	-	Controls vs FLGwtplusAD*** Controls vs FLGnullplusAD*

Level of significance < 0.05; *p < 0.05; **p< 0.01, ***p< 0.005 in comparison with healthy controls; Ntotal= 66.

For both TEWL and LDF, maximum responses occurred on Day 3, except for the FLGnull$_{plus}$AD group LDF, where a maximum was reached on Day 2 (Fig. 4). All patient groups displayed a higher LDF and TEWL response compared with healthy controls at the highest SLS concentration (Fig. 4).

When analysing the reaction to different doses of SLS, there was a conspicuous difference on Day 2 primarily between those with the AD phenotype, FLGwt$_{plus}$AD and FLGnull$_{plus}$AD compared with the healthy controls Table 4.

To evaluate the total response of each individual throughout each dose of SLS, area under the curve (AUC) was calculated and the results are shown in Table 5.

No significant differences between groups on Day 3 were seen in either AUC-LDF or AUC-TEWL.

Clinical scores

All 4 groups displayed a maximum reaction to 1.00% SLS. No significant difference was detected between the groups at the lowest dose of SLS. At the concentration of 0.50% SLS, a significant difference between healthy controls and FLGnull$_{plus}$AD, P= 0.0056 was detected on Day 2. At the maximum SLS dose, a significant difference was found between healthy controls and FLGwt$_{plus}$AD and controls and FLGnull$_{plus}$AD (p= 0.040 and p= 0.00029 respectively). No difference at either concentrations occurred on Day 3.

Table 5

Reactivity to SLS by ΔAUC on Day 2 and Day 3, evaluated by transepidermal water loss (TEWL) and laser Doppler flowmetry (LDF)

ΔAUC Day 2, Ntotal	TEWL 50% percentile	TEWL Interquartile range	LDF 50% percentile	LDF Interquartile range
Controls	10,3	8.35-13.39	30,31	21.02-52.84
FLGnull$_{non}$AD	14,00	8.91-16.20	60,02	15.62-101.83
FLGwt$_{plus}$AD	17,46 ***	11.49-20.50	64,86 *	33.22-94.05
FLGnull$_{plus}$AD	13,31	10.48-16.77	66,42 ***	47.70-133.27
ΔAUC Day 3, Ntotal				
Controls	18,44	7.08-21.19	34,26	19.50-58.16
FLGnull$_{non}$AD	18,56	13.63-24.39	58,13	32.72-104.56
FLGwt$_{plus}$AD	23,40	15.51-32.34	57,48	43.17-69.90
FLGnull$_{plus}$AD	19,41	11.88-23.98	41,08	30.55-57.72

Level of significance < 0.05; *p < 0.05; **p< 0.01, ***p< 0.005 in comparison with healthy controls.
The results are presented in quartiles. Ntotal$_{Day2}$ and $_{Day3}$= 66.

Skin regeneration

Laser Doppler Flowmetry and TEWL

For LDF on Day 8, no significant differences between groups were seen at any of the three concentrations. For TEWL, a significant difference between healthy controls and AD individuals irrespective of filaggrin mutation status was seen at the 1.00% SLS site (p= 0.015 and p= 0.024).

For regeneration R, full regeneration for the healthy controls was seen after exposure to the lowest dose of SLS (0.25%) and poorer regeneration when exposed to 1.00% SLS, displaying a dose-response regeneration. The same pattern was seen in TEWL; however, none of the 4 groups was fully regenerated even at 0.25% SLS (Table 6).

No significant differences in R between groups were detected in any of the 3 concentrations for either LDF or TEWL. For AUC, no significant difference between groups was seen when analysing on ΔAUC LDF, whereas ΔAUC-TEWL significantly differed between healthy controls and $FLGwt_{plus}AD$, p= 0.007.

Clinical Score

A significant difference occurred at both 0.50 and 1.00% SLS between the groups healthy controls and $FLGnull_{plus}AD$ and between $FLGwt_{plus}AD$ and $FLGnull_{plus}AD$, p< 0.005.

Table 6

The regeneration calculated from medians by the formula: $R= 100\cdot (\Delta_{max}-\Delta_8)/ \Delta_{max}$

SLS dose %	Healthy controls Ntotal= 19		$FLGnull_{non}AD$ Ntotal= 10		$FLGwt_{plus}AD$ Ntotal= 18		$FLGnull_{plus}AD$ Ntotal= 18	
Quartiles	50% percentile	Quartile range	50% percentile	Quartile range	50% percentile	Quartile range	50% percentile	Quartile range
LDF								
0.25	100.54%	91.58–122.07	91.11%	84.32–94.99	90.09%	67.17–104.33	93.75%	73.33–101.72
0.50	91.69%	81.27–103.74	90.62%	78.61–96.63	81.37%	63.64–93.43	90.23%	77.13–100.54
1.00	88.56%	31.37–95.12	81.41%	65.04–94.00	60.64%	26.50–89.11	83.15%	43.18–85.90
TEWL								
0.25	87.12%	71.89–96.26	84.87%	66.83–94.87	79.69%	70.48–86.96	89.14%	59.13–115.79
0.50	86.21%	76.67–94.63	81.96%	76.70–100.93	70.38%	64.25–92.77	87.00%	74.78–101.03
1.00	84.35%	71.48–89.90	81.20%	72.75–96.90	75.28%	59.30–83.72	76.75%	64.83–83.81

LDF = laser Doppler flowmetry, TEWL= Transepidermal Water Loss

Kruskal-Wallis statistics was used to compare all groups and the individual comparisons were performed by Man-Whitney statistics. The results are presented in quartiles. Regeneration R is presented as a percentage of Δ_{max}. Ntotal= 65.

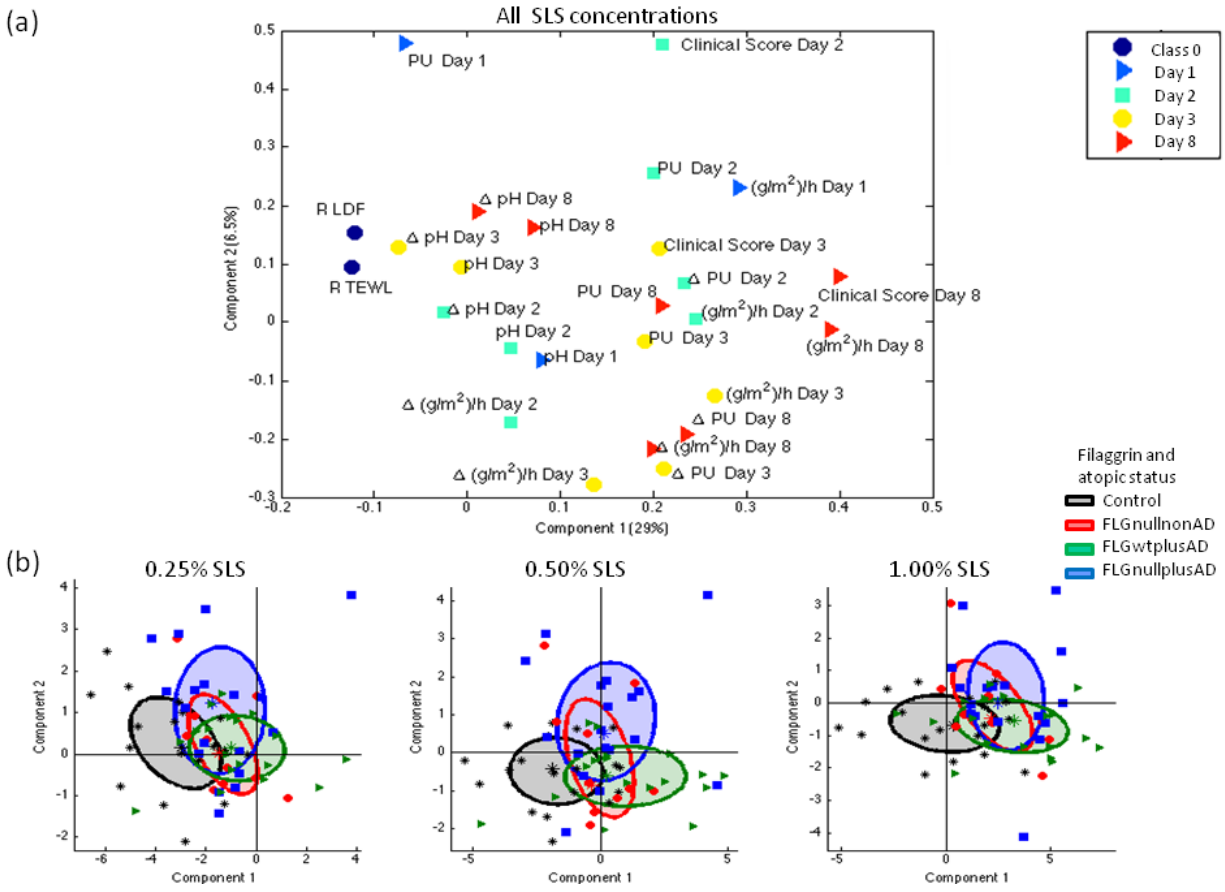
Multivariate analysis, pattern analysis

Figure 5a illustrates the loading plot with two components from the ASCA model reflecting the dominating variation in relation to the study design. Component 1, reflecting overall reactivity as all variables loads in the same direction, except for regeneration, which, by construction, inversely relates to its input responses (Figure 5a).

Component 2 is primarily driven by LDF Day 1 and Clinical Score Day 2 on the positive side and an increase in TEWL from baseline to Day 2, 3 and 8 on the negative side. Figure 5b illustrates the corresponding scores plot for increasing SLS concentrations. Increasing SLS concentrations resulted in higher responses on component 1 and thus increase in severity ($p_{SLS} < 10^{-4}$).

Figure 5

Variation in baseline and SLS responses according to filaggrin genotype and atopic status



a) Loading plot from ASCA analysis displaying the most informative pattern in terms of study design. Color and markersize indicate responses (and derived responses) according to day. B) scoreplot of the individual patients distributed across four AD/filagrin groups (marked by color and markers) for SLS concentrations: B1) 0.25%, B2) 0.50% and B3) 1.00% The ellipses cover 47% (±1 standard deviation in both directions) of the individual classes.

In line with the univariate measures, the control group obtained the lowest values across all SLS concentrations, whereas the three case groups obtained similar levels on component 1. In component 2 there is significant separation between the FLGnull$_{plus}$AD and the remaining groups ($p = 0.005$), which primarily was driven by a higher baseline LDF, especially among homozygote/compound heterozygote in the FLGnull$_{plus}$AD group (N=8) (results not shown). When eliminating the LDF Day 1 from the multivariate model, the inference lessened between the FLGnull$_{plus}$AD and the remaining groups, though still significant ($p = 0.02$). In the multivariate model we found that ΔTEWL is the driving discriminator (most pronounced at Day 2 and 3 and for the lower SLS concentrations – see univariates).

Discussion

This study shows that having filaggrin mutations, atopic dermatitis or both results in a higher reaction and poorer regeneration of the skin after SLS provocation compared to normal skin. Filaggrin mutations and AD are closely related and it is known that individuals with AD have a sensitive skin barrier [27-30].

Baseline results

Surprisingly, we did not find any significant difference in baseline TEWL, LDF or pH between our population groups measured on non-lesional skin sites. This is in keeping with other recent studies showing no significantly difference in TEWL at baseline between a control population and subjects with AD, irrespective of filaggrin status[20;31], and between patients with AD with or without filaggrin mutations on both lesional and non-lesional atopic skin sites[32]. Before stratifying for FLGnull, studies have shown increase in TEWL among AD in comparison to controls[33]. When stratifying for FLGnull a further significant increase in TEWL has been shown[34]. Interestingly, a study on Irish children with moderate to severe AD showed an increase in TEWL according to genotype, with the highest TEWL among FLG homozygotes, suggesting increasing TEWL according to filaggrin mutations [35].

Other studies have shown significantly higher TEWL values among FLGnull$_{non}$AD and FLGnull$_{plus}$AD compared with healthy controls [17;18;34]. Thus, an inconsistent result on baseline TEWL exists in the literature. A reason for these inconsistencies could be due to three things: difference in population sizes, anatomical differences in measurement locations and the fact that AD is a very heterogeneous disease not only based on differentiation by filaggrin mutations.

SLS reaction

SLS is a detergent, and thereby an irritant affecting the skin barrier. SLS is widely used in dermatological research in experimentally induced irritant contact dermatitis [9;37]. In our study all subjects displayed dose-dependent barrier impairment and inflammation after SLS provocation. This is in line with a study

showing similar dose-dependent skin impairment after application of increasing doses of SLS in healthy subjects [37] and more recently, with the findings of Petersen et al showing SLS dose-dependent local inflammation measured by LDF [40;41].

In our patient population we measured a more severe skin reaction on Day 2 and Day 3 compared to healthy controls. When viewing the SLS reactions separately, the vascular response on Day 3 suggests that having either phenotype or genotype induces an equal vascular response to irritancy (Fig. 4). For the total reactivity ΔAUC, a significantly higher response was seen on Day 2 among patients with AD irrespective of filaggrin mutations in both ΔAUC LDF and TEWL in comparison to healthy controls. This was in accordance with our clinical assessments, showing that those with the AD phenotype displayed a more severe clinical response to 1.00% SLS than did the healthy controls. This again suggests a reduced threshold to irritants due to AD. Because no significant difference was detected between healthy controls and FLGnull$_{non}$AD and between FLGnull$_{plus}$AD and FLGwt$_{plus}$AD, filaggrin mutations alone do not seem to contribute significantly to the irritancy level in this study, as also reported in other studies [17;20]. An explanation of this could be functional filaggrin defects[32;42].

FLGnull$_{non}$AD had increased response in comparison to controls, though non-significant, which could be due to the small group number (N=10).

Both in mouse studies and larger population studies, the notion that filaggrin deficiency leads to a more susceptible barrier function has been proposed [7;14;43-45]. However, we were not able to show any increased reactivity among individuals with filaggrin mutations irrespectively of AD status by univariate analysis. Other studies also fail to show any increased reactivity according to filaggrin genotype [17;18]. A possible explanation could be that these studies are based on small study groups and that homozygote and heterozygote individuals are often assessed together. One study in ichtyosis vulgaris patients showed significant higher TEWL in homozygotes compared to that of healthy controls [19].

In our study the patient population reacted similarly and therefore no significant difference was detected among the population at either the individual concentrations or at total reactivity by AUC. However, a significant difference was detected compared with the healthy controls.

Regeneration

Only few studies have assessed post-irritation skin regeneration in relation to filaggrin mutations. One study showed a reduction of mRNA expression of profilaggrin in biopsies taken on 1.00% SLS skin sites after 6 hours with succeeding increased regulation[21]. In one clinical study, TEWL measurements were performed to evaluate effect of 1.00% SLS throughout a 72-hour observational period. No significant differences in barrier recovery rate were

seen between the groups. Subjects with AD displayed the highest TEWL and vascular response compared with those without AD. Subjects with filaggrin mutation but without AD were not included in this study [18].

In our study regeneration was assessed from 2 different angles: the Day 8 measure and by the factor "R". The Day 8 TEWL measurements were significantly different between those with the atopic phenotype and the healthy controls, and in the clinical assessment between FLGnull$_{plus}$AD and healthy controls and between FLGnull$_{plus}$AD and FLGwt$_{plus}$AD. Full regeneration was not obtained on Day 8 for any patient group (Fig. 4). Our results suggest that regeneration depends on phenotype more than genotype. For regeneration R, no significant difference was found between groups. That the FLGwt$_{plus}$AD group had the poorest regeneration could be due to a systemic downregulation of filaggrin caused by AD alone, as suggested by others[35;46], given that FLGwt$_{plus}$AD subjects have a functional filaggrin deficiency which induce the same poor barrier as FLGnull$_{plus}$AD.

Mulitvariate analysis

A plural of studies have been made illustrating filaggrin and AD in univariate analysis; however since filaggrin deficiency is more complex, we applied a multivariate technique. Here we could significantly distinguish the FLGnull$_{plus}$AD and the FLGwt$_{plus}$AD from all the remaining groups, showing that these two groups have different reaction patterns (Figure 5). FLGwt$_{plus}$AD seemed to have the overall highest reactivity driven by component 1 and FLGnull$_{plus}$AD seemed to be driven by a higher inflammatory alertness, thereby suggesting that this group are early responders (with higher clinical score Day 2, LDF Day 1 and Day 2 and higher TEWL Day 1), which significantly segregates from all the other groups. This segregation was driven by component 2, which primarily depends on LDF Day 1 (Figure 5); however, this segregation was still significant when eliminating the LDF Day 1 from the model. The fact that ΔTEWL in the multivariate model was the driving discriminator, indicates that a high dermal baseline inflammation (LDF Day 1) tends to attenuate the SLS effect as measured by TEWL primarily for the lowest stimulation doses (SLS 0.25%, 0.50%) whereas when stimulating with SLS 1.00% this protective effect is lost.

This study is the first to examine pattern analysis of skin reaction and regeneration up to 145 hours after removal of Finn chamber with SLS stratified by the filaggrin mutation genotype and AD phenotype. The strength of this study is the population size, compared with that of other clinical studies on pheno-and genotype. It is a limitation that the FLGnull$_{non}$AD were not healthy individuals, but eczema patients with either irritant contact dermatitis or allergic contact dermatitis. However, we were unable to recruit sufficient individuals with FLG mutations but without skin disease. A control chamber would have been preferred, but was however not applied.

Conclusion

We found that both LDF and TEWL increased markedly in a dose-dependent manner in response to SLS. This suggests a dose-dependent alteration of the skin barrier function, with notable distinction between the patient population and the healthy control group. Moreover, the skin regeneration significantly differed between the patient population with AD and the healthy controls. This study forwards the current knowledge that having filaggrin mutation, AD or both decreases the skin barrier function and regeneration ability. Additionally, those with the AD phenotype, irrespective of filaggrin mutation status, exhibited the greatest response and the poorest regeneration. Subjects with $FLGnull_{plus}AD$ where characterised by multivariate analysis as being more rapid responders primarily driven by having higher inflammatory alertness both compared to the healthy controls, $FLGnull_{non}AD$ and $FLGwt_{plus}AD$.

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3.3 Manuscript III

Bandier J, Ross-hansen K, Carlsen BC, Tanassi J, Johansen JD, Heegaard NH. *Quantification of epidermal filaggrin in human skin and its response to SLS exposure over time.* Journal of Investigative Dermatology. Submitted 2015.

Title:
Quantification of epidermal filaggrin in human skin and its response to skin irritation

Authors: Bandier J[1], Ross-Hansen K[1], Carlsen BC[1], Tanassi JT[2], Johansen JD[1] and Heegaard NHH[2,3]

[1]National Allergy Research Centre, Department of Dermato-Allergology, Copenhagen University Hospital Gentofte, Hellerup, Denmark.
[2]Department of Autoimmunology and Biomarkers, Statens Serum Institut, Copenhagen, Denmark.
[3]Department of Clinical Biochemistry & Pharmacology, Odense University Hospital, University of Southern Denmark, Odense, Denmark.

The work was done in
Hellerup and Copenhagen, Denmark

Corresponding author:
Josefine Bandier
National Allergy Research Centre,
Department of Dermato-Allergology
Copenhagen University Hospital Gentofte
Kildegaardsvej 28, 20A, 1st floor
2900 Hellerup, Denmark
Email: Josefine.Bandier@regionh.dk
Phone: 39 77 73 40
Fax: +45 39 77 71 18

Short title: Filaggrin protein quantification in epidermis

Abbreviations in order of appearance

AD – atopic dermatitis
SLS – sodium lauryl sulphate
ICD – irritant contact dermatitis
NMF – natural moisturizing factor
FLGwt – wild type filaggrin
FLGhet – heterozygous for filaggrin mutations
FLGhom – homozygous for filaggrin mutations
IQR – inter quartile range
UV – ultra violet
FLG – filaggrin gene
ICDRG –international contact dermatitis research group
EB – extraction buffer
PBS – phosphate buffered saline, pH 7.4
ELISA – enzyme-Linked immunosorbent assay
TTN – Tris Tween
AU – arbitrary units
RT – room temperature

Abstract

Filaggrin is a key protein for skin integrity. Even though links between filaggrin mutations and atopic dermatitis (AD) are well established, the actual amount of filaggrin protein and its turnover in the epidermis has been less studied. To date, filaggrin quantification has primarily been at the mRNA level or through estimates of degradation products. To assess the actual amounts of filaggrin protein in the epidermis, we developed an ex vivo quantitative immunoassay and applied it to skin samples from 67 individuals representing 4 groups, i.e., with and without filaggrin mutations and with and without AD. Biopsies were taken before and after exposure to 0.50% sodium lauryl sulphate (SLS). In total 262 samples were analysed. At baseline we found a 50% reduction in filaggrin protein content between filaggrin wild type and filaggrin heterozygous carriers without AD and a reduction of 100% between filaggrin heterozygote and homozygous carriers. Additionally, we found a clear distinction in epidermal filaggrin content according to AD phenotype and filaggrin genotype. In all groups an immediate increase followed by a decrease in filaggrin content in response to SLS was seen. Notably, homozygous carriers displayed the largest variability of epidermal total protein suggesting disruption of skin homeostasis.

Introduction

The skin is a dynamic organ with a complex molecular structure. The epidermal protein filaggrin, which is expressed in the outermost skin layers, is essential for the structural organisation and water binding capacity of the stratum corneum. Lack of filaggrin, caused by null mutations in the filaggrin gene, is associated with dry skin (Harding *et al.*, 2013). Filaggrin mutations are common, affecting 8–10% of adults from the general population (Thyssen *et al.*, 2010;Palmer *et al.*, 2006) and are the strongest known genetic factors associated with the pathogenesis of atopic dermatitis (AD) (Paternoster *et al.*, 2012;Weidinger *et al.*, 2007).

AD is an established risk factor for irritant contact dermatitis (ICD), and the recent focus on genetics in AD has led to the notion that filaggrin mutations increase susceptibility to ICD. This is supported by studies showing that individuals with AD and filaggrin mutations have increased risk of developing chronic ICD (De Jongh *et al.*, 2008) and that both AD and filaggrin mutations are independent risk factors (Visser *et al.*, 2013). The highest risk has been associated with the coexistence of filaggrin mutations and AD, as seen in a consecutive clinical study on patients with chronic ICD on the hands (Visser *et al.*, 2013).

In the epidermis, Filaggrin degrades into its constituent amino acids, which are a prominent part of the natural moisturising factor (NMF) (Rawlings and Harding, 2004). Thus, quantification of NMF in the stratum corneum has been used as a proxy for the amount of filaggrin (Kezic *et al.*, 2009). Accordingly, the amount of filaggrin degradation products (histidine, glutamic acid and derivatives hereof) in the stratum corneum correlates with the number of functional filaggrin alleles, i.e. hetero- or homozygous mutation status (Kezic *et al.*, 2009). Also, estimates of filaggrin breakdown products indicate that AD without filaggrin deficiency causes decreased filaggrin content in the skin (Kezic *et al.*, 2011). Whether such a decrease follows homeostatic epidermal changes associated with the AD phenotype, or if it is a result of epigenetic variation ultimately affecting filaggrin transcription, expression or processing is presently unknown.

The total lack of filaggrin in R501X homozygous carriers has been supported by immunohistochemical staining (Sandilands *et al.*, 2009). Whether heterozygous mutation carriers only express half the amount of filaggrin protein compared to wild type carriers is not known.

In addition to the NMF proxy and qualitative estimates by immunehisto-chemical methods, the content of filaggrin in the skin has previously also been indirectly estimated by determination of mRNA levels of profilaggrin (Torma *et al.*, 2008). However, none of these studies represent actual filaggrin protein quantification. Thus, the aim of this study was to develop a method for filaggrin quantification in skin samples and to apply it to samples from

healthy controls and from patients with and without filaggrin deficiency.

Since individuals with AD may have a functional filaggrin deficiency as suggested by another study (Pellerin *et al.*, 2013), we also evaluated the filaggrin amount with respect to presence or absence of AD. Further, since profilaggrin mRNA decreases six hours after experimental exposure to sodium lauryl sulphate (SLS) (Torma *et al.*, 2008), we investigated the dynamics of epidermal filaggrin content after SLS exposure.

Results

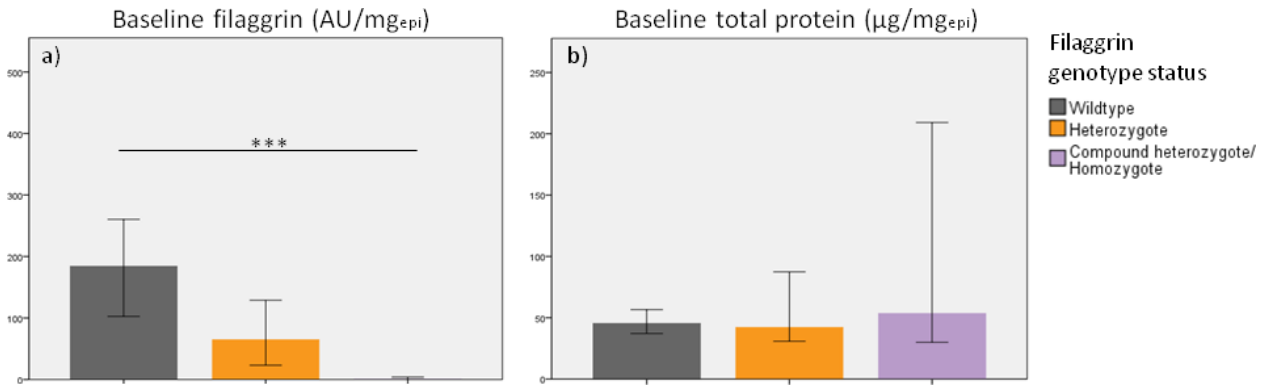
Baseline results

Quantification of the epidermal total protein and filaggrin content according to filaggrin genotype

Proteins were extracted from the epidermal layer of 4 mm skin biopsies, taken from the left upper arm. Filaggrin was quantified by an in-house developed solid-phase immunoassay and expressed as the amount of filaggrin/dry epidermal weight.

Figure 1

Baseline quantification of epidermal filaggrin and total protein according to genotype



a) The amount of filaggrin (AU/mg epidermis) at baseline, b) the total protein amount in the epidermis at baseline (µg/mg epidermis).
Wild type: Carriers without 2 functional filaggrin gene copies.
Heterozygote: Carriers with one functional filaggrin copy.
FLGhom: Carriers with double-allele null mutations either compound heterozygous or homozygous.
The filaggrin mutations analysed are the three most common in the European population, 501X, 2282del4, and 2447X.
Ntotal= 67; FLGwt, N= 39, FLGhet, N= 20 and FLGhom, N= 8.
Significance was evaluated by linear-by-linear associations. Level of significance < 0.05; *p < 0.05; **p< 0.01, *** p< 0.005.
The results are presented as medians. Error bars represent 95% CI.

The total protein amount was also measured in all samples. We found (Figure 1a) that wild type carriers (FLGwt) exhibit the highest amount of filaggrin (N= 39). In contrast, homozygous carriers for the filaggrin mutations (FLGhom) had no measurable filaggrin (N= 8), whereas heterozygous carriers (FLGhet) with one functional allele (N= 20) had intermediate filaggrin protein levels. Thus, a statistically significant dose-dependent relation between epidermal filaggrin amount and the number of functional alleles was found by Mann-Whitney, p< 0.001. The median decrease in filaggrin between the groups was 65% between FLGwt and FLGhet (184.0, IQR (60.2–373.7) to 64.5, IQR (23.1–134.7)), and 99.5% between FLGhet and FLGhom (64.5, IQR (23.1–134.7) to 0.42, IQR (0.29–1.7).

The total protein content did not differ significantly between wild-type, hetero- and homozygous carriers (Kruskal-wallis, p= 0.872), but the variability increased with decreasing filaggrin content (Figure 1b).

Since AD has been associated with a lower amount of filaggrin breakdown products in the skin, the filaggrin protein levels could both reflect AD disease and filaggrin mutation status. Therefore, we stratified for absence of AD, analysing the difference between controls (N=20) and FLGhetnonAD (N=10). Here we found a decrease (49%) in filaggrin content between FLGwt (N=20) and FLGhet (N=10) (195.2, IQR (108.1–486.3) to 100.2, IQR (23.8–172.2)).

Quantification of the epidermal total protein content and filaggrin according to AD and genotype

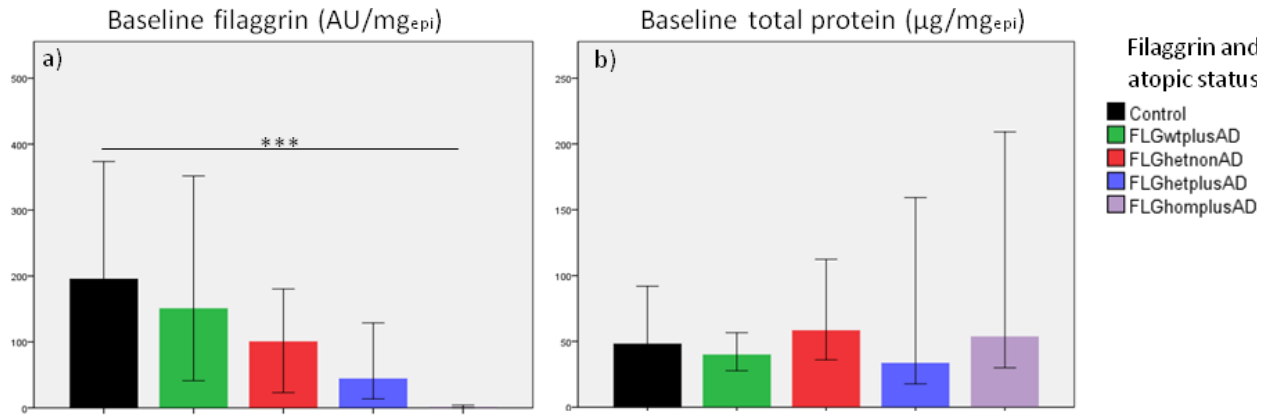
To analyse whether AD individuals could have a decrease in filaggrin content which could not be explained by mutational status, we next grouped the samples according to both AD and filaggrin genotype status, healthy control (N= 20), FLGwtplusAD (N= 19), FLGhetnonAD (N= 10) and FLGhetplusAD (N= 10), FLGhomplusAD (N= 8) Figure 2.

Figure 2a clearly outlines a decreasing amount of filaggrin in relation to both filaggrin carrier status and AD, and a significant linear-by-linear association (p< 0.001) was seen with regards to filaggrin content (Figure 2a). The impact of AD itself was examined by looking at the difference in filaggrin content between groups with equal carrier status (Controls vs. FLGwtplusAD and FLGhetnonAD vs. FLGhetplusAD). No significant differences between these groups were found (Figure 2a and Table 1).

Concerning the total protein amount, no significant difference was seen between groups (Kruskal Wallis, p= 0.980). Nevertheless, an increased variability in the total protein content was consistently associated with the inherited total lack of filaggrin rather than with the AD-associated filaggrin decrease (Figure 2b).

Figure 2

Baseline quantification of epidermal filaggrin and total protein content, according to atopic dermatitis and genotype



a) The amount of filaggrin (AU/mg epidermis) at baseline, b) the total protein amount in the epidermis at baseline (µg/mg epidermis).
Controls and nonAD: individuals without atopic dermatitis (AD). PlusAD: individuals with AD. AD was assessed according to the UK Working Party's diagnostic criteria for atopic dermatitis. Controls and FLGwt are individuals with 2 functional filaggrin gene copies.
FLGhet are carriers with one functional filaggrin gene copy.
FLGhom are carriers with double-allele null mutations either compound heterozygous or homozygous. The filaggrin mutations analysed are the three most common in the European population, 501X, 2282del4, and 2447X.
Control, N= 20, FLGwtplusAD, N= 19, FLGhetnonAD, N= 10, FLGhetplusAD, N=10 and FLGhomplusAD, N=8. Significance was evaluated by linear-by-linear associations. Level of significance < 0.05; *p < 0.05; **p< 0.01, ***p< 0.005.
The results are presented as medians. Error bars represent 95% CI.

Response to skin irritant

Quantification of the epidermal total protein content and filaggrin

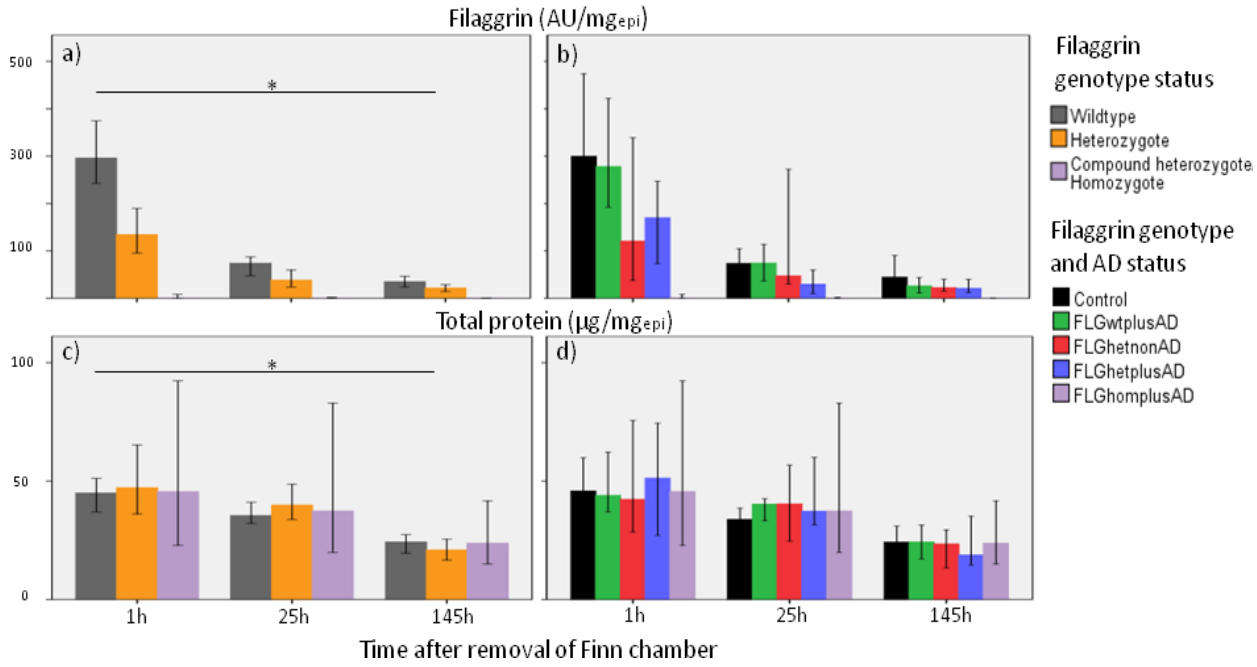
A Finn chamber with 0.50% SLS was applied on the inner surface of the upper arm to elicit ICD. Biopsies were taken 1, 25 and 145 hours after removal of the Finn chamber to evaluate the immediate and the prolonged effect of SLS on epidermal filaggrin content. After

SLS irritation, the dose-dependent pattern of decreasing filaggrin content according to filaggrin mutation carrier status was still apparent (Figure 3a). In addition, accumulation of filaggrin protein was evident 1 hour after removal of the Finn chamber (Table 1), followed by a consistent decrease from 1 hour to 25 hours after removal of SLS.

This decrease was most pronounced among wild type and heterozygous carriers (Figure 3a and 3b).

Figure 3

Quantification of epidermal filaggrin and total protein content according to filaggrin genotype and atopic dermatitis in relation to SLS irritation over time



a) Filaggrin units (AU/mg epidermis) at different time points after SLS irritation, according to genotype.
b) Filaggrin units (AU/mg epidermis) at different time points after SLS irritation, according to genotype and AD phenotype.
c) Total epidermal protein amount (µg/mg epidermis) at different time points after SLS irritation, according to genotype.
d) Total epidermal protein amount (µg/mg epidermis) at different time points after SLS irritation, according to genotype and AD.

- FLGwt= Carriers with 2 functional filaggrin gene copies at 1 hour (N= 37), 25 hours (N= 38) and 145 hours (N= 37) after removal of Finn chamber with SLS.
- FLGhet= Carriers with one functional filaggrin gene copy at 1 hour (N= 20), 25 hours (N= 20) and 145 hours (N= 19) after removal of Finn chamber with SLS.
- FLGhom= Carriers with no functional filaggrin gene copies, N throughout trial = 8.
- Controls= carriers with 2 functional filaggrin gene copies and no prior eczema at 1 hour (N= 19), 25 hours (N= 19) and 145 hours (N= 19) after removal of Finn chamber with SLS.
- FLGwtplusAD= Carriers with 2 functional filaggrin gene copies but with atopic dermatitis (AD) at 1 hour (N= 18), 25 hours (N= 19) and 145 hours (N= 18) after removal of Finn chamber with SLS.
- FLGhetnonAD= Carriers with 1 functional filaggrin gene copies but no AD, N throughout trial= 10.
- FLGhetplusAD= Carriers with 1 functional filaggrin gene copies and with AD, at 1 hour (N= 10), 25 hours (N= 10) and 145 hours (N= 9) after removal of Finn chamber with SLS.
- FLGhomplusAD= Carriers without functional filaggrin gene copies and with AD, N throughout trial= 8.
- Controls and nonAD: Carriers without AD. PlusAD: individuals with AD. AD is assessed according to the UK Working Party's diagnostic criteria for atopic dermatitis.
The filaggrin mutations evaluated in this trial was 501X, 2282del4, and 2447X.
The results are presented as medians. Error bars represent 95% CI.

Even though carriers of the same filaggrin mutation status with AD have slightly lower filaggrin content than those without AD (except for 1 hour after removal of Finn chamber) the differences did not reach statistical significance at any time point. Hence, the wild type carriers' responses were similar irrespective of the presence of AD (Figure 3b).

Comparing 145 hours after chamber removal to baseline by Wilcoxon's test, a significant decrease in both filaggrin protein amount and total protein amount was seen in all of the three genotype groups ($p< 0.02$) (Figure 3a and 3c). No significant difference in total protein content between groups was found.

Comparing the filaggrin amount 145 hours after chamber removal to baseline according to AD, the decrease was significant for all groups except for the FLGhetnonAD group: 77.4%, p= 0.005 for controls, 82.7%, p= 0.001 for FLGwtplusAD, 78%, p= 0.03 for FLGhetnonAD, 52,3%, p= 0.173 for FLGhetplusAD and 59.5%, p= 0.017 for FLGhomplusAD (Table 1).

The total protein amount was significantly decreased at 145 hours compared with baseline, except for the FLGhetplusAD which was lower, but not significantly so (p=0.139). No statistically significant variation in the total protein content was seen between groups over time (Figure 3d). Interestingly, the finding of higher variances among homozygous carriers was reproducible after irritation, especially 1 and 25 hours after removal of the Finn chamber (Figures 3c and 3d).

Table 1

Distribution of filaggrin AU/mg epidermis according to AD and filaggrin mutation carrier status

	Filaggrin (AU/mgepidermis)				
	Control	FLGwtplusAD	FLGhetnonAD	FLGhetplusAD	FLGhomplusAD
Time after removal of chamber	(25th and 75th percentile)				
Baseline	195 (108-486)	150 (41-352)	100 (24-172)	44 (16-93)	0.42 (0.29-1.70)
1 hour	300 (224-474)	278 (188-450)	120 (55-219)	170 (90-228)	0.65 (0.22-2.21)
25 hours	74 (24-105)	74 (36-113.7)	48 (35-128)	30 (10-57)	0.33 (0.23-0.72)
145 hours	44 (24-91)	26 (11-44)	22 (17-32)	21 (12-31)	0.17 (0.16-0.33)

Discussion

Filaggrin is an important protein in the upper layers of the epidermis (Ross-Hansen *et al.*, 2014). Its significance in different skin conditions has previously been evaluated by genotyping (Smith *et al.*, 2006;Meldgaard *et al.*, 2012), gene expression (Torma *et al.*, 2008), immunhistochemical staining of skin cross sections (Sybert *et al.*, 1985;Jensen *et al.*, 2004;Smith *et al.*, 2006;Kurokawa *et al.*, 2006;Howell *et al.*, 2009) and by degradation product measurements (Kezic *et al.*, 2009). In the present study, we developed a quantitative immunoassay for filaggrin protein in human ex vivo samples, allowing the evaluation of how filaggrin mutations and external stimuli affect the epidermal content of this protein.

At baseline, we demonstrate a clear dose-response relation of filaggrin content according to mutation carrier status. In FLGhom, filaggrin was practically absent or at least at the detection limits of the assay. This is in keeping with immune-histochemical findings (Sybert *et al.*, 1985).

Further, at baseline a 50% decrease in filaggrin content was seen in heterozygous compared with wild type carriers without AD. When dividing the individuals into geno- and phenotypes, AD disease was associated with a trend toward an additional decrease in filaggrin baseline levels. Although this was statistically non-significant the finding is in accordance with NMF measurements (Angelova-Fischer *et al.*, 2014). It has been speculated that the filaggrin decrease in AD may be caused by the effects of inflammatory and proinflammatory cytokines on filaggrin expression and/or processing (Howell *et al.*, 2009;Pellerin *et al.*, 2013;Deleuran *et al.*, 2012;Gutowska-Owsiak *et al.*, 2012;Gutowska-Owsiak *et al.*, 2011). On the other hand, unknown epigenetic variations affecting filaggrin turnover directly or indirectly may also account for an additive, inherited decrease in the epidermal filaggrin levels.

We find that skin irritant stimulation causes increased filaggrin levels in all groups. Since physical occlusion augments the skins humidity, the processing of filaggrin into water binding amino acids could possibly be superfluous under these conditions. The degradation of filaggrin into NMFs is a natural process occurring when ambient humidity drops below 80%; the lower the humidity, the faster the degradation (Scott and Harding, 1986). Thus, the initial increase of filaggrin could be due to the accumulation of filaggrin protein (i.e. decreased degradation) rather than changes in the expression. The increase could also reflect an accelerated release of filaggrin from hyaline granules to protect the skin barrier in response to irritancy, but these alternatives cannot be differentiated from the experimental data in this study. However, in a Swedish study, mRNA expression of profilaggrin in response to 1% SLS did not increase initially (Torma *et al.*, 2008). In this study, the first measurement was performed 6 hours after removal of the chamber, where a 50% reduction in profilaggrin expression was found. After 24

hours the expression level was normalised and then upregulated, possibly reflecting a compensatory increase in the repair phase after barrier disruption. Such increase in profilaggrin expression after barrier disruption is supported by a study on the reepithelialisation phase of wound healing (Kurokawa *et al.*, 2006), and also akin to a study on mouse skin where it was shown, that increased expression of filaggrin accelerates barrier recovery (Presland *et al.*, 2004).

Comparing our findings with Torma et al (2008), the decrease in filaggrin protein content was seen later (25 hours after chamber removal), and was not followed by an increase, but rather a continuing decrease in response to irritation . The compensatory mechanisms after SLS barrier disruption include an increase in the filaggrin gene expression (Torma *et al.*, 2008). Further, our results may indicate an even higher degradation rate of filaggrin protein due to the SLS induced xerosis, and thereby release of the amino acids to counteract the barrier disruption.

Interestingly, when comparing groups of the same mutation carrier status but with different phenotypes, FLGhetnonAD and FLGhetplusAD, we saw that the individuals with AD had slightly less filaggrin than individuals without AD, except for 1 hour after irritant removal. This decrease of filaggrin in response to SLS could indicate a functional filaggrin deficiency as suggested by Pellerin (Pellerin *et al.*, 2013), but it could also be explained by an increased utilisation of amino acids to compensate for the xerosis among individuals with AD.

Compellingly, the FLGhomplusAD group had the greatest variance in total epidermal protein, which could indicate alterations in the epidermal plasticity. An increase in epidermal thickness has previously been shown in a study of living skin equivalents (Aho *et al.*, 2012), where filaggrin transcription was eliminated in an in vitro skin model. A clinical study has additionally shown increased hyperkeratosis among double allele-carriers after cellophane stripping (Gruber *et al.*, 2011). Our results are however stratified by the amount of protein per mg of epidermal weight, suggesting a change in the skin homeostasis besides epidermal thickening.

The limitations of the present study are: FLGhetnonAD carriers were not healthy individuals but dermatitis patients with either irritant or allergic contact dermatitis. Healthy individuals with filaggrin mutations would have been ideal for dissecting the exact contribution of AD and filaggrin mutations; however these individuals are difficult to recruit. The patients did not present with dermatitis at the sampling site nor did they have widely spread active dermatitis. Also the number of patients in the FLGhetnonAD group was relatively low (N=10). Further, it would have been preferable to have applied a control chamber without SLS, to confirm or reject the humidity hypothesis. Finally, it would have been interesting to have measured the gene expression level and the NMFs simultaneously with the protein quantification.

Despite these reservations, this is, to our knowledge, the first study to determine the quantity of filaggrin in human epidermis at the protein level before and after barrier disruption. Additionally, we were able to assess the effect of both AD status and filaggrin mutation carrier status.

In summary, we have established a method to quantify the protein levels of filaggrin in ex vivo skin samples. We have shown significant reduction in the filaggrin content in direct proportion to the number of functional filaggrin alleles, and suggest that AD may further affect filaggrin protein content. We have shown that following irritation with SLS under occlusion, the filaggrin content increases initially but is succeeded by a continuous decrease long after ended exposure. Homozygous carriers displayed the greatest variance in total protein content in the epidermis, indicating that the lack of filaggrin may be associated with alterations in skin homeostasis.

Materials and methods

Participants

The study population comprised 67 individuals. Healthy controls were recruited through online advertising (N= 20).

The patient population was recruited at the department of Dermato-Allergology at Gentofte Hospital, Denmark (N= 47), and considered for inclusion if assessed for both AD at the department and genotyped for filaggrin mutation carrier status (R501X, 2282del4 and 2447X) by multiplex genotyping analysis (Meldgaard *et al.*, 2012). Additional inclusion criteria were: age 18-69 years and Caucasian. At inclusion all were re-assessed for AD by means of the UK working party´s Diagnostic Criteria (Williams *et al.*, 1994). Participants were excluded if they had active or widespread eczema (the healthy controls did not, however, have eczema at any point in their life) or chronic inflammatory diseases besides eczema, if they had been treated with UV-light within 3 weeks of trial, with topical steroids 2 weeks before trial, had applied emollients on the test areas or had received immunomodulating medical treatment. The population has been described elsewhere (Bandier *et al*., 2015).

The distribution of AD and filaggrin mutations in our population is shown in table 2 and from this distribution, 2 different groupings were created: Three groups correspond to genotype (FLGwt, FLGhet and FLGhom), and a group of five was formed by including history of AD and considering mutational zygosity (Control, FLGwtplusAD, FLGhetnonAD, FLGhetplusAD and FLGhomplusAD). The ten individuals within the patient population with nonAD and FLGhet had contact dermatitis (irritant or allergic according to ICDRG guidelines (Wilkinson *et al.*, 1970)), but not AD.

The study was carried out from end October 2011 to mid March 2012 and conducted in accordance with the principles of the Declaration of Helsinki. The study was approved by the local ethical committee (H–1–2011–011) and written informed consent was obtained from all participants.

Table 2

Distribution of study population by filaggrin mutation carrier status and atopic dermatitis

Filaggrin and atopic status	Filaggrin mutation status			Ntotal
	FLGwt	FLGhet	FLGhom	
nonAD	20[1]	10[2]	0	30
plusAD	19	10	8	37
Ntotal	39	20	8	67

[1]Healthy controls.
[2]NonAD, but with either allergic or irritant contact dermatitis.

Clinical setup

The 4 mm biopsies were taken 9 cm distally from the centre of the axillary fossa on the inner surface of the left arm. The biopsies were stored in Eppendorf vials with 800 µL storage buffer (10 mM potassium phosphate, 2 mM Na_2EDTA, pH 7) (Ross-Hansen *et al.*, 2014). The samples were immediately placed at -80°C until use.

To elicit ICD experimentally, 200µL 0.50% SLS aqueous solution (Sigma-Aldrich, Steinheim, Germany, 99% purity) was applied in an 18 mm Finn chamber with filter disc from Vitaflo Scandinavia (Navamedic AB) . The chamber was left for approximately 24 hours and then removed. The baseline biopsies were obtained right outside the Finn chamber, and 3 biopsies were taken on SLS induced lesional skin within the Finn chamber.

All participants were seen before application of SLS (baseline) and 1, 25 and 145 hours after removal of Finn chamber.

During the study, 1 participant dropped out after the first day and before the last day, 1 patient refrained biopsy at the last day and 1 sample was lost during quantification. Their data were included as missing those days and forward in the trial. In total 262 biopsies were analysed.

The extraction process

The epidermal protein extraction was based on the previously described procedure (Ross-Hansen *et al.*, 2014). Briefly, the 4 mm skin samples were incubated at 56°C for 10 min and the epidermis peeled off and weighed in a pre-weighed Eppendorf vial. The sample was then minced into small pieces and transferred back to the vial with 30 µL extraction buffer (EB) and stored at -80°C until further use. Two small teaspoons quartz sand and 100 µL EB were added and the samples were sonicated at 50-60% of max output for 2 times 30 sec while chilled on ice. Between each sample, the tip of the sonicator was rinsed with 100 µL EB. Vials then rotated for 1h at RT and the sonication process was repeated. The samples were centrifuged at 3,200g for 30 min at RT and the supernatants were transferred to new tubes filling one third of tubes.

In the delipidation process the samples were mixed with a pipette and then rotated for 1 hour. The centrifugation step was repeated. Then 200 µl supernatant was transferred with a pipette into an Eppendorf vial and 100 µL phosphate buffered saline, pH 7.4 (PBS) was added. The extracts were transferred into dialysis cassettes after discarding the lipid solvent phase and interphase and dialysed overnight in PBS at 4°C. The supernatant volumes were adjusted up to 450 µL with PBS. Total protein concentration was estimated by 280 nm absorbance and normalized by epidermal weight.

Filaggrin quantification:

An in-house enzyme-linked immunosorbent assays (ELISA) was developed as follows: MaxiSorp microtiter plates (Nunc) were coated with 100 µL of polyclonal rabbit anti-FLG (Sigma HPA030189, 0.5 g/l, dilution 1:250 in carbonate–bicarbonate buffer, pH 9.6) and incubated at 4°C overnight. The plates were washed 3 times with TTN buffer (0.05M Tris, 0.3M NaCl and 1% Tween 20, pH= 7.5). An extraction pool (mixture of extracts of 10 different skin samples from individuals undergoing an abdomen pendens operation) were used to generate a standard curve consisting of seven calibrators (assigned 100, 50, 25, 12.5, 6.25, 3.125 and 1.56 Units). All samples were diluted in TTN with 0.2 % BSA and 100 µl of diluted standard and 100 µl samples were added in duplicates into the appropriate wells and incubated for 1 h at room temperature (RT). After 3 repetitive washings with TTN-buffer, 100 µL of monoclonal mouse anti-Filaggrin (Abcam cat.no.ab3137, 1:500 dilutions) were added to each well and the plates were incubated for 1 h at RT. After another TTN-buffer wash, each well was incubated with 100 µL of alkaline phosphatase-conjugated goat anti mouse IgG (Sigma) (1:2000) in PBS for 1 h at RT. After TTN buffer wash, 100 µL of freshly prepared 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma) was added into each well, and incubated for 20 minutes at RT. Enzyme activity was read on ELISA reader (VERSAmax) at 405 nm with 650 nm as a reference. Concentrations (in arbitrary units, AU) for all samples were calculated based on reference to the seven-point standard curve and after multiplication with the dilution factor. Final concentration is given in AU/mg epidermis.

Data analysis and statistics

Normal distribution of data was assessed by Shapiro-Wilk's test, showing skewed distribution ($p > 0.05$), thus non-parametrical tests were used for analysis (Kruskal-Wallis test and Mann Whitney). Wilcoxon signed rank test was used for repeated measures with 2 conditions and thereby to evaluate changes over 2 time points within groups. Friedman's test was performed to analyse time variations. Chi^2-test, linear-by-linear was used to analyse for significant variations across different groups.

The level of significance was set at $\alpha = 5\%$.

Conflicts of interests

The authors state no conflict of interests

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4 CONSIDERATIONS AND COMMENTS ON METHODOLOGY

This section is an elaboration on the methodology and considerations not covered or only briefly described in Manuscripts I, II and III.

4.1 Epidemiological study (Manuscript I)

The epidemiological study is based on population data collected in a cross-sectional study from June 2006 to May 2008. The invited individuals were aged 18–69 years. They participated in a general health examination and were patch tested. The population has been thoroughly described elsewhere[114]. Upon examination, a questionnaire was answered by the participants concerning irritant exposure at work (Figure 4).

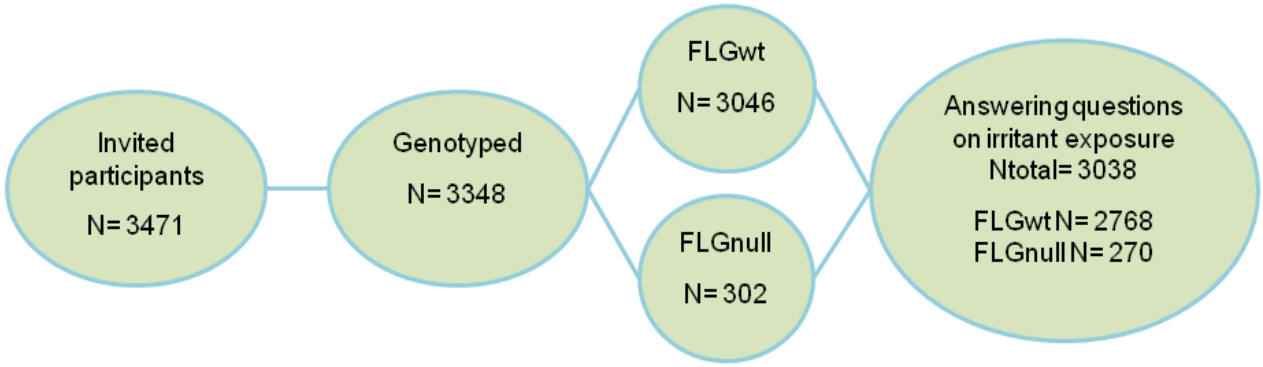

Figure 4: An overview of the epidemiological data based on genotype and irritant exposures in the epidemiological study (Manuscript I). N= the number of subjects in the groups. FLGwt= individuals without filaggrin mutations and FLGnull= individuals with filaggrin mutations. We genotyped for three common filaggrin mutations: R501X, 2282del4 and R2447X.

Evaluation of occupational exposures to irritants was assessed by the following question: “Have you, at your past or present workplace, been exposed to any of the following exposures several times a week” (yes/no). If an individual confirmed exposure to at least one of the following seven listed exposures, he or she was included in the analysis as being exposed to irritants in an occupational setting:

- Wet work
- Use of protective gloves more than 2 hours/day
- Hand washing at least 20 times daily
- Cooking or food handling
- Solvents
- Cleaning agents
- Water vapour or moist environments

Wet work was not defined in the questionnaire and did not include exposure time. Thus, this may have been understood differently by the participants than when the term is used based on the definitions in the German Approved Code of Practice no. 531. Here wet work is defined as regular work with the hands (approximately 2 h daily) in a wet working environment or use of occlusive gloves over the same period or frequent and intensive hand washing over 20 times[115]. As 7% (213/3038) answered that they had been exposed to wet work, without affirmative answer to hand washing or glove use, we included them as exposed to wet work of unspecified nature. Had the wet work been defined according to definition[115], this would possibly have reduced the number of individuals confirming exposure to this parameter. However, since the distribution of wet work was similar among mutation and non-mutation carriers (Manuscript I), our results are not skewed, and therefore our results would not have been affected by excluding wet work from our analysis. When evaluating irritant exposure according to the filaggrin genotype, all irritants were equally distributed among mutation and non-mutation carriers (Table 1, Manuscript I), except for “water vapour or moist environment”, which was actually more common among filaggrin mutation carriers and therefore could not have influenced our final results. Quantification of the extent of irritant exposure by adding a time limit is addressed in only two out of the seven irritants, which limits information of exposure load across mutational status.

In the questionnaire, eczema was defined as follows: “Eczema is an itching skin disorder showing redness, dryness and possibly vesicles and exudation. Eczema is present in the same area for some time”. Hand eczema was established if participants answered yes to the question “Have you ever had hand eczema”. Self-reported hand eczema has been validated in a Danish study on hairdressing apprentices, with an overall sensitivity of 70.3% and specificity of 99.8%[116]. We chose to divide the population by age of onset of hand eczema before and after the age of 15 years to identify a group who probably had eczema preceding the occupational exposure. However, it is possible that a few individuals had already been exposed to irritants before the age of 15 years, but we consider the risk to have minor impact on our results.

We were not able to stratify by domestic irritant exposure because no such questions were included. Information about this exposure would have been interesting because a study among dermatological patients has shown that individuals with excessive work-related hand washing > 10 times daily were more likely to have similar exposure privately[117]. Additionally, there were no questions included on whether the individuals considered their dry skin when choosing not to be exposed to irritants. This would possibly have confirmed the de-selection based on phenotype.

Selection bias could exist concerning the population participating in the study, thereby affecting how well the population represents the entire Danish population. Firstly, 7931 individuals were invited and 43.8% (3471/7931) participated in the study; this participation rate could indicate that selection bias may occur.

The questions concerning skin diseases constituted only a small part of the total questionnaire; this minimises the risk of an over-representation of participants with skin diseases. This would be a concern if using skin specific questionnaires.

AD can be diagnosed by either the Hanifin and Rajka criteria[118] or by the UK diagnostic criteria[119]. In this thesis AD is defined by using the UK Working Party criteria[119-122], which is validated solely on clinical signs and symptoms, showing a specificity of 96% and a sensitivity of 69–85%[122]. However, this validation is mainly based on children, which could prompt misclassification[123]. The risk of recollection bias could exist when answering the UK validated questions since the questions concern age of onset. This recollection bias would be considered greater among individuals with mild AD in childhood, as demonstrated by a Swedish study showing that disease activity in adult life (onset > the age of 15 years), severity and disease attention from surrounding network were determining factors of remembering AD in childhood[124]. Individuals with filaggrin mutations have shown an increased risk of hand eczema persistency and a more severe disease course[63]. The persistency and severity of disease would entail individuals with filaggrin mutations to be more prone to remembering their skin symptoms than non-mutation carriers are. It could therefore be speculated that even despite the early debut of disease among mutation carriers[125] their recollection bias is reduced.

In addition, recollection bias concerning work exposure could exist since the occupational exposure was based on either past or present workplaces with exposure several times a week. This could reduce the recollection of minor exposures, causing more severe exposure to be reported primarily. However, this recall bias is considered identical in both groups and therefore without consequence for our data.

4.2 Clinical study (Manuscript II)

The clinical study was conducted at The Department of Dermato-Allergology, Copenhagen University Hospital Gentofte, Hellerup, Denmark and comprised 67 individuals, divided into 4 groups depending on AD status and filaggrin mutation status:

- Healthy control individuals without AD and without filaggrin mutations ($FLGwt_{non}AD$).
- Individuals with filaggrin mutations but without AD ($FLGnull_{non}AD$).
- Individuals without filaggrin mutations but with AD ($FLGwt_{plus}AD$).
- Individuals with filaggrin mutations and with AD ($FLGnull_{plus}AD$).

The participants are described in more detail in Manuscript II. The distribution of participants throughout the trial is illustrated in Figure 5.

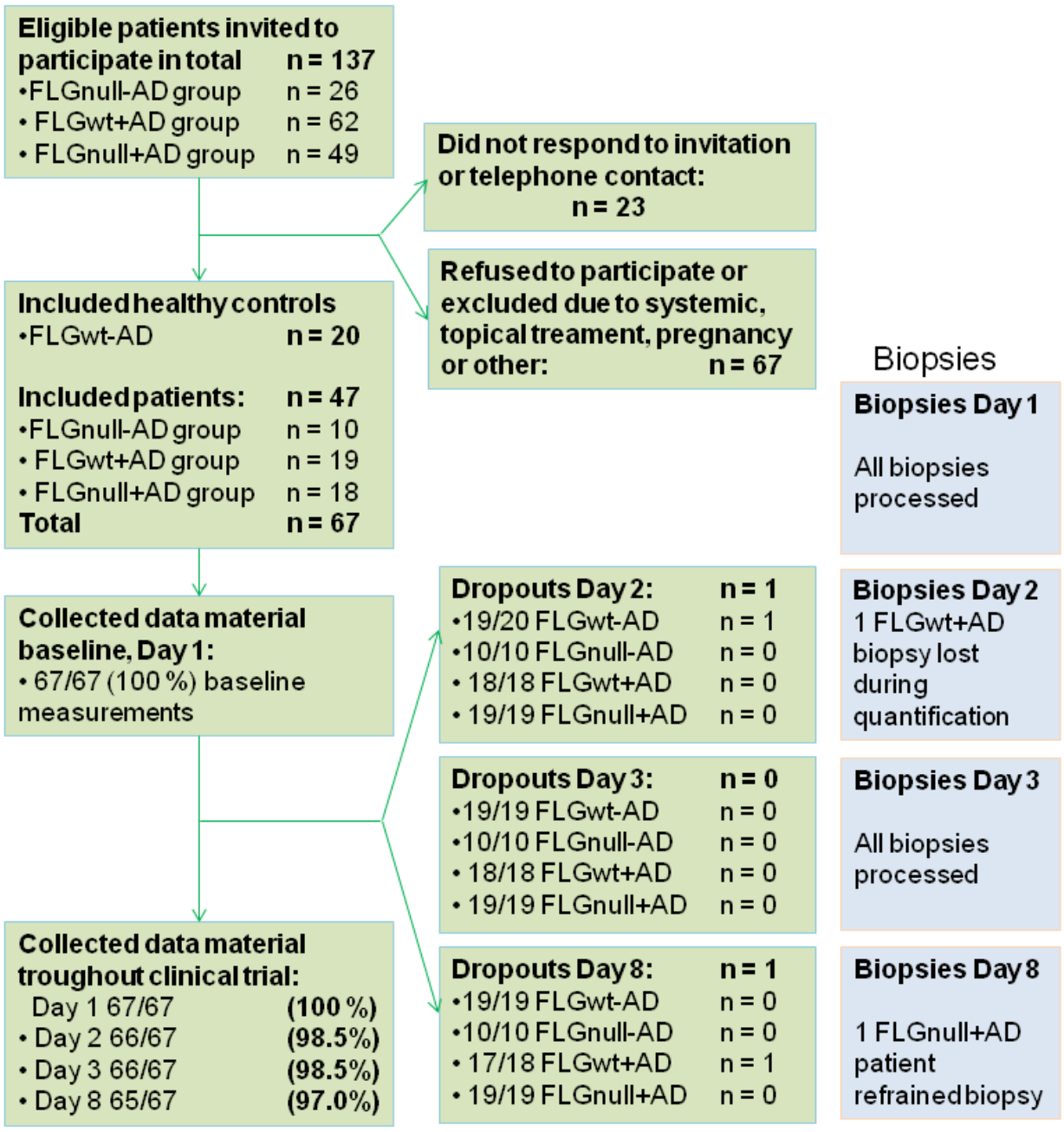

Figure 5: Flow chart of the study population which participated in the clinical experimental study and the biochemical study (Manuscript II and III).

4.2.1 Considerations concerning the study population

The fact that the patients were genotyped prior to inclusion could create bias, because a medical doctor at the dermatological department had already concluded that a filaggrin mutation screening was relevant according to the patient's medical history and clinical presentation. Another factor of importance is that the individuals without AD, but with filaggrin mutations, were not healthy individuals, but eczema patients, with 60% (6/10) having allergic contact dermatitis and 40% (4/10) having irritant contact dermatitis. The ideal setup would have been to recruit healthy individuals with filaggrin mutations from the general population; however, this would have been a very time consuming step requiring screening of multiple individuals as only 8–10% of healthy individuals possess filaggrin mutations[34;55]. Accordingly, this was not possible to perform in our set-up due to time factors. This is something that would be worthwhile pursuing in the future. We were able to include only 10 individuals with filaggrin mutations without AD, which creates a power problem in Manuscript II.

Our patient population was recruited from our department, which constitutes a different population concerning severity and disease course compared with patients obtained from a general practitioner or through a specialist dermatology clinic. We tried to even out the population groups by adding different exclusion criteria as described in Manuscript II.

All patients were Caucasian, because it has been shown that the filaggrin mutation spectrum varies according to different populations across the world[126-129]. The distribution of sex and age was comparable between groups.
It would have been interesting to have included SCORAD (SCORing Atopic Dermatitis) of the patients or percentage involvement of eczema to establish whether the severity and regeneration was related to this.

4.2.2 Considerations on skin barrier function assessment

The considerations concern the methods used in Manuscript II and are listed alphabetically.

- Clinical Scoring: The scoring was performed by J. Bandier, who also assessed the individuals for AD by the UK criteria[119-122]. To compensate for this lack of blinding towards the AD characteristics when assessing the clinical score, the assessment of AD was performed on the first day and was then kept inaccessible until the trial had ended. The clinical scoring was performed without inter-individual variation. The scoring system is valid only up till 96 hours after irritation; however, we used the same scoring throughout the trial. The scoring we used did not allow for further subdivision into morphology of reaction (erythema, scaling, oedema, roughness, fissures)[93]. This limits the possibility of including the putative diversity of the clinical irritant response. Pictures were taken of all reactions (799 pictures) as documentation. No later reviews of the scores were made, because of the difficulty of estimating weak erythema, oedema and roughness from pictures.

- Filaggrin sequencing: The coverage of these three mutation is not 100% but only 83% of known filaggrin mutation[130]. This could possibly underestimate the filaggrin mutation frequency and skew data. decreasing the likelyhood of a significant result.

- Laser Doppler flowmetry, LDF: Three measurements were made, each of approximately 30 sec. Within each measurement a steady state region of interest of approximately 15–20 sec was selected. From the three regions of interest an average was created; an example of one region of interest is illustrated in Figure 6.

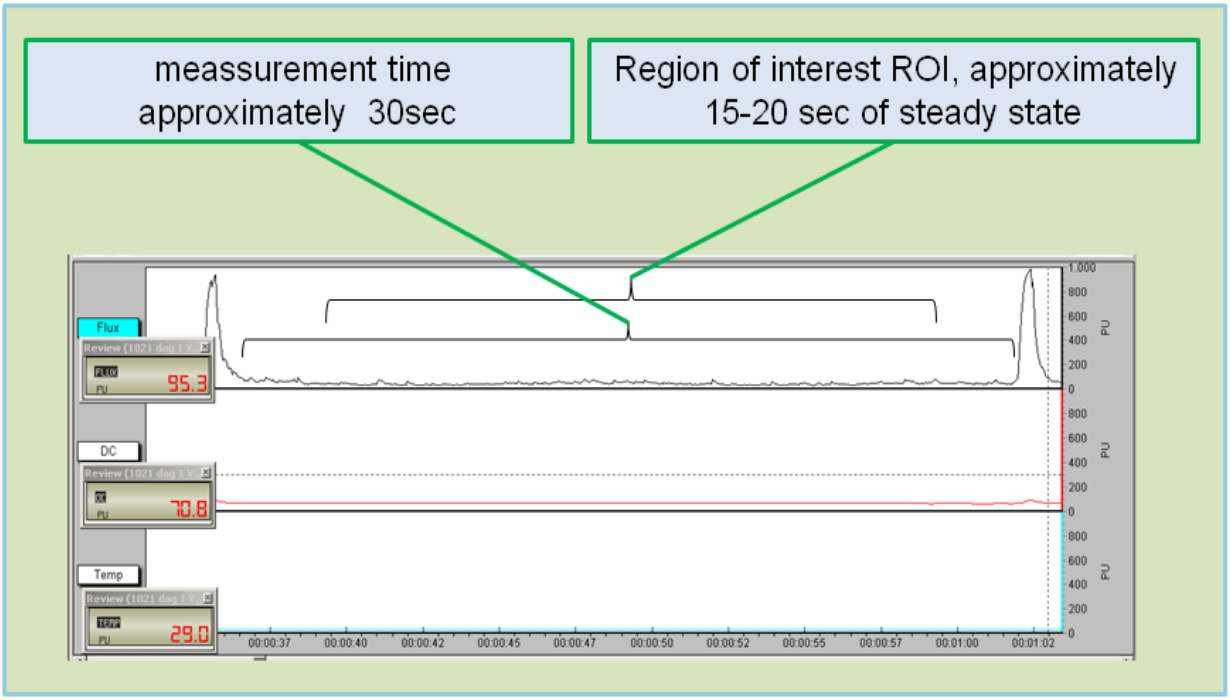

Figure 6. Illustration of LDF measurements with an example of one region of interest.

Although laser Doppler flowmetry is a well-established, reliable and effective method for measuring microcirculation, limitations exist. An example is movement artefacts caused by movement of optical fibres[131]. This could lead to a change in blood flow unrelated to the physiological changes. We compensated for this by using a larger fibre. Moreover, calibration problems can occur. Thus, we frequently calibrated the probe according to manufactory guidelines. Laser Doppler probes are pressure sensitive, and increased pressure could alter the measured blood flow, because the vessels could be compressed. We minimised this by using a larger probe area with flexible leads and avoiding direct contact with the probe and by only one person performing all tests. The DC is the amount of reflected light, and this was constant during all our measurements (Figure 6, middle panel), indicating that the pressure was constant.

- PH: This measurement is determined by the dissociation of water into $H_2O \rightarrow H^+ + OH^-$. PH is then defined as the negative logarithm of the H^+ ions; a pH of 0 being strongly acidic and a pH of 14 being strongly alkaline. The measurements were performed directly on the skin after washing the probe in distilled water. Due to the fact that the definition of pH is based on the dissociation of water molecules and that the skin is neither aqueous nor in aqueous equilibrium, the acidic measurement in the semi-hydrophobic milieu is not directly applicable. Thus the skin pH is a relative pH value[11]. Calibration of the probe was performed with pH solution 4 and 7, and the electrode was immersed in a saturated KCl solution between measurements and rinsed in distilled water prior to all measurements to secure proper readings.

- SLS: The advantages of using SLS in clinical and experimental settings are that it is inexpensive, well described in the literature, reproducible and stable in its irritant potential. Additionally, SLS is non-allergenic and thus by definition qualifies as an irritant[93;132;133]. The concentration of 1% SLS was chosen since this is what has been used in many previous studies[93;103;111;134;135]. From the 1 % SLS, we made a dilution to yield 0.50% and 0.25% SLS in aqueous solutions. The reactions from SLS are not directly transferable to reactions by other irritants[136] or to clinical irritant contact dermatitis. Additionally, irritant exposures in daily life often occur repeatedly and with varying concentration and skin contact, thus single exposure does not suffice to predict skin reaction to repeated exposure[137]. Evaluation time after SLS response by TEWL has shown that a steady state is reached after one hour post Finn chamber removal[138]. Thus all measurements were performed one hour after removal of the Finn chamber. A control chamber without SLS would have been ideal to elude potential artifactual measurements. However, this was not included in the experimental setup. All participants reacted with a significantly high reaction to 0.25% SLS compared with baseline, thus we could not evaluate difference in threshold responses.

- Trans Epidermal Water Loss, TEWL: The TEWL probe is sensitive to changes in temperature and humidity; therefore, the room conditions should be controlled. Seasonal variations are known to influence TEWL[139], thus the study was conducted in a narrow seasonal time span (end of October 2011–beginning of March 2012). All individuals were told to limit exercise before measuring, and were acclimatised and relaxed 15–20 minutes prior to measuring. No talking was allowed during measuring and the faces of the individuals were turned away from the probe to avoid expiration bias. TEWL was measured for 30 sec, and from the last 10 seconds of steady state an average was generated. An example of the TEWL output is shown in Figure 7.

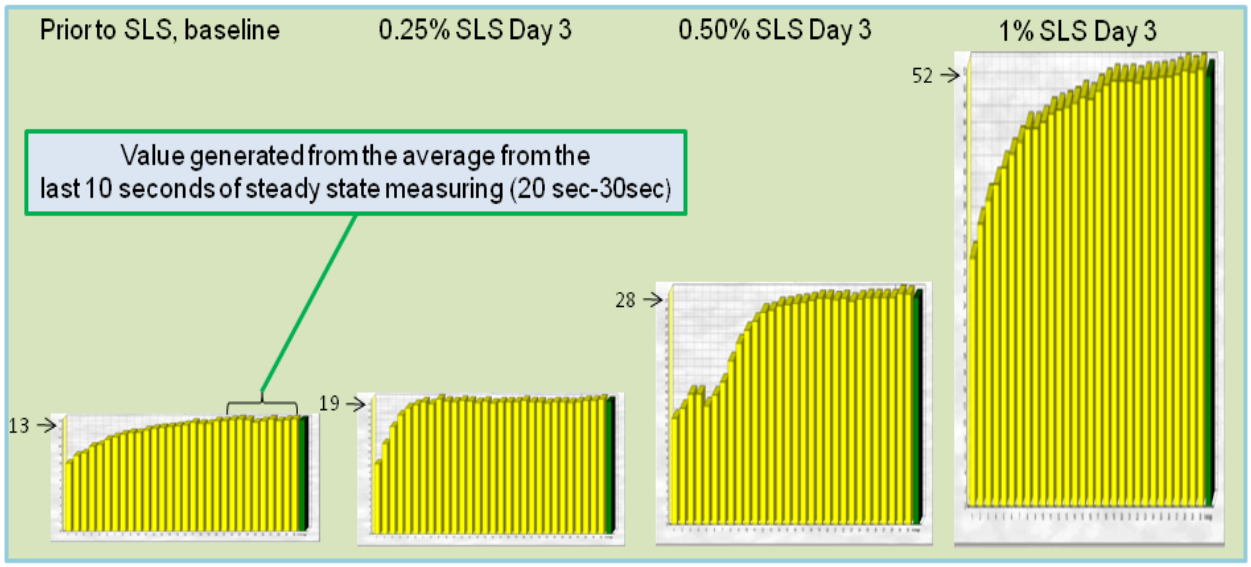

Figure 7. Example of the output of TEWL measurements at baseline prior to SLS application, and measurements on Day 3 for three different SLS concentrations, illustrating increasing TEWL corresponding to increasing SLS.

4.3 Biochemical study (Manuscript III)

The individuals included in Manuscript II were the same as those in Manuscript III. However, the left arm was employed instead of the right arm. We applied 200µL 0.50% SLS in an 18 mm Finn chamber, according to recommended chamber capacity[93].

4.3.1 Quantitative immunoassay

This method was developed at Statens Serum Institut and is, to the author's knowledge, the first method ever published to quantify epidermal filaggrin in individuals of known AD phenotype and filaggrin genotype status. The method is based on extracting epidermal proteins from 4 mm skin biopsies taken centrally 9 cm distally from the axillary fossa, followed by specific quantification by ELISA. Evaluation of protein content was performed before and after SLS exposure. The baseline biopsy from non-lesional skin was taken laterally and proximally to the 18 mm Finn chamber and the following 3 biopsies were taken from within the area covered by the chamber. The biopsies were placed in Eppendorf vials with storage buffer and immediately stored at -80º C. The biopsies were transported on dry ice between Gentofte Hospital and Statens Serum Institute. In total, 262 biopsies were analysed, since one person dropped out after baseline, one after Day 3, one individual refused to have a biopsy taken on the last day and one sample was lost during laboratory processing (Figure 5). Both the extraction and the quantification are described in Manuscript III.

Filaggrin's effect in human skin has primarily been evaluated by genotyping, measuring gene expression and by measuring NMF in tape strips[23;54;111;140]. Thus we wanted to quantify filaggrin in the epidermis at protein level both before and after SLS exposure. To establish a method with high reproducibility, we first extracted protein from surgical waste skin obtained from 10 different individuals undergoing surgery for pendulous abdomen. We took multiple biopsies from each skin sample and confirmed by both silver staining and immunoblot the presence of an epidermal protein with a molecular weight corresponding to that of filaggrin monomers[5]. Specific bands appeared on the immunoblot developed with monoclonal antibodies against filaggrin (Figure 8). To ensure optimal reproducibility of our method, we measured total protein concentration by 280 nm absorbance from several biopsies from each of the 10 individuals. This showed that the extraction was highly reproducible with some inter-individual variability, but little intra-individual intersample variability. This was also confirmed by ELISA for filaggrin.
We could then proceed with processing and quantifying the samples as described in Manuscript III, because we could extract filaggrin from our samples and we had shown antibody specificity.

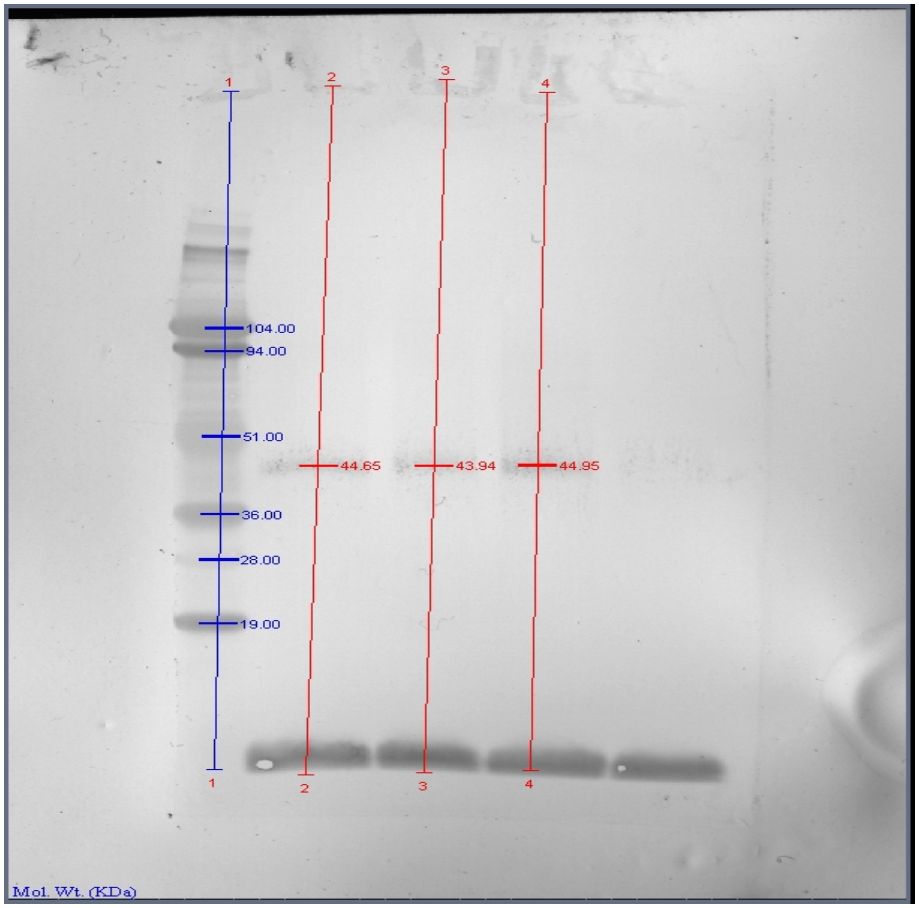

Figure 8. Immunoblot, verifying filaggrin presence in the epidermal extracts. Lane 1 containing the molecular weight (kDa) markers and lanes 2, 3 and 4 epidermal extracts from three different individuals. The red horizontal line corresponds to the position of the filaggrin band.

4.3.2 Considerations on extraction

The extraction process was a modification from Palosuo et al and Ross-Hansen et al[141;142], who both utilised skin from surgical waste, i.e., larger-sized epidermal material. Pestle and mortar grinding was challenging with small skin biopsies (4 mm in diameter). Thus, instead, we finely chopped the epidermis with disposable surgical knives to diminish loss of material; knives were rinsed with extraction buffer. Due to the epidermis being a very rigid and cohesive structure, many extraction steps were necessary. These included grinding in quartz sand, ultrasonication on ice and rotating the samples at room temperature, all contributing to releasing the soluble proteins. Due care was taken to minimize sample loss, e.g. after each processing step, we rinsed with extraction buffer. We cannot rule out that a minimal amount of extract was lost during the different processing steps. However, all samples were handled similarly and by the same person. Throughout the extraction process, consideration of sample volume was taken, standardising the entire extraction process to equal volumes. In the extraction phase, we added organic solvent for delipidation (2-bromo-2-chloro-1,1,1-trifluoroethane or halothane), which is labelled as toxic. Our samples were dialysed overnight to replace the residual halothane with buffer.

4.3.3 Considerations on quantification

As variation in the ELISA plates can occur, we chose to separate samples so we could analyse time variation without considerations concerning plate variance. Thus, the four groups (controls, $FLGwt_{plus}AD$, $FLGnull_{non}AD$ and $FLGnull_{plus}AD$) were randomly distributed on 4 different plates with all 4 samples from one individual being analysed on the same plate. Filaggrin genotype severity was not taken into consideration when distributing the samples.

We utilised 96-well flat bottom plates, allowing equal distribution of coating antibody on the plate bottom. The filaggrin concentration was evaluated in relation to a 7-point standard curve made from the extract pool on each plate. This minimised possible contributions from variation between the 4 different ELISA plates.

The filaggrin specificity of the ELISA was confirmed by the immunoblot, Figure 8. All processing steps were performed using the same buffers and the same extract pool to minimise variation.

We used a sandwich ELISA with indirect detection of filaggrin protein, Figure 9. This ensured signal amplification and specificity. In this colorimetric assay, we added a chromogenic substrate to the wells. The cleavage of 4-nitrophenyl phosphate disodium salt hexahydrate by alkaline phosphatase situated on the secondary antibody created a measurable colour shift proportional to the amount of target protein in the well. The colour shift was measured spectrophotometrically. To ensure that the secondary enzyme-labelled antibody (the 3rd antibody) was not cross-reacting with the capture antibody, we used capture and detection antibodies from different host species (rabbit and mouse).

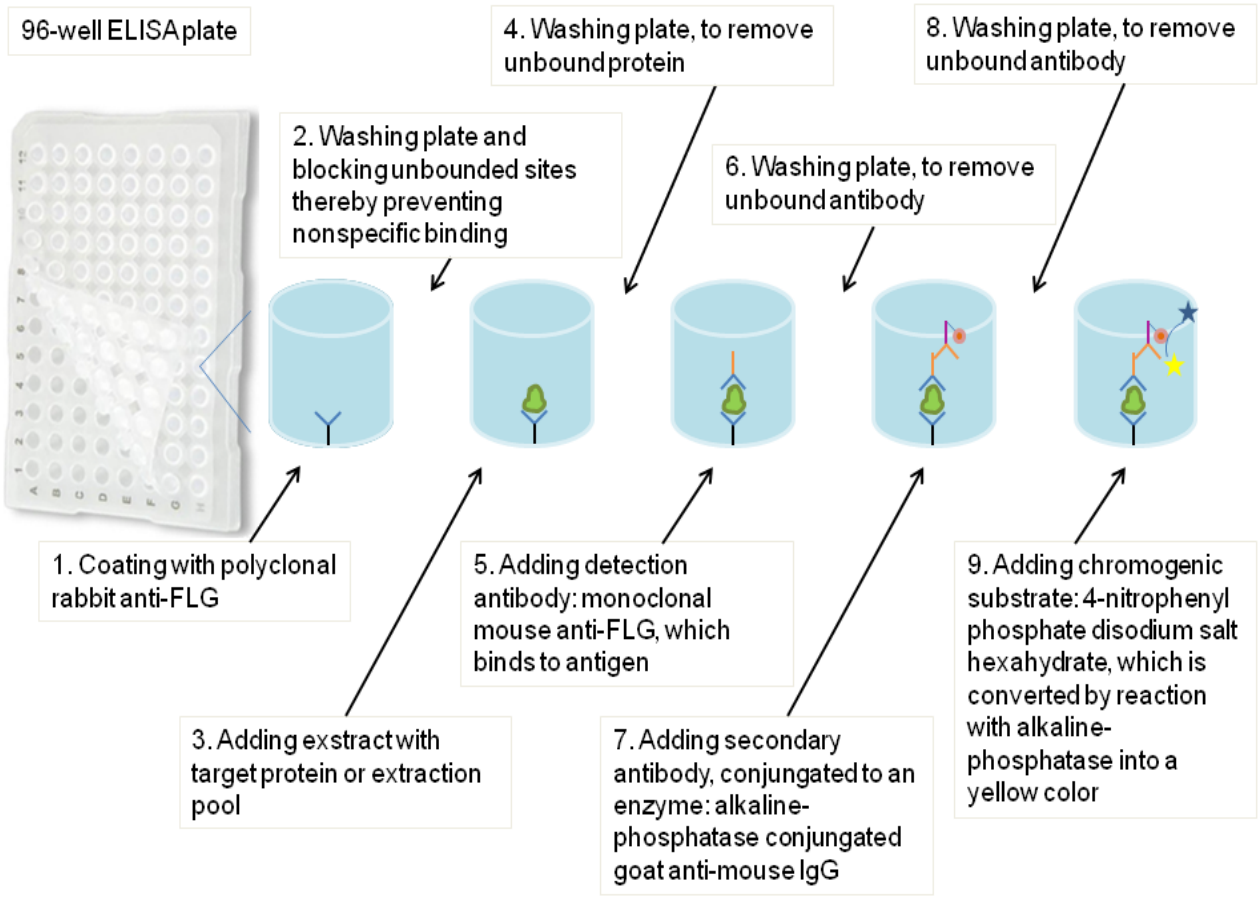

Figure 9. Illustration of the 9 different steps in the ELISA, which were performed prior to filaggrin quantification by spectrometry.

5 DISCUSSION

5.1 Irritant contact dermatitis on the hands and filaggrin mutations

Hand eczema is an important occupational skin disease. It carries a high risk of becoming a chronic disease[143] with widespread consequences including impaired quality of life[49;144].
One of the most frequent causes of hand eczema is contact with skin irritants[145]. However, some individuals can have multiple irritant exposures and never develop eczema, whereas others are more susceptible. Preventive strategies aiming at identifying susceptible individuals have been of increasing interest over the few of years.

In studies not stratifying for filaggrin mutations, it has been established that there is an increased risk of occupational hand eczema in individuals with AD[59;146]. Further, children with both AD and hand eczema are at high risk of hand eczema persistency with multiple recurrences into adulthood[45]. In a recent Danish cohort study, significant factors for developing adulthood hand eczema were identified as hand eczema and AD in childhood, and wet work and handling young children in adulthood[147]. Hand eczema was also found to be related to sick leave, pension, and need for rehabilitation[147]. Studies from other countries have shown similar effects of occupational hand eczema[143;145;148].
AD is an established risk factor for early onset of occupational hand eczema[59;149]. It is also known that Danes with AD avoid entering the hairdressing trade[150;151], which is a high risk trade for occupational hand eczema[149]. These studies did not consider filaggrin mutation status, but it could be speculated that a behavioural change based on filaggrin mutational status exists.

Our epidemiological study was the first to demonstrate a healthy worker effect among persons with both hand eczema onset before the age of 15 years and with filaggrin mutations. This finding suggests that individuals with filaggrin mutations have a certain skin phenotype, making them protect their hands. Additionally, filaggrin mutation carriers also reported a more frequent use of gloves, supporting this notion. Interestingly, the individuals in this study were not informed about their filaggrin mutation status, and their precaution was not based on this knowledge. This indicates that their avoidance of jobs with risk of irritant exposures is based on their skin phenotype. Such a genotype/phenotype driven behavioural pattern has also been found in a recent Dutch prospective cohort study investigating hand eczema in nursing apprentices genotyped for filaggrin mutations. Here, 90% of the individuals with filaggrin mutations and AD applied hand cream at least once daily compared with 68% of the individuals with only AD and 62% among individuals with only filaggrin mutations[110]. This increase in emollient application could imply that individuals with both AD and filaggrin mutations have a more severe barrier disruption and xerosis, leading them to apply emollients more frequently. However, it could also be speculated whether this increased use of emollients could be due to added awareness and compliance induced by an increased number of doctor consultations,

underlying the increased severity of disease. This study further identified a history of AD as the most prominent factor for increasing the risk of developing hand eczema during vocational training[110]. A hand eczema history and wet work exposures were also important risk factors[110], similar to the risk factors reported for development of adulthood hand eczema among AD individuals in studies not stratified by filaggrin mutations[45;147]. Visser et al. further found no effect of having filaggrin mutations in the absence of AD in relation to hand eczema. Only the combination of filaggrin mutations and AD increased the risk of hand eczema (OR 3.6)[110]. This study underlines the hypothesis that the effect of filaggrin mutations is primarily detectable among adult AD individuals and does not seem to exert an individual risk factor, which has also been implied by other studies[63;152]. Evidence of filaggrin mutations as an independent risk factor of developing hand eczema was recently found in consecutively sampled adult patients diagnosed with chronic irritant contact dermatitis on the hands[106]. The highest risk of developing chronic irritant hand eczema was seen among individuals with concomitant AD and filaggrin mutations, but both AD and filaggrin mutations were independent risk factors[106]. However, the finding that filaggrin mutations were independent risk factors was not seen in a prior Dutch study nor replicated in the before mentioned follow-up study[105;110].

Our epidemiological study suggests that incident hand eczema developed in adulthood does not depend on filaggrin mutations, whereas filaggrin mutations are important in the early development of hand eczema. This finding has been confirmed in a recent Slovenian prospective study on AD patients[153].

The importance of age of onset of eczema has been implied previously[60-63]. A British study showed that infants with filaggrin mutations were significantly more likely to have a clinically dry skin, a higher TEWL, and an increased risk of eczema compared with non-mutation carriers[60]. Several studies have confirmed that filaggrin mutations predispose to early manifestation and eczema persisting into adulthood[61-63].

A study characterising individuals in the general population in Copenhagen has suggested that homozygous filaggrin mutation carriers who do not develop hand eczema in childhood do not seem to have an increased risk of developing contact dermatitis on the hands when they pass the age of 18 years.

All of this forwards the notion that filaggrin mutations are factors affecting the development of eczema in early childhood and in chronic eczema, linked to a more severe AD phenotype.

5.2 SLS skin reactions under experimental conditions

Studies on the acute reaction to a skin irritant considering filaggrin mutations have been performed in animals using flaky-tail mice, which are mice with truncated profilaggrin instead of normal profilaggrin[108;109] and in clinical experimental studies.

In animal studies comparing wild type mice and flaky tail mice, a decreased threshold towards an irritant was seen in the flaky tail mice, but only at the lowest concentrations[108]. This suggests that a high irritant concentration could exert such a powerful reaction that mutation status and

thus barrier integrity becomes of minor importance. In another animal study a stronger irritant reaction in the flaky tail mice was found compared with that in the wild type mice, this study did not include multiple irritant concentrations[109].
In human experimental studies the results have not been as convincing as in the animal studies. In a Danish/German study 1% SLS was applied to the skin of individuals stratified by filaggrin mutations and AD, and the response was evaluated after 24 hours. No difference in ΔTEWL was observed between groups[103]. This finding was similar to the findings in a German study with a similar experimental design but with additional assessments of barrier recovery[74]. In this study no difference in either TEWL or chromametry between groups in either skin reaction or recovery was found[74].

In our experimental study we found that the patient population displayed a higher LDF and TEWL response compared with healthy controls, especially at the highest SLS concentration (Manuscript II). The maximum response occurred mostly on Day 3, supporting a continuing penetration of SLS even after chamber removal[154]. However, individuals with filaggrin mutations and concomitant AD had a maximum LDF on Day 2. In our study, we first analysed the data univariately, here we found significantly different Δreactions on day 2 between healthy controls and individuals with AD irrespective of filaggrin mutations. We found no significant difference in Δvalues between the patients groups, similar to that of the German and German/Danish studies[74;103]. This suggests that filaggrin mutations alone do not contribute significantly to skin irritancy when analysing in a univariate manner. The regeneration by both ΔAUC and R was the poorest among individuals with AD, which was also confirmed by multivariate analysis.
When analysing the data by multivariate technique, individuals with AD with and without filaggrin mutations could, in contrary to the univariate analysis, be statistically separated from each other, as filaggrin mutation carriers had a different reaction pattern then that of the other groups. This pattern was characterised by a higher inflammatory alertness (increased LDF) at baseline and a high inflammatory response Day 2 and higher clinical score Day 2. In the German/Danish study the highest baseline erythema was found among individuals with both filaggrin mutations and AD, supporting this higher inflammatory alertness[103].
As our study includes only an increase in LDF, the inflammatory response is not specified. Thus specific immunological questions cannot be answered by this study. Further, the skin reactions to SLS do not represent skin irritability in general, since irritation to one specific irritant does not predict the skin reactivity to another irritant[136].

Our study also indicates that individual assessment of different measures or even using conventional multi-variate statistically analysis makes it difficult to separate adult AD individuals with and without filaggrin mutations from each other. This requires more complex analysis such as a pattern analysis technique.
Whether the association between filaggrin mutations and chronic irritant contact dermatitis is based on a higher inflammatory alertness and/or a disturbed regeneration could be speculated but could not be proved in this study.

5.3 Functional and acquired filaggrin deficiency

Ever since the link between filaggrin mutations and AD was established[34], the perception has been that filaggrin mutations cause a leakier barrier, allowing increased penetration of allergens and irritants and a reduced inflammatory threshold, as shown in mouse studies[108;155]. Among individuals with ichtyosis vulgaris increased paracellular permeability has been found[156]. In response to SLS, an increased penetration of SLS could possibly prompt a rapid IL-1a release from keratinocytes, initiating inflammation[157;158]. However, how a leaky skin barrier due to filaggrin mutations develops into irritant contact dermatitis is still not understood[83].

Epidermal barrier disruption may affect the immune system and lead to a cytokine shift towards a Th2 response[156]. The different cytokines involved in the Th2 response can attenuate the level of filaggrin[35;38;39;159;160] leading to a functional filaggrin defect[36]. Thereby both the immunological response and filaggrin mutations can disrupt the epidermal skin barrier.

High non-specific inflammatory alertness in individuals with AD and filaggrin mutations could cause a functional filaggrin deficiency as suggested in recent studies[36;161].

We therefore investigated the filaggrin protein amount in skin biopsies before and after SLS application (Manuscript III).

We found, that when comparing filaggrin wild type with heterozygote individuals and heterozygote with homozygote individuals a reduction in filaggrin protein content of respectively 65% and 100% was seen. In the absence of AD, a 50% reduction in filaggrin protein content between wild type and heterozygous individuals was seen. This is the first study to show this reduction in protein level. We found that the filaggrin content was lower among individuals with AD compared with those without AD, albeit the amount primarily depended on mutation status. When adding 0.50% SLS an increase in filaggrin protein was seen followed by a decrease to lower than baseline values.

The increase in filaggrin content 1 hour after removal of Finn chamber could be speculated to be a sign of hyaline granule release of filaggrin or decreased filaggrin degradation due to occlusion. Conversely, the prolonged effect of 0.50% SLS suggests increased filaggrin processing, explaining the filaggrin decrease. Interestingly, when adding the 0.50% SLS, the filaggrin content clustered according to genotype rather to phenotype.

The level of filaggrin in the skin cannot alone explain the skin reactions to SLS, since the most severe skin reactions and the poorest regeneration was found among individuals with only AD. Filaggrin mutations and especially with concomitant AD were primarily separated statistically from the other groups by the higher inflammatory alertness and early response. The increased inflammatory reactivity among filaggrin mutation carriers further supports the outside-inside-outside barrier cycle[162], which could partly explain the chronicity seen among individuals with filaggrin mutations. Importantly, this degenerative cycle could possibly be bypassed if individuals avert their childhood eczema[57], which makes the primary prevention of disease of high priority. Fortunately, our epidemiological study suggests that tertiary prevention is already in place among some individuals with the filaggrin genotype.

6 CONCLUSIONS

This thesis focuses on skin barrier susceptibility to irritants and the influence of genetic predisposition. It contributes to the field of knowledge on filaggrin mutations and irritant contact dermatitis. The main findings in our research are presented below:

- Among individuals with early onset of hand eczema, filaggrin mutations promote avoidance of occupational exposure to irritants (Manuscript I).

- Exogenous exposures and AD are more prominent triggering factors of hand eczema in adulthood than are filaggrin mutations (Manuscript I).

- In a clinical experimental setting, the acute reaction to SLS is more severe and the regeneration the poorest in patients with AD irrespective of filaggrin mutations in comparison with healthy control individuals (Manuscript II).

- None of the patient groups had obtained full regeneration with either TEWL or LDF 145 hours after SLS skin barrier disruption.

- Individuals with filaggrin mutations and concomitant AD have a higher inflammatory alertness both at baseline and in the early response to SLS (Manuscript II).

- A method has been established to analyse the exact amount of filaggrin in skin samples (Manuscript III).

- The quantity of filaggrin in epidermis was directly proportional to the number of functional alleles. Comparing filaggrin wild type with heterozygote individuals and heterozygote with homozygote individuals a reduction in filaggrin protein content of respectively 65% and 100% was seen. Comparing heterozygous individuals without AD with healthy control individuals, a 50% reduction in filaggrin content was found (Manuscript III).

- We showed that on non-lesional skin sites, individuals with the same mutational status had lower filaggrin levels if they had concomitant AD, suggesting a decrease in filaggrin content due to AD (Manuscript III).

- Following SLS irritation, a marked effect on the filaggrin content in the epidermis was seen, with a substantial and long-lasting decrease in filaggrin content to levels below baseline values (Manuscript III).

7 PERSPECTIVES AND FUTURE STUDIES

Irritant contact dermatitis is a multifactorial heterogeneous disease, which is difficult to assess, because it is clinically ill-defined and so far no biomarkers have found their way into clinical practice. Further, irritant contact dermatitis can be caused by different exposures of varying potency and of varying intervals. The irritant skin exposures can be domestic as well as occupational. Apart from these factors, the reaction is also dependent on individual susceptibility.
Differences in real-life-setting irritant exposures and experimental exposures are obvious, complicating comparisons and application of findings from experimental to clinical conditions.

Filaggrin mutations are risk factors for the early development of eczema in childhood. The development of irritant contact dermatitis in adulthood largely depends on the AD phenotype and environmental exposures, but filaggrin mutations still seem to play a role in the chronicity of eczema. Individuals with filaggrin mutations and especially those with concomitant AD, seem to have a higher inflammatory alertness and thereby a more immediate response to irritants. Therefore, it may be advisable to do routine risk assessments among young adults before they choose their occupation, give information about avoiding skin hazards based on AD and also to implement skin protection programmes[163]. Guidance on avoidance and/or protective behaviour should not be based on routine genotyping but primarily on the phenotype. Perhaps specific attention should be given to moisturising as this has recently been shown to reduce the risk of developing AD among infants with a high risk of developing eczema[164;165] and thereby enforces the primary prevention of disease.

Further studies involving filaggrin quantification would be intriguing, with investigation of gene expression, immunohistochemistry, protein quantification and degradation simultaneously.
The fact that in our clinical experimental setting none of the patient groups had fully regenerated in either skin response or filaggrin content 145 hours after irritant exposure calls for a longer observational period.
Lowering the SLS irritant dosage in the same experimental setting could also be fascinating, so that evaluation could be made of the threshold response to an irritant. This could not be done in our study, because all groups had a marked reaction to 0.25% SLS. Additionally, evaluating repetitive exposures could be of clinical relevance since irritant exposure in daily life is rarely a single exposure but multiple exposures.

As filaggrin mutations have primarily been investigated in relation to AD, it would be compelling to also evaluate skin irritant responses in healthy individuals with filaggrin mutations without the modification of AD.

As suggested by the large variance in the total protein content (Manuscript III), compensatory mechanisms among filaggrin mutational carriers could be interesting to evaluate further. Moreover, it would be interesting to investigate epigenetic regulatory mechanisms supplementary to the single genetic parameter for barrier disruption as used in our studies.

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9 SUMMARIES

9.1 Summary in English

The outer layer of the skin, the epidermis, provides a self-renewing semi-permeable skin barrier, protecting our inner bodily environment from water loss and protecting us from external environmental insult. One of these external hazards is skin irritants, such as detergents. Detergents are found in many of our daily products. Not all individuals react similarly to detergents, some are more susceptible than others, such as individuals with a history of atopic dermatitis (AD). Recently, mutations in the filaggrin gene have been identified as a major risk factor for atopic dermatitis, early onset and severe disease. Filaggrin is an important protein in the epidermis. Mutations in the filaggrin gene cause either partial or total lack of filaggrin in the epidermis, which leads to changes in the epidermal architecture and skin hydration, causing drier skin, which is more susceptible to exogenous stressors.

To date, filaggrin mutations have been assessed through blood samples, buccal swabs, skin biopsies and tape strips, analysing on mRNA expression, DNA and degradation products or quantified by immunohistochemical staining. However, filaggrin mutations have never been assessed at the protein level in the skin.

It has been shown that carriers of filaggrin mutations have an increased risk of occupational irritant contact dermatitis with poor prognosis. However, the interactions between genes and exposure to skin irritants are still not clear and the effect of irritants on the filaggrin contents in the skin has not been investigated. It is known that the atopic dermatitis may lead individuals to take precautions against exposures to irritants, but no studies exist on the influence of filaggrin genotype.

This thesis consists of an epidemiological, a clinical-experimental and a biochemical study.

The aim was first to explore potential behavioural changes related to the genetic filaggrin risk profile and AD.

We used data from a population-based epidemiological study on 3471 adults, 3348 were genotyped. Regarding filaggrin mutations in combination with lifetime prevalence of hand eczema, we saw a larger proportion of mutation carriers than non-carriers avoiding occupational irritant exposure (63.3% versus 50.5%, respectively). Among individuals with early debut of hand eczema before the age of 15 years (assumed to be prior to occupational exposure), the same pattern was seen with occupational irritant exposure of 50.6% among non-mutation carriers, 28.6% among heterozygote carriers and 0% among filaggrin homozygote individuals. This avoidance was not confounded by a varying prevalence of AD. However, AD was a significant associating factor for adulthood onset of hand eczema.

In the clinical-experimental study we investigated whether individuals with filaggrin mutations exerted a higher irritant response to skin irritants, represented by the detergent, which could potentially explain their avoidance.
The study comprised 67 individuals divided into four groups according to atopic dermatitis and filaggrin mutational status. We analysed skin susceptibility to a known skin irritant, sodium lauryl sulphate (SLS), at three different doses (0.25, 0.50 and 1% SLS). Laser Doppler flowmetry, transepidermal water loss, pH and clinical evaluation were used to evaluate the degree of barrier disruption. The reactions were assessed before application and 1 hour, 25 hours and 145 hours after removal of the detergent, enabling us to assess both the acute response and the following regeneration.
The patient population had a more pronounced reaction and a poorer regeneration compared with that of the healthy control individuals. The response to the irritant was the most severe among individuals with atopic dermatitis without filaggrin mutations and the regeneration was the slowest. No significant difference between the patient groups was found univariately. The data were further analysed by pattern analysis technique Anova Simultaneous Component Analysis. This showed that individuals with filaggrin mutations and AD could be significantly separated from the rest of the patient groups and the healthy controls individuals. The separation was due to a higher inflammatory alertness (baseline LDF) and a higher early inflammatory response to SLS among filaggrin mutation carriers, notably among those with concomitant AD.

Lastly, we investigated the amount of filaggrin protein in epidermis from skin biopsies from the aforementioned population. We took one 4mm biopsy from a non-lesional skin site and three biopsies at the 0.50% SLS site at 1, 25 and 145 hours after removal of Finn chambers. To analyse the protein level, we separated epidermis from the biopsies and established a novel method to extract epidermal filaggrin from the epidermis. We then constructed an enzyme-linked immunosorbent assay to quantify the amount of filaggrin protein in the epidermis. This method was used on the skin biopsies from the experimental study and we analysed the results according to filaggrin mutational status and atopic dermatitis. In total 262 biopsies were analysed.
At baseline we found that the filaggrin content decreased in a dose dependent manner according to mutational zygocity. We found a reduction in filaggrin content between filaggrin wild type and heterozygote individuals of 65% and a reduction of 100% between heterozygote and homozygote individuals. In the absence of AD, a 50% reduction in filaggrin protein content between wild type and heterozygous individuals was seen.
When comparing individuals of the same mutational severity but with and without atopic dermatitis, a decrease in epidermal filaggrin content according to atopic dermatitis status was found. This suggests a functional filaggrin deficiency with a disease-mediated reduction in the filaggrin protein level, although this did not reach statistical significance.
The amount of filaggrin increased in the biopsies taken 1 hour after removal of the SLS chamber, but decreased to levels below baseline at all the following time points.

In conclusion: The amount of filaggrin was consistently the lowest among individuals with filaggrin mutations. When comparing groups of the same zygosity with and without AD, a decrease in filaggrin content was seen at baseline due to atopic dermatitis, albeit non-significant. At 145 hours after removal of SLS the filaggrin protein content was still below baseline values, suggesting an increased filaggrin turnover in response to SLS to repair the skin barrier disruption.
Individuals with filaggrin mutations and early onset of hand eczema preselect their occupational irritancy load depending on their genetic premise. This avoidance might be explained by a severe disease or explained partly by a higher inflammatory alertness and higher early response to irritancy, as shown in our clinical experimental study following exposure to SLS, although this was most notable among individuals with concomitant AD.

Irritants such as detergents can cause irritant contact dermatitis and also induce an effect on the level of filaggrin protein in the skin. Increased awareness of irritant avoidance and skin protection to prevent and diminish irritant contact dermatitis is therefore without doubt an important issue.

9.2 Summary in Danish (dansk resume)

Det yderste lag af huden, overhuden, fungerer som en selvfornyende barriere, som beskytter vores indre kropslige miljø mod vandtab og samtidig beskytter os mod ydre miljømæssige påvirkninger. Detergenter findes i mange af vores dagligdagsprodukter og er samtidig kendt for at kunne irritere huden. Ved kontakt med detergenter, vil nogle individer være mere modtagelige overfor at udvikle en hudirritation end andre. Dette gælder særligt personer med børneeksem.

Mutationer i det gen, der koder for hudproteinet filaggrin, er for nylig blevet identificeret som værende en væsentlig risikofaktor for det at udvikle børneeksem, med tidlig debutalder og mere alvorlig sygdom. Filaggrin er et vigtigt protein i huden og mutationer i genet, der koder for filaggrin forårsager enten en delvis eller en total mangel på filaggrin i huden, hvilket medfører ændringer i hudens arkitektur samt hydrering. Mangel på filaggrin medfører således en mere tør hud, som er mere modtagelig for eksterne stimuli.

Hidtil har man undersøgt filaggrinstatus ved hjælp af blodprøver, mundskrab, hudbiopsier og tape strips og analyseret ved mRNA-ekspression, DNA, nedbrydningsprodukter og immunhistokemisk farvning. Imidlertid er filaggrin mutationer aldrig blevet kvantificeret på proteinniveauet i huden.

Det har vist sig, at individer med filaggrinmutationer har en øget risiko for arbejdsrelateret irritativt kontakteksem med dårlig prognose. Samspillet mellem gener og eksponering for hudirriterende agens er stadig uklart, og det er ikke blevet undersøgt, hvorvidt et hudirriterende stof kan påvirke mængden af filaggrin i huden. Det er kendt, at personer med børneeksem træffer visse forholdsregler for at beskytte sig mod erhvervsmæssig irritantudsættelse, men der findes ingen undersøgelser, om hvorvidt dette også gør sig gældende for individer med filaggrinmutationer.

Denne afhandling består af et epidemiologisk, et kliniskeksperimentelt og et biokemisk studie.

Det første studie havde til hensigt at udforske mulige adfærdsændringer relateret til filaggrinmutationer samt børneeksem.

Studiet tog udgangspunkt i en populationsbaseret epidemiologisk undersøgelse af 3.471 voksne individer i Danmark, hvoraf 3348 var genotypet for filaggrinmutationer. Blandt dem, der rapporterede håndeksem på et tidspunkt i livet, var der flere individer med filaggrinmutationer der undgik erhvervsmæssig irritantudsættelse end blandt dem uden mutationer (henholdsvis 63,3% versus 50,5%). Blandt personer der debuterede med håndeksem før 15-års alderen (altså før en eventuel erhvervsmæssig eksponering), så vi det samme mønster, idet 50,6% af individer uden filaggrinmutationer, 28,6% blandt heterozygote mutationsbærere og 0% blandt filaggrin homozygote individer rapporterede arbejdsrelateret hudirritant udsættelse. Dette indikerer en adfærdsændring på baggrund af det at have filaggrinmutationer. Denne ændring kunne ikke forklares ved en varierende forekomst af personer med børneeksem. Børneeksem var derimod en signifikant faktor for udviklingen af håndeksem i voksenalderen.

I det kliniskeksperimentelle studie undersøgte vi, om personer med filaggrin mutationer havde en voldsommere hudreaktion på en detergent, hvilket kunne forklare ovenstående adfærdsændring.
Undersøgelsen omfattede 67 personer, som blev opdelt i fire grupper i henhold til børneeksem og filaggrinmutationsstatus. Vi analyserede hudens reaktion over for en kendt hudirriterende detergent, natrium lauryl sulfat (SLS), ved tre forskellige doser (0,25, 0,50 og 1% SLS). Graden af barrierepåvirkning blev vurderet ved laser Doppler flowmetry, transepidermalt vand tab, pH og kliniske vurderinger. Huden blev vurderet ved baseline samt 1 time, 25 timer og 145 timer efter fjernelse af detergenten, således at vi kunne vurdere både den akutte reaktion og den efterfølgende regenerering.
Vores patientpopulation reagerede ensartet og med en mere udtalt hud reaktion og en ringere regenerering i sammenligning med raske kontrol individer. Den kraftigste reaktion og den ringeste regenerering var blandt individer med børneeksem uden filaggrinmutationer. Patientgruppernes reaktioner kunne ikke adskilles statistisk fra hinanden. Data blev yderligere analyseret ved mere komplekse metoder, Anova Simultaneous Component Analysis. Her så vi et højere inflammatorisk respons før og umiddelbart efter fjernelsen af detergenten og ligeledes et mere svært eksem blandt individer med mutationer, og særligt blandt dem, der også havde børneeksem, hvilket adskilte dem fra de øvrige grupper.

I det biokemiske studie undersøgte vi mængden af filaggrinprotein i overhuden fra hudbiopsier taget fra den ovennævnte population. Vi tog en 4mm stansebiopsier før irritantudsættelse og tre biopsier i 0,50% SLS kammeret efter hhv. 1, 25 og 145 timer efter fjernelse af detergenten.
For at analysere proteinniveauet adskilte vi overhuden fra biopsierne, hvorefter vi etablerede en metode til at udvinde filaggrin fra overhuden. Vi fremstillede et enzym-linked immunosorbent assay, hvorved vi kunne kvantificere mængden af filaggrinprotein i overhuden fra hudbiopsier fra det eksperimentelle studie, og vi analyserede resultaterne efter filaggrinmutationsstatus og børneeksem. I alt blev 262 biopsier analyseret.
Ved baseline fandt vi, at filaggrin indholdet faldt på en dosisafhængig måde, i henhold til hvor muteret individerne var. Vi fandt en reduktion i filaggrinindhold mellem vildtype og de heterozygote individer på 65% og en reduktion i filaggrin indholdet på 100% mellem heterozygote og homozygote individer. Vi fandt ligeledes en reduktion i filaggrin indholdet mellem vildtype og heterozygote uden børneeksem på 50%.
Ved sammenligning af individer af samme mutationsgrad, men med og uden børneeksem, fandt vi et fald i indholdet af filaggrin i relation til det at have børneeksem. Dette indikerer en funktionel filaggrinnedsættelse grundet børneeksem, selvom dette ikke var statistisk signifikant. Mængden af filaggrin steg i biopsierne taget 1 time efter fjernelse af kammeret med detergenten, men faldt derefter til et niveau under baseline på alle efterfølgende tidspunkter.

Konklusion: Den laveste mængde af filaggrin i overhuden var blandt dem med filaggrin-mutationer, men en reduktion i mængden af filaggrinprotein sås ligeledes i relation til det at have børneeksem. Dog var denne forskel ikke statistisk signifikant. Ved 145 timer efter fjernelse af detergenten, var indholdet af filaggrinprotein fortsat under baselineværdierne, hvilket tyder på en øget filaggrinomsætning efter hudirritation for derved at genopbygge hud barrieren.
Personer med filaggrinmutationer og tidlig debut af håndeksem fravælger erhvermæssig irritantudsættelse på baggrund af deres gener. Denne undgåelse kan eventuelt forklares ud fra deres svære hudsygdom eller forklares ved at disse personer har et højere inflammatorisk beredskab ved udsættelse af en detergent, som vist i vores kliniskeksperimentelle studie. Det inflammatoriske beredskab var dog mest udtalt blandt personer med samtidig børneeksem.

Hudirriterende stoffer såsom detergenter kan forårsage irritativt kontakteksem og ligeledes påvirke niveauet af filaggrin protein i huden. Øget bevidsthed om hudbeskyttelse samt undgåelse af irritanter og derved forebygge og mindske risikoen for irritativt kontakteksem er derfor uden tvivl vigtigt.