FACULTY OF HEALTH AND MEDICAL SCIENCES
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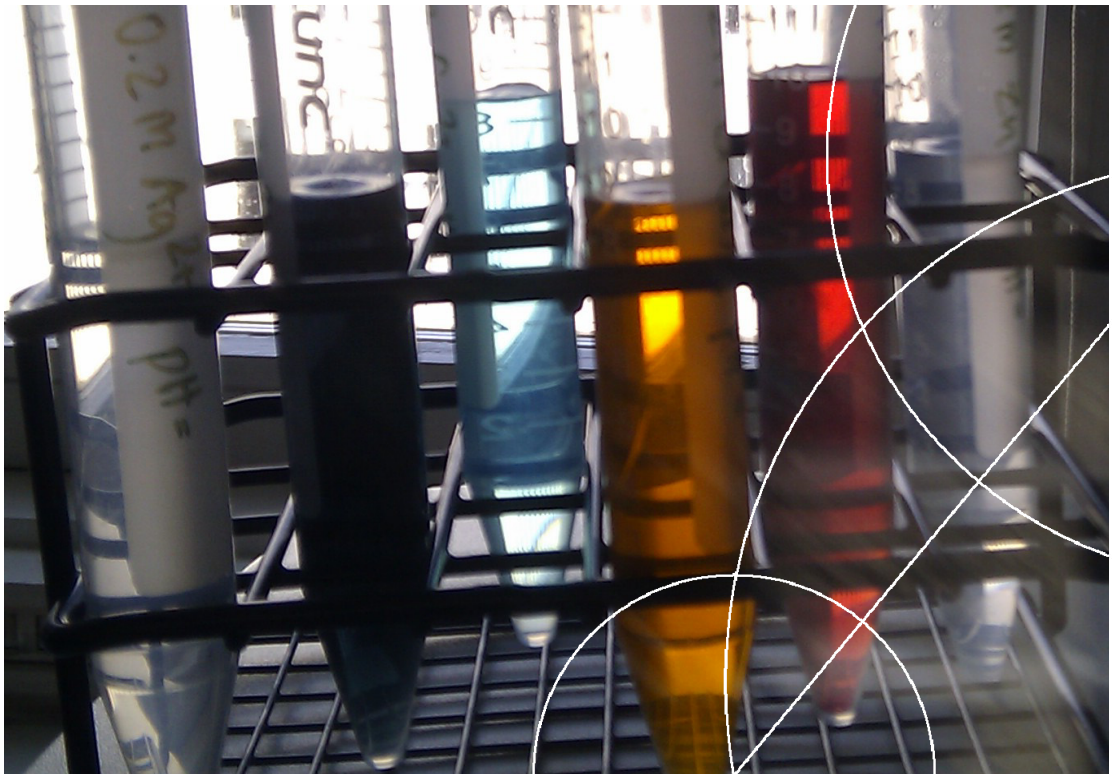
PhD thesis

Skin barrier and contact allergy: genetic risk factor analyses



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Skin barrier and contact allergy: genetic risk factor analyses

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I Ross-Hansen K, Menné T, Johansen JD, Carlsen BC, Linneberg A, Nielsen NH, Stender S, Meldgaard M, Szecsi PB, Thyssen JP. Nickel reactivity and filaggrin null mutations–evaluation of the filaggrin bypass theory in the general population. Contact Dermatitis. 2011; 64: 24-31

II Ross-Hansen K, Linneberg A, Johansen JD, Hersoug LG, Brasch-Andersen C, Menné T, Thyssen JP. The role of glutathione S-transferase and claudin-1 gene polymorphisms in contact sensitization: a cross-sectional study. Br J Dermatol. 2012; 2013; 168: 762-770

III Ross-Hansen K, Ostergaard O, Tanassi J, Thyssen JP, Johansen JD, Menné T, Heegaard NHH. Filaggrin is a predominant member of the nickel binding proteome of human epidermis. J Invest Dermatol. 2013; Submitted

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PREFACE

This dissertation is based on scientific work carried out from May 2010 to April 2013 at the National Allergy Research Centre, Copenhagen University Hospital Gentofte and Department of Clinical Biochemistry, Immunology and Genetics, Statens Serum Institut. The studies received financial support from the Danish Board of Health, the Danish Environmental Protection Agency, the Copenhagen County Research Foundation, Aase and Einar Danielsens Foundation, Aage Bang Foundation, The Hørslev Foundation, the Velux Foundation, ALK-Abelló A/S, Denmark, and the Danish Scientific Research Council. Mekos Laboratories and ALK-Abelló A/S, Denmark kindly donated some of the TRUE tests. All are gratefully acknowledged.

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Gentofte, April 2013
Katrine Ross-Hansen

ABBREVIATIONS

AD	Atopic dermatitis
CLDN1	Claudin-1 gene
EDTA	Ethylenediaminetetraacetic acid
FLG	Filaggrin gene
GST	Glutathione S-transferase gene
GWAS	Genome-wide association study
ICDRG	International Contact Dermatitis Research Group
IMAC	Immobilized metal affinity chromatography
MHC	Major histocompatibility complex
MS	Mass spectrometry
OR	Odds ratio
PCA	Pyrrolidone carboxylic acid
RR	Relative risk
ROAT	Repeated open application test
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel
SNP	Single nucleotide polymorphism
TCR	T cell receptor
TLR4	Toll-like receptor 4
UCA	Urocanic acid
UPLC	ultra-high pressure liquid chromatography

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ABSTRACT

Background Contact allergy is frequent in the general population and arises from prolonged or repeated skin contact with chemical substances. The environmental risk factor is obvious, yet some studies report on associations between genetic variance and an increased risk of developing contact allergy.

Objectives To evaluate the effect of specific gene polymorphisms on the risk of developing contact allergy by a candidate gene approach. These included polymorphisms in the glutathione S-transferase genes (*GSTM1*, *-T1* and *-P1* variants), the claudin-1 gene (*CLDN1*), and the filaggrin gene (*FLG*) in particular.

Methods Epidemiological genetic association studies were performed on a general Danish population. Participants were patch tested, answered a questionnaire on general health and were genotyped for *GST*, *CLDN1* and *FLG* polymorphisms. Filaggrin's nickel binding potential was evaluated biochemically by extracting epidermal proteins from human surgical waste samples and *stratum corneum* scrapings followed by binding studies using immobilized metal affinity chromatography.

Results As suggested by Kaplan-Meier event history analyses, *FLG* null mutations lowered the age of onset of nickel dermatitis, when ear piercing status was regarded. Nickel patch test readings indicated that proportionally more mutation carriers than wild types had stronger reactions. Epidermally derived filaggrin binds nickel. The *GST* gene polymorphisms did not associate with contact allergy among adult Danes. The *CLDN1* polymorphisms rs9290927 minor allele, rs893051 minor allele and rs17501010 major allele were associated with increased risk of different contact allergies.

Conclusions Epidemiological and biochemical data suggest that *CLDN1* and *FLG* gene polymorphisms predispose to contact allergy.

INTRODUCTION

The skin functions as the body's first line of defence against external physical, biological and chemical challenges and the internal challenge of water loss [2]. The skin's complex design and plasticity makes it readily adaptable to our ever changing surroundings. Hence, the human being can reside at varying temperature, humidity and level of sun exposure. Due to the modern living conditions, the load of chemicals in our environment is substantial.

Contact allergy

Repeated or prolonged skin contact with chemical substances can cause contact allergy. The condition is persistent and frequent in the general population [3]. The clinical disease is allergic contact dermatitis [4] (fig. 1a and b), which is a T cell mediated, delayed-type hypersensitivity reaction (type IV). Mechanistically, there are two distinct phases: the induction phase (sensitization) and the effector phase (elicitation) [4]. Sensitization is the primary event, where allergen penetration into the skin activates the skin-residing immunological 'guard' cells, the Langerhans' cells. These cells subsequently prime allergen-specific T cells. Elicitation is when the secondary encounter with the same allergen causes activation of the allergen-specific T cells triggering a local inflammatory response [4]. Contact allergens include many and different substances that must be able to both penetrate the skin and conjugate to skin molecules in order to be recognized by and activate the immune cells.

The clinical diagnostics of contact allergy rely primarily on patch testing. For routine allergy screening in Europe, a minimum recommended test series of the most frequent local allergens is employed. The series currently comprises 28 test substances and is designated the European baseline series [5]. A positive patch test reaction (fig. 1c) indicates that contact sensitization has occurred, and it is identified by a set of criteria dictated by the International Contact Dermatitis Research Group (ICDRG) [6]. Positive patch test readings are rated: +1, +2 or +3 according to the degree of redness, infiltration and vesicles present in the test area; +3 is the strongest positive reaction [6]. In addition to a negative patch test reaction, meaning no reaction at all, reactions can be classified as doubtful or irritant. They are regarded as negative reactions in terms of contact sensitization. Patch tests are applied on the upper back, they are

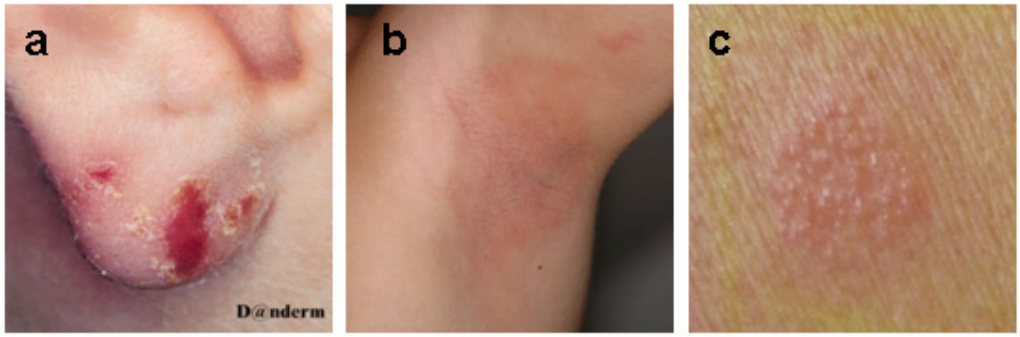

Figure 1. Contact allergy. A) Allergic nickel contact dermatitis on the earlobe from wearing imitation jewellery. B) Allergic fragrance contact dermatitis in the armpit from deodorant application. C) A positive patch test reaction to nickel.

preferably occluded for 48 hours, and the readings are ideally performed at day three as the earliest and day seven as the latest [7].

The prevalence of contact allergy in a given population is affected by the characteristics of the test population, the local allergen exposure pattern and the diagnostic methodology [8]. Hence, epidemiological data are often time-typical, geographically confined and can be hard to compare. In an estimation from different general population studies, the median prevalence of contact allergy was 21.4% (range 12.5–40.6%) [3]. Thus, contact allergy is frequent in the general population. The studies were mainly from North America and Western Europe, and they were performed between 1966 and 2007. In Denmark, it has been estimated that 10 - 15% of the adult population is contact sensitized [9]. The most prevalent allergy is nickel, which accounts for as much as 5.9%. In addition, contact allergy to fragrance mix I is frequent (1.6%) in the Danish population [9]. Nickel is generally a frequent cause of contact allergy, and it has been estimated that up to 17% of women and 3% of men are sensitized in the general population [3, 10].

Nickel has repeatedly been reported to be a frequent contact sensitizer [3, 10]. The phenomenon has been shown in many populations and for many years [3, 10]. Nickel is present in the Earth's crust, and it is essential for plant and microbial metabolism [11, 12]. Whether nickel is essential for humans remains unclear, but excessive exposure can cause both carcinogenicity and toxicity in addition to contact allergy [12]. Even though nickel is common in our natural environment, it is the skin contact with consumer items that release nickel ions, which is the main source of both sensitization and elicitation [10]. Thus, the major sources of nickel allergy and allergic nickel

dermatitis have varied over time according to the time-typical consumer habits. These include the use of stocking suspenders in the 1950-1960s, the wearing of jeans with nickel-releasing buttons and zippers in the 1970-1980s and ear piercing, which became popular in the 1980s [10]. In 1990, the Danish nickel regulation came into force, which restricted the release of nickel from certain items intended for direct and prolonged skin contact to 0.5 μg nickel/cm^2/week [13]. With this regulation, nickel allergy was reduced significantly among young women [14]. The effective intervention in Denmark caused EU to implement a similar nickel directive in 2001 (94/27/EC). Today, the EU legislation prohibits excessive nickel release from items intended for continuous skin contact for more than half an hour or for repeated skin contact for a total of one hour per day. The nickel release limit equals the preceding Danish limit value, except for piercing objects, which are now even stricter regulated with a release limit of 0.2 μg nickel/cm^2/week (Regulation 552/2009). Since the European intervention, nickel sensitization has decreased significantly among young women in Germany [15].

The skin barrier and allergen penetration

Allergen penetration of the skin is a prerequisite for activation of the skin immune apparatus. In terms of percutaneous allergen penetration, the skin barrier can be seen as a composite membrane. Structurally, the outer skin compartment, the epidermis, is characterized by four morphologically distinct layers [16]. Their appearances arise from a stage-specific gene expression, which is associated with a continuous, inside-out keratinocyte turnover. Hence, the keratinocytes progress from a basal stem cell layer, *stratum basale*, through the *stratum spinosum, stratum granulosum* and *stratum corneum* (fig. 2). The *stratum corneum*, the outermost layer, consists of flattened, cornified cells, which are ultimately shed [16]. These cells are extremely resistant due to a heavily cross-linked protein shell, the cornified envelope, which replaces the cell membrane during cornification [16, 17]. In the process of cornification, tissue-specific lipids are deposited in the extracellular space; they complete the barrier being the essential 'mortar between the bricks' of the *stratum corneum*. These lipids are mostly ceramides, cholesterol, fatty acids and cholesterol esters [17]. The process of molecular passage through the *stratum corneum* is basically governed by passive diffusion [18]. Hence, the dose of allergen per unit skin area is important for experimental induction and elicitation of contact allergy [19, 20]. However, the percutaneous absorption rate also varies with the chemical characteristics of the given allergen due to the composition of the outermost skin compartment.

Considering the structure of the *stratum corneum*, the route of allergen penetration can be either transcellular, intercellular or occur through the appendages (e.g. hair follicles) [18]. The primary penetration pathway will depend on parameters such as allergen size, polarity and lipophilicity. Thus, a high molecular weight, lipophilic substance would be prone to diffuse through the intercellular, lipid part of the *stratum corneum*, whereas a low molecular weight, ionic substance probably penetrates transcellularly in addition [18]. Some compounds acquire their sensitizing potential within the skin by metabolical activation. This is for example the case for the hair dye chemical p-phenylenediamine (PPD) and the fragrance compound eugenol [21].

The skin penetration kinetics of nickel has been studied more intensively than most allergens. Nickel ions, like many other metal ions, are electrophilic in nature, which causes reactivity towards certain protein constituents. Accordingly, nickel ions accumulate in *stratum corneum* on skin deposition [22]. Both *in vivo* and *in vitro* penetration studies have indicated a gradual nickel depot build-up in the *stratum corneum* following applications of different nickel salts [23-25]. Thus, *in vitro* diffusion experiments on excised human full thickness skin were found associated with lag-times of up to 50 h [26]. The nickel content which was retained in the different skin compartments after 96 h of artificial nickel exposure was altogether higher than in the recipient

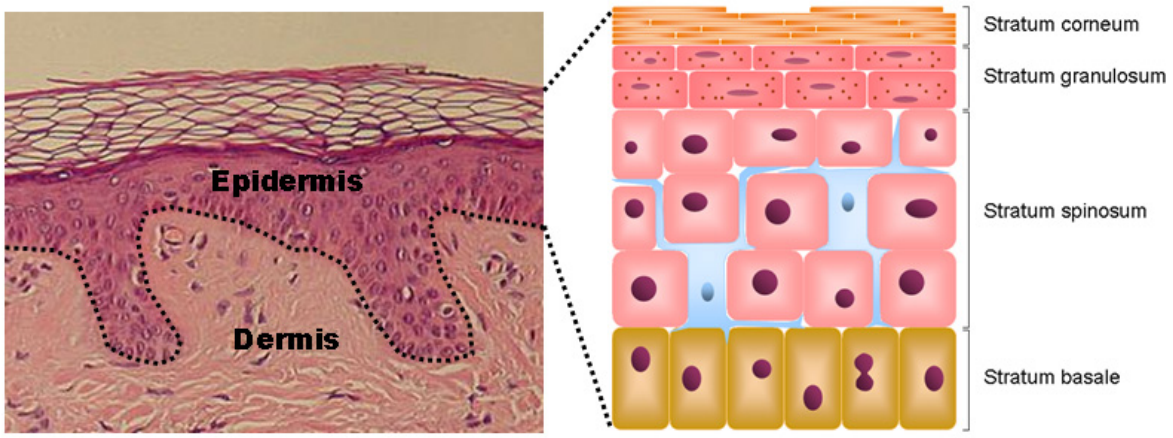

Figure 2. Cross-sections of the skin. Left (Modified from [1]): Human skin section stained with hematoxylin and eosin. Right: Schematic representation of the cellular layers of epidermis. The keratinocytes progress from the basal stem cell layer into stratum spinosum, where the Langerhans' cells reside (blue). The keratinocytes of stratum granulosum are characterized by distinctive protein-filled granules. The stratum corneum is comprised by flattened, terminally differentiated cells, which are shed continuously.

phase [24]. Moreover, nickel was primarily recovered from the topmost layers in sequentially, tape-stripped *stratum corneum* samples following a single open nickel exposure *in vivo* [25]. The epidermal affinity for nickel has been studied by binding studies on powdered tissue [27]. Whereas the freeze-dried epidermis did bind nickel, the binding could be reversed by chelating agents such as ethylenediaminetetraacetic acid (EDTA) [27]. Experimentally, nickel skin penetration kinetics is also influenced by the choice of vehicle, the nickel salt counter-ion and whether open or closed application is performed [23-25]. The degree of nickel penetration through hair follicles is undetermined. Nevertheless, follicular reactions are common in nickel patch testing suggesting that diffusion through the appendages does take place - at least under occlusion [28].

Genetics and contact allergy

Barrier perturbation of both exogenous and endogenous character could potentially increase the degree of percutaneous allergen transfer; perhaps it could even affect the mode of immune activation. Exogenously, factors such as physical insults on epidermis or concomitant irritant exposure could influence the rate of allergen influx. Endogenously, genetic variation in the genes related to epidermal integrity may cause individual susceptibility to contact allergy. Moreover, once the allergens have penetrated the skin barrier, the xenobiotic elimination processes and the immunological response mechanisms are probably partly individually conditioned. Classically, the genetics of contact allergy have been examined in family or twin studies. In this way Walker *et al.* (1967) demonstrated experimentally that the propensity to become contact sensitized with the weak allergen p-nitroso-dimethylaniline was increased in children of parents, who were successfully sensitized. In contrast, the risk of becoming sensitized to the strong allergen 2,4-dinitrochlorobenzene was found genetically unrelated. Thus, they concluded that a high allergenic potential overruled genetic susceptibility [29]. Studying the risk of contact sensitization in relatives, the results may reflect a similar xenobiotic exposure pattern within a family entity rather than true heredity. Therefore, only few classical reports on the genetics of contact allergy are considered convincing.

More recent studies concentrate on candidate genes, which based on their certain function and genetic divergence potentially could cause varying susceptibility to contact allergy. Hence, a few genes functioning in cellular detoxification mechanisms have been found associated with individual predisposition [30]. These include Glutathione S-transferase (GST) deletion alleles of

the M1 and T1 variants, which were associated with thiomersal and chromate sensitization [31, 32]. Additionally, increased risk of PPD sensitization was associated with the genetically determined 'rapid' form of N-acetyltransferase 2 and low levels of circulating angiotensin-converting enzyme [30]. Further, genetic polymorphisms in the promoter of tumor necrosis factor may be important for the susceptibility to contact allergy on a more general level, although the results are not completely unambiguous [30]. Lastly, certain promoter variants of interleukin-16 have been found overrepresented in contact sensitized individuals compared with healthy controls [30].

The genetic predisposition for nickel contact allergy has been studied in the classical manner in two Danish twin cohorts with conflicting results [33, 34]. Menné and Holm (1983) showed that nickel allergy was pairwise more frequent in monozygotic than dizygotic twins [33]. Conversely, Bryld *et al.* (2004) reported that nickel allergy was environmentally conditioned rather than genetically [34]. Interestingly, the manner of nickel exposure differed between the studies. Whereas the participants of the early study were exposed to nickel primarily through skin contact with buttons and stocking suspenders, the participants of the later study were mainly sensitized by ear piercing. Considering the nickel binding capacity of the *stratum corneum*, the physical penetration of epidermis associated with nickel containing studs would bypass the retention of nickel ions in the outermost skin layers. Consequently, the nickel ions would be presented directly to the immune apparatus, and a potential hereditary effect of *stratum corneum* nickel binding would have been confounded in the analyses. In 2007, null mutations in the gene coding for the epidermal protein filaggrin (*FLG*) were found associated with nickel allergy combined with costume jewelry intolerance in a German cohort [35]. Based on filaggrin's biochemical characteristics, Thyssen *et al.* speculated that it contributed to the nickel binding in *stratum corneum* [36]. Accordingly, they found that *FLG* null mutations were associated with a higher risk of developing nickel allergy among Danish women without ear piercings, whereas there was no genetic effect when evaluating on the entire population [37].

Filaggrin

In 2006, *FLG* null mutations were identified as the cause of the common skin disorder *ichthyosis vulgaris* [38]. Soon after, it became evident that the null mutations were also major risk factors for atopic dermatitis (AD), eczema in general and eczema associated with asthma [39, 40]. The associations have been replicated in many populations, and much research has been directed at

characterizing filaggrin's role in general barrier integrity. Remarkably, filaggrin appears to have numerous functions in epidermis.

FLG is situated in a cluster of genes on chromosome 1q21 called the epidermal differentiation complex [41, 42]. The transcript unit comprises 3 exons of which the first is non-coding, the second is initiating translation and the third is encoding nearly all of the protein product, profilaggrin [43] (fig. 3). Profilaggrin is a ~400 kDa polyprotein that requires extensive processing to yield the functional protein units [44]. This large and highly phosphorylated molecule is expressed in the cells of *stratum granulosum*, where initially it is stored in the form of cytoplasmic protein aggregates; namely the keratohyalin granules that gave the epidermal layer its name [44] (fig. 2 and 3). Activation of profilaggrin involves degranulation, dephosphorylation and enzymatic breakdown [45]. Fluctuations in Ca^{2+} concentration regulate keratinocyte differentiation and proliferation through Ca^{2+}-binding proteins that respond to the changes. For example, the epidermal S100 proteins that undergo conformational changes and activate on Ca^{2+}-binding [46]. The profilaggrin N-terminal encompass an A and a B domain (fig. 3). The A domain contains two motifs resembling the S100 protein Ca^{2+}-binding domain [47]. Indeed, Ca^{2+} removal induces conformational changes in recombinant profilaggrin *in vitro*, and the binding may function in granule formation and/or break down [48]. The N-terminal is cleaved of by enzymatic processing, on which it translocates to the nucleus due to the nuclear localization signal of the B domain [49-51]. The proposed nuclear functions of the N-terminal domain is either promotion of denucleation or to provide a signal, which aids in balancing the lower layers keratinocyte proliferation and differentiation [51]. Apparently, the C-terminal domain is critical for profilaggrin processing to functional filaggrin monomers, before which the C-terminal is cleaved of from the parent molecule [45, 52]. Each profilaggrin molecule encompasses 10 to 12 filaggrin repeats, which are probably released by more than one protease cleaving of the linker seqences [45]. Although the filaggrin monomers without exception consist of 324 amino acids, the amino acid composition vary up to 40% in a single individual [53]. Initially, the key function of the filaggrin monomer is supposedly to mediate orderly keratin aggregation in the transition zone between *stratum granulosum* and *stratum corneum* [53, 54]. Indeed, filaggrin associate with keratin filaments *in vitro* and cause macrofibril formation [54]. Despite the huge variation in filaggrin amino acid composition, sequence analyses indicate a conserved secondary structure consisting of β-turn motifs, which suggestively mediate keratin filament alignment by functioning as an 'ionic

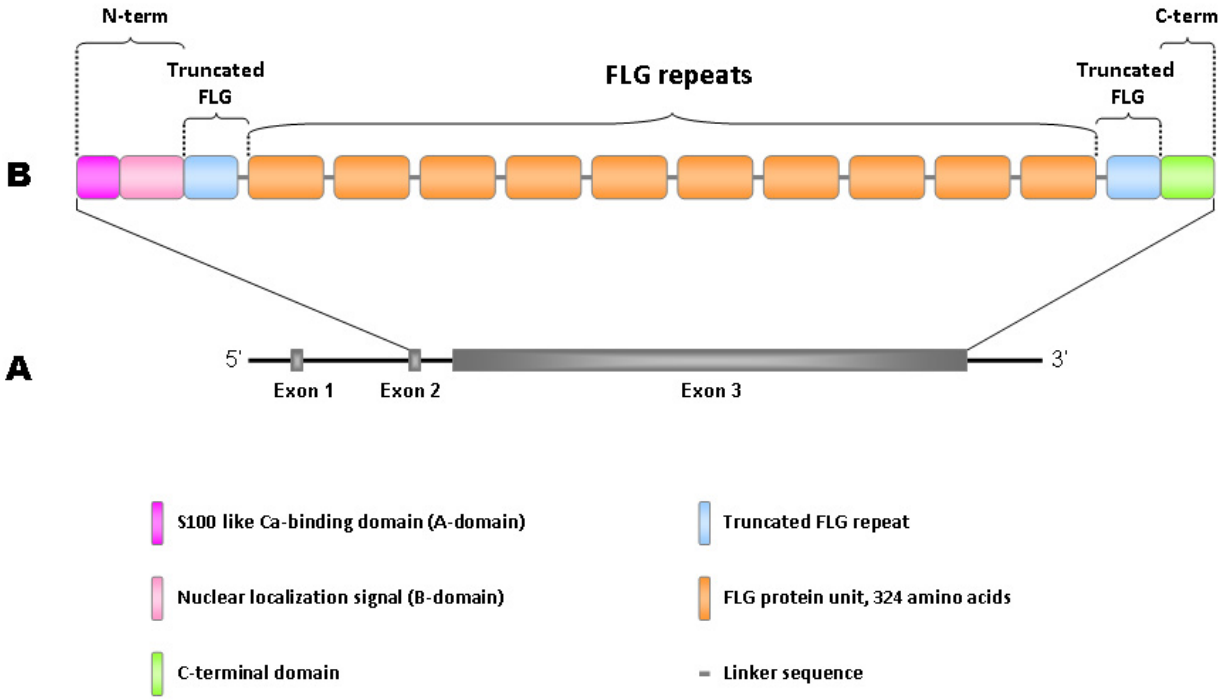

Figure 3. Genomic and protein structure of filaggrin (FLG). A: Genomic organization of *FLG* depicting the three exons, which constitute the mature *FLG* mRNA. Exon 1 is untranslated. Exon 2 and 3 encode profilaggrin. B: Schematic organization of the profilaggrin protein domains. The number of functional Filaggrin repeats varies from 10 to 12.

zipper' [53]. In the *stratum corneum*, filaggrin is deiminated, which disrupts the association with keratin and promotes the protelytic breakdown to free amino acids [55, 56]. As for the processing of profilaggrin to filaggrin monomers, filaggrin degradation involves various proteases [57, 58]. Together with salts, free hygroscopic amino acids and amino acid derivatives constitute the 'natural moisturizing factors' (NMFs) [59]. Many of the amino acids originating from filaggrin are hygroscopic and contribute substantially to the water binding potential of the *stratum corneum* [59, 60]; a second key function of filaggrin. One such major NMF is pyrrolidone carboxylic acid (PCA), which besides water binding purportedly contributes to the acidification of the *stratum corneum* [61]. Furthermore, *trans*-urocanic acid (UCA), a derivative of the major filaggrin amino acid histidine, is involved in epidermal absorption of ultraviolet beta radiation [62].

Considering that null mutations in the *FLG* lead to complete loss of functional filaggrin protein production from the affected allele they are remarkably common. In populations of European

ancestry up to 10% of all individuals carry at least one null mutation, and two mutations that are particularly prevalent (R501X and 2282del4) account for up to 80% of these [63]. Asian populations have their own mutation spectra, and genotyping of one highly selected African population has revealed a single and region-specific *FLG* null mutation there [63, 64]. The dry skin phenotype associated with *FLG* null mutations is inherited in a semidominant manner, in which homozygous carriers have the most severe and heterozygous carriers have less severe clinically recognizable features [38]. In AD, both genotypes are overrepresented [40, 65], especially in early-onset and persistent disease [66]. Since the pathogenesis of AD includes an environmental component and since AD is often followed by development of allergies, asthma and hay fever, it has been hypothesized that a weakened epidermal barrier due to filaggrin deficiency might potentiate the effect of or enhance the penetration of environmental allergens [67].

OBJECTIVES

This thesis is based on epidemiological and biochemical studies. The overall objective was to identify barrier related risk factors of contact allergy based on a candidate gene approach. In particular, the role of *FLG* null mutations for nickel penetration and sensitization was investigated. The aims were:

- To study whether *FLG* null mutation carriers report nickel dermatitis at an earlier age than non-mutation carriers (Manuscript I)

- To examine if the strength of nickel patch test reactions depend on *FLG* mutation status (Manuscript I)

- To investigate the associations between selected *GST* gene polymorphisms and contact allergy in an adult Danish population (Manuscript II)

- To investigate the associations between selected claudin-1 gene (*CLDN1*) polymorphisms and contact allergy in an adult Danish population (Manuscript II)

- To evaluate the effect of *FLG* null mutations combined with the *GST* or *CLDN1* polymorphisms on the risk of developing contact allergy (Manuscript II)

- To determine whether human epidermal filaggrin binds nickel and evaluate the nickel binding potential of other epidermal proteins (Manuscript III)

MANUSCRIPT I

Ross-Hansen K, Menné T, Johansen JD, Carlsen BC, Linneberg A, Nielsen NH, Stender S, Meldgaard M, Szecsi PB, Thyssen JP. Nickel reactivity and filaggrin null mutations–evaluation of the filaggrin bypass theory in the general population. Contact Dermatitis. 2011; 64: 24-31

Nickel reactivity and filaggrin null mutations – evaluation of the filaggrin bypass theory in a general population

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Summary

Background. It was recently shown that filaggrin null mutation carrier status was associated with nickel allergy and self-reported intolerance to costume jewellery. Because of the biochemical characteristics of filaggrin, it may show nickel barrier properties in the stratum corneum.

Objectives. To investigate whether subjects with filaggrin null mutations report nickel dermatitis at an earlier age than wild-type individuals, and to analyse whether null mutation carriers have stronger patch test reactivity to nickel sulfate than do wild-type individuals.

Materials. A total of 3471 Danes (18–69 years of age) answered a questionnaire about general health, and underwent patch testing and filaggrin genotyping.

Results. The mean number of years at risk of developing nickel dermatitis was significantly lower for the filaggrin null genotype than for the wild-type genotype when ear piercing status was considered. In positive patch test readings, the proportion of null mutants increased with increasing reaction strength.

Conclusions. Filaggrin null mutations may lower the age of onset of nickel dermatitis. The hypothesis that ear piercings obscure the effect of filaggrin null mutations on the development of nickel allergy in statistical analyses was supported. An association between the null genotype and increased nickel sensitivity was indicated by patch test reading and questionnaire data.

Key words: allergy, dermatitis, filaggrin, nickel, null mutations, patch test reactivity.

Filaggrin (FLG) is an epidermal protein found in the stratum corneum, where it has a structural as well as a moisturizing function (1). *FLG* null mutation homozygosity is rare, whereas approximately 8% of adult Danes are heterozygous carriers of the two most frequent Caucasian null mutations (2, 3). Novak et al. recently showed that *FLG* null mutation carrier status was associated with nickel allergy and self-reported intolerance to costume jewellery (4).

We have suggested that *FLG* null mutation status may increase nickel penetration through the epidermis (5). As skin is a complex biological matrix, penetration of metal ions is determined by a variety of factors (6). It is known that nickel accumulates in the stratum corneum (7) and, on the basis of binding studies, Fullerton et al. hypothesized that a gradual build-up of local nickel reservoirs could lead to saturation and ultimately free passage of nickel ions through the skin (8). Because of the biochemical characteristics of FLG, which is relatively

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histidine-rich (9), and the fact that histidine strongly chelates nickel, the presence of filaggrin may be important for the nickel-binding capacity of the stratum corneum. Thus, FLG proteins may prevent free nickel ion passage to deeper skin compartments, where interaction with the immune system can provoke nickel sensitization and elicit an allergic response (5). According to the recently proposed bypass theory, a piercing event (with a nickel-releasing piercing post) would bypass the skin barrier effect of filaggrin proteins, as the barrier is perforated (5). Thus, the epidemiological effect of *FLG* null mutations is difficult to study in pierced individuals (2).

Given that nickel and, perhaps, other metal ions are held in the stratum corneum by direct interaction with FLG, its derivatives and, probably, other proteins, *FLG* null mutation carriers may not only have a higher risk of developing contact allergy to nickel and/or other metals (5), but, in a lifetime perspective, also have an earlier onset of nickel allergy and dermatitis, and a stronger patch test response to nickel sulfate. We therefore set out to investigate whether subjects with *FLG* null mutation status report nickel dermatitis at an earlier age than subjects with wild-type *FLG* status. Furthermore, we investigated whether the strength of patch test reactivity to nickel sulfate was stronger in null mutation carriers than individuals with wild-type *FLG*.

Materials and Methods

Study population

A total of 7931 Danes (age range 18–69 years) in Copenhagen, drawn from the Danish Civil Registration System, were invited to participate in a cross-sectional study; 3471 (43.7%) subjects underwent a general health examination between June 2006 and May 2008 (10). A total of 3460 (99.7%) participants were patch tested, and 3346 (96.4%) were *FLG* genotyped for the two most common Caucasian mutations: R501X and 2282del. More women than men, and more older than younger individuals, participated (mean age: 47.5 years) (11). The study was approved by the Ethics Committee of Copenhagen County (KA-20060011), and a written informed consent form was obtained from all participants.

Questionnaire

A questionnaire concerning general health, lifestyle and socioeconomic factors was sent to the participants with the invitation letter. A description of eczema was included: 'Eczema is an itching skin disorder showing redness, dryness, and possibly vesicles and exudation. Eczema is present at the same area for some time.' An affirmative answer to the question 'Have you ever had eczema under the buckle of your watch strap, under the button of your jeans, or from wearing ear pins or earrings?' was regarded as a nickel dermatitis event. Age of onset was then defined by the question 'If yes, how old were you at the first occurrence?' Ear piercing status was assessed through the question 'Have you ever had your ears pierced?', and if the answer was affirmative, participants were asked 'If yes, how old were you the first time you had them pierced?' A history of atopic dermatitis was defined according to the UK Working Party's diagnostic criteria as a history of an itchy skin condition plus a minimum of two of four minor criteria (12). Thus, the major criterion was an itchy skin condition, and the minor criteria were: a history of flexural involvement, a history of asthma or hay fever, generalized dry skin within the last year, and onset before the age of 2 years.

Patch testing

Nickel patch testing was performed with the standardized ready to apply TRUE test® (Mekos Laboratories, Hillerød, Denmark). Patch tests with instructions for self-application to the upper back 2 days before presentation for health examination were mailed to the participants. Trained healthcare personnel performed patch test readings, and the reactions were photographed 1–1.5 hr after removal (supervised by J.P.T. and A.L.). The photographs were later reviewed by T.M., N.H.N., A.L. and J.P.T. to confirm that the recommendations of the International Contact Dermatitis Research Group (ICDRG) had been followed (13). Positive allergic reactions were scored as +, ++, or +++, according to the ICDRG criteria. Thus, homogeneous redness and infiltration in the entire test area was scored as a + reaction. Homogeneous redness, infiltration and vesicles in the test area were scored as a ++ reaction, and homogeneous redness, infiltration and coalescing vesicles in the test area as a +++ reaction. In cases where a patch did not have direct skin contact on the patch test reading, or if participants had removed the nickel patch from the test series before application because of known allergy status, data were registered as missing.

FLG genotyping

Blood samples were taken on the day of the health examination, and genomic DNA was purified from white blood cells. Regions covering the two major mutations R501X and 2282del4 were amplified by polymerase chain reaction (PCR) from genomic DNA, and the PCR products were subsequently hybridized to microbeads carrying either mutation-specific or wild-type-specific probes.

Detection was performed on a BioPlex 200 (BioRad, Hercules, CA, USA).

Statistical analysis

Characteristics were compared with the χ^2-test. A logistic regression analysis was performed with self-reported nickel dermatitis as the dependent variable and with sex, ear piercing status (yes/no), *FLG* genotype (null/wild type) and self-reported atopic dermatitis (yes/no) as the explanatory variables. An interaction term between atopic dermatitis and *FLG* genotype was inserted in a similar regression analysis, to test whether nickel dermatitis depended on *FLG* status in participants with atopic dermatitis. Finally, we performed a logistic regression analysis to confirm that self-reported nickel dermatitis among participants who reported atopic dermatitis was nickel-specific. In this analysis, adjustment was additionally performed for nickel allergy (positive patch test reaction to nickel). Associations were expressed as odds ratios (ORs) with 95% confidence intervals (CIs).

For the different combinations of atopic dermatitis and *FLG* status, we constructed a variable with the following groups: (i) presence of atopic dermatitis and *FLG* null; (ii) presence of atopic dermatitis and *FLG* wild type; (iii) absence of atopic dermatitis and *FLG* null; and (iv) absence of atopic dermatitis and *FLG* wild type. The χ^2 trend test (linear-by-linear association) was used to test for statistically significant differences across the groups of the constructed variable.

The event history distributions were generated using Kaplan–Maier survival statistics. In the analyses, self-reported nickel dermatitis was estimated without the bypass effect. Thus, participants who reported a piercing event were right censored in the analyses, provided that piercing took place before the onset of nickel dermatitis. Participants therefore contributed with risk years from birth and until one of the following events: (i) onset of nickel dermatitis; (ii) first ear piercing event; or (iii) the year of the study, provided that neither of the former events had occurred. The mean onset of nickel dermatitis was calculated with the Kaplan–Meier survival method, including only participants with a history of nickel dermatitis. Owing to an excess of events early in life and a higher proportion of censored observations later in life, the Tarone–Ware significance test was used, and a p-value <0.05 was considered to be significant.

Patterns of patch test reactivity to nickel sulfate (0.20 mg/cm^2) for the *FLG* wild-type and null genotypes, respectively, were investigated with the χ^2-test (linear-by-linear association).

All statistical analyses were performed with SPSS (SPSS, Chicago, IL, USA) for Windows (release 15.0).

Results

Of the 3471 individuals enrolled in the study, 3439 (99.1%) responded to the question on nickel dermatitis. Stratification by sex, *FLG* genotype, ear piercing status and atopic dermatitis is shown in Table 1. More women (55.3%) than men (44.7%) responded. The observed *FLG* genotype frequencies did not deviate significantly from the assumption of Hardy–Weinberg equilibrium (2), and FLG_{null} (8.0%) refers to the combined genotypes including both homozygotes and heterozygotes for both null mutations (R501X and 2282del4). More than half of the participants reported ear piercing (53.1%), of whom 85.7% were women (1564/1825).

Among the 3439 respondents, 830 (24.1%) reported nickel dermatitis at some point in their lives [Table 1; 84.7% of women versus 15.3% of men; OR_{CRUDE} 6.50 (95% CI 5.30–7.97)]. *FLG* null mutation status was not associated with a higher risk of reporting nickel dermatitis ($OR_{CRUDE} = 1.10$, 95% CI 0.82–1.46), whereas a history of ear piercing ($OR_{CRUDE} = 4.70$, 95% CI 3.91–5.65) and a history of atopic dermatitis ($OR_{CRUDE} = 2.40$, 95% CI 1.91–3.02) were strongly associated with self-reported nickel dermatitis. Nickel allergy, as an indicator of self-reported nickel-allergic reactions, had, as expected, the strongest association ($OR_{CRUDE} = 12.84$, 95% CI 9.18–17.95). In a logistic regression analysis with nickel dermatitis as the dependent variable, and sex, atopic dermatitis, ear piercing status and *FLG* mutation status as the independent variables, similar associations were found (Table 1). Further analyses revealed a modest but significant interaction term between *FLG* status and atopic dermatitis ($p = 0.045$). We therefore assessed the effect of the *FLG* genotype in participants who reported atopic dermatitis. In this subgroup, we observed a positive but statistically non-significant association between self-reported nickel dermatitis and the *FLG* null mutation ($OR_{ADJUSTED} = 1.65$, 95% CI 0.88–3.09, $p = 0.121$). To confirm that the positive association observed for the participants with *FLG* null mutation status was related to nickel allergy, and that it was not just a proxy for increased skin reactivity, the regression analysis was extended to include nickel allergy as an explanatory variable. This analysis revealed a positive association for both nickel allergy ($OR_{NICKEL\ ALLERGY} = 10.56$, 95% CI 2.38–46.74, $p = 0.002$) and *FLG* mutation status ($OR_{NULL} = 1.47$, 95% CI 0.76–2.83, $p = 0.257$).

The prevalence of self-reported nickel dermatitis was further investigated in participants without ear piercings stratified by *FLG* genotype in combination with atopic dermatitis (Table 2). An upward trend was found, from the lowest prevalence among wild-type

Table 1. Characteristics of the 3471 participants from the cross-sectional study in western Copenhagen and their risk of developing nickel dermatitis

				ORs for the explanatory variables Former/latter within variable (95% CI)			
					Adjusted OR[c]		
		All, % (n/n_{tot})	Nickel dermatitis[a], % (n/n_{total})	Crude OR[b]	All participants ($n = 3299$)	Participants who reported atopic dermatitis ($n = 333$)	Participants who reported no atopic dermatitis ($n = 2966$)
Sex	Women	55.3 (1903/3439)	36.9 (703/1903)	6.50*	4.03*	4.58*	4.03*
	Men	44.7 (1536/3439)	8.3 (127/1536)	(5.30–7.97)	(3.15–5.19)	(1.97–10.63)	(3.09–5.25)
Ear-piercing	Yes	53.1 (1825/3434)	36.0 (657/1825)	4.70*	2.03*	1.41	2.11*
	No	46.9 (1609/3434)	10.7 (172/1609)	(3.91–5.65)	(1.61–2.56)	(0.65–3.05)	(1.65–2.70)
FLG genotype	Null	8.0 (265/3304)	25.7 (68/265)	1.10	0.95	1.65**	0.79
	Wt	92.0 (3039/3304)	23.9 (727/3039)	(0.82–1.46)	(0.70–1.30)	(0.88–3.09)	(0.55–1.15)
Atopic dermatitis[d]	Yes	10.0 (346/3439)	40.8 (141/346)	2.40*	1.84*	—	—
	No	90.0 (3093/3439)	22.3 (689/3093)	(1.91–3.02)	(1.43–2.38)		

OR, odds ratio; CI, confidence interval; Null, carrier of at least one of the two most common null mutations (R501X and 2282del4); Wt, wild-type.
*Significant difference in χ^2-tests, $p < 0.001$.
**Difference in χ^2-tests, $p = 0.121$.
[a]An affirmative answer to the question: 'Have you ever had eczema under the buckle of your watch strap, under the button of your jeans, or from wearing ear pins or earrings'?
[b]Effect of the noted variable on the development of nickel dermatitis (each separately).
[c]Effect of the noted variable on the development of nickel dermatitis in binary regression analyses.
[d]Defined by the UK Working Party's diagnostic criteria for atopic dermatitis (one major and at least two of four minor criteria).

Table 2. The prevalence of self-reported nickel dermatitis among participants without ear piercings with stratification for the combination of filaggrin (*FLG*) genotype and status for atopic dermatitis

	Combined atopic dermatitis and *FLG* genotype[a]				
	− Atopic dermatitis *FLG* Wt% (n/n_{total})	− Atopic dermatitis *FLG* null% (n/n_{total})	+ Atopic dermatitis *FLG* Wt% (n/n_{total})	+ Atopic dermatitis *FLG* null% (n/n_{total})	χ^2 *p*-trend[b]
Proportion reporting nickel dermatitis	9.8 (133/1357)	12.2 (11/90)	17.9 (14/78)	30.0 (6/20)	<0.001

− Atopic dermatitis, absence of atopic dermatitis; + Atopic dermatitis, presence of atopic dermatitis; Wt, wild-type; Null, carrier of at least one of the two most common null mutations (R501X and 2282del4).
[a]Division of the participants in the four denoted groups depending on their *FLG* genotype status and atopic dermatitis status.
[b]Linear-by-linear association.

carriers without atopic dermatitis (9.8%), to null carriers without atopic dermatitis (12.2%), to wild-type carriers with atopic dermatitis (17.9%), to the highest prevalence, which was seen for null carriers with atopic dermatitis (30.0%). The trend was statistically significant (linear-by-linear association, $\chi^2 = 12.14$, $p < 0.001$).

Figure 1 shows the Kaplan–Meier plots, indicating the mean time at risk until the first event of nickel dermatitis (excluding the bypass effect). Even with no effect from ear piercings, women had a significantly shorter mean time at risk than men (Fig. 1a: Tarone–Ware, $\chi^2 = 111.03$, $p < 0.001$), with values of 58.8 years (95% CI 57.4–60.1) and 65.4 years (95% CI 64.8–66.1), respectively. The mean time at risk for the *FLG* null genotype was significantly shorter than for the wild-type genotype (Fig. 1b: Tarone–Ware, $\chi^2 = 4.30$, $p = 0.038$), the mean values being 60.3 years (95% CI 57.5–63.0) and 63.3 years (95% CI 62.6–64.0), respectively. When men and women were analysed separately with respect to *FLG* genotype (Fig. 1c and d), the mean values for *FLG* null mutants and wild-type genotypes were not significantly different, although the difference was more distinct for men ($\chi^2 = 2.95$, $p = 0.086$) than for women ($\chi^2 = 1.55$, $p = 0.213$).

The mean age at onset (bypass effect excluded) was 21.4 years (95% CI 20.0–22.8) (Table 3). The *FLG* null genotype consistently showed a lower mean age at onset of nickel dermatitis than the wild-type genotype (Table 3).

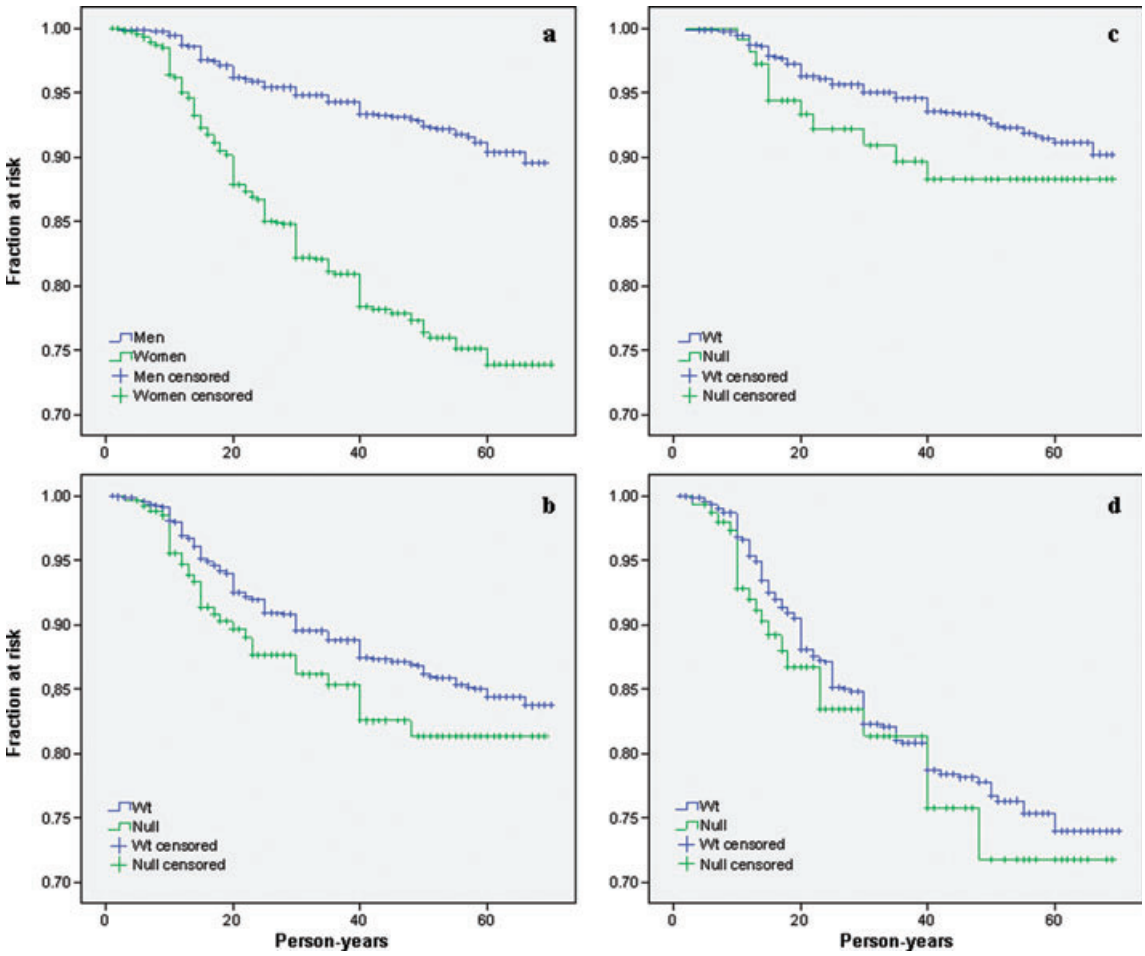

Fig. 1. Kaplan–Meier event history functions. (a) Respondents stratified by sex. (b) Respondents stratified by *FLG* genotype. (c) Male respondents stratified by *FLG* genotype. (d) Female respondents stratified by *FLG* genotype. Person-years: years at risk for each individual until nickel dermatitis debut (event, step down), first ear piercing (censored, +), or age, if neither of the former occurred (censored, +). Wt, wild-type; Null, carrier of at least one of the two most common null mutations (R501X and 2282del4).

Table 3. Mean age at onset (years) of nickel dermatitis stratified by filaggrin (*FLG*) genotype in participants without ear piercings

	Overall (95% CI)	*FLG* Wt (95% CI)	*FLG* null (95% CI)	*p*-value[a]
Total	21.4 (20.0–22.8)	21.7 (20.2–23.3)	18.3 (14.4–22.2)	0.091
Men	25.8 (22.9–28.7)	26.5 (23.3–29.6)	20.6 (14.7–26.6)	0.173
Women	19.4 (17.8–20.9)	19.6 (18.0–21.3)	17.2 (12.1–22.3)	0.172

Wt, Wild-type; Null, carrier of at least one of the two most common null mutations (R501X and 2282del4); CI, confidence interval.
[a]Comparison of *FLG* wild type with *FLG* null (Tarone–Ware).

To investigate whether the *FLG* null genotypes had an augmented response following skin contact with nickel, the strength of patch test reactivity was analysed with respect to *FLG* genotype (Table 4). Among the 107 participants who had a + reaction, 94.4% of the reactions were accounted for by the *FLG* wild-type genotype. Among the ++ reactions, the proportion of *FLG* wild type was 89.2%, whereas the proportion among the +++ reactions was only 66.7%. The reverse tendency was observed for the *FLG* null genotype, which accounted for 5.6%, 10.8% and 33.3% of the +, ++ and +++ reactions, respectively. Statistically, the reactivity patterns (relative proportions) of the two genotypes were not significantly different, although a low *p*-value was obtained (linear-by-linear association, $\chi^2 = 3.181$, $p = 0.075$).

Table 4. Patch test reactivity to nickel sulfate (0.20 mg/cm^2) stratified by filaggrin (*FLG*) genotype

		FLG Wt%	*FLG* null%	Total%
Patch test reactivity[a]	+	94.4 (101)	5.6 (6)	100.0 (107)
	++	89.2 (74)	10.8 (9)	100.0 (83)
	+++	66.7 (2)	33.3 (1)	100.0 (3)
χ^2 *p*-trend[b]			0.075	

Wt, Wild-type; Null, carrier of at least one of the two most common null mutations (R501X and 2282del4).
[a]Readings were performed according to the International Contact Dermatitis Research Group criteria: +, homogeneous redness and infiltration in the entire test area; ++, homogeneous redness, infiltration and vesicles in the test area; +++, homogeneous redness, infiltration and coalescing vesicles in the test area.
[b]Linear-by-linear association.

Discussion

This study suggested that *FLG* null mutations may increase the risk of self-reported nickel dermatitis in subjects with atopic dermatitis, and also that null mutations may lower the age at onset of nickel dermatitis.

Interestingly, the observed difference in the mean time at risk between *FLG* wild-type and null genotypes was more distinct among men than among women (Fig. 1c and d). The explanation for this finding is uncertain, but we speculate that the difference was stronger for men, as they are generally less exposed to nickel from, for example, jewellery than are women. Thus, the nickel-binding capacity of FLG could be saturated in a larger proportion of women with wild-type *FLG*, resulting in a higher prevalence of nickel dermatitis (as nickel then penetrates the skin). This would tend to dilute the difference between wild-type and null mutation genotypes that can be observed in women. Our findings may support the notion that a certain number of nickel ions can be held in the stratum corneum, the amount being proportional to the amount of FLG present (as well as other nickel-binding proteins). Thus, a lower threshold for nickel sensitization and elicitation is to be expected in subjects with *FLG* null mutation status. The merged curves (Fig. 1d) could reflect such a heavy degree of cutaneous exposure to nickel among women in their early 20s to age 40 years. Thus, at lower exposure levels, the curves would be distinctly separated with respect to *FLG* genotype (as seen for men; Fig. 1c). This might be the future scenario, when the cohort effect, resulting from individuals sensitized before the nickel legislation in Denmark and Europe, disappears (14, 15). The mean and median debut year for nickel dermatitis among non-pierced individuals in the present study was 1977 (data not shown).

Data on participants without ear piercings may be used to evaluate the role of FLG as the barrier against nickel sensitization through intact skin. Data on participants who are ear pierced represent a combination of scenarios, where FLG's role is to protect the individual against sensitization but also elicitation. Alternatively, the effect of FLG may be undermined if sensitization and elicitation happen both as a result of, and at the site of, the piercing. Analyses support the hypothesis that ear piercings obscure the FLG effect in association analyses with nickel allergy. In an event history analysis, where the bypass effect was disregarded, the female genotype curves (corresponding to Fig. 1d) were coincident (data not shown).

The mean age at onset of nickel dermatitis among non-pierced participants also supports the possible association between *FLG* null mutation status and nickel dermatitis, as a lower age was consistently seen for the *FLG* null genotype (Table 3). The observed statistical insignificance is probably attributable to inadequate statistical power. Disregarding the bypass effect, the mean age at onset was 22.1 years (95% CI 21.2–22.9) (data not shown). This is reminiscent of the results from a Finnish study of the general population in the late 1970s, where Peltonen et al. found that the mean age of onset for nickel contact dermatitis was 23.1 years (16). The similarity in mean age estimates probably reflects a cohort effect resulting from the large proportion of Danes who experienced nickel allergy and dermatitis during the 1970s and 1980s. Thus, if only young subjects had been questioned, the mean age could possibly have changed, as the frequency of ear piercing, age at first piercing and fashion trends may have changed markedly over the three decades.

Patch test reading data suggested an association between the *FLG* null genotype and a lower threshold to nickel, as the proportion of null mutants among positive patch test readings (+, ++, and +++) increased with increasing reaction strength (Table 4). The +++ data should, however, be interpreted with caution, considering the low counts. A dose–response relationship apparently exists, and may be registered in conventional patch testing. The amount of nickel used for the purpose of patch testing is much higher than the amount of nickel (per area) that an individual will be exposed to in our environment. The results presented in Table 4 indicate that *FLG* null carriers have a lower elicitation threshold than wild-type individuals, and the finding could therefore prove to be of clinical significance.

The two null mutations R501X and 2282del4 examined in our study were previously found to be strong predisposing factors for atopic dermatitis (17). We found a significant interaction term between *FLG* genotype and atopic dermatitis in the regression analysis with nickel

dermatitis as the dependent variable (Table 1). In terms of the development of nickel dermatitis, this result means that *FLG* deficiency may increase the risk of self-reported nickel dermatitis in subjects with atopic dermatitis. Crude data analysis suggested that the prevalence of self-reported nickel dermatitis among individuals without ear piercings was dependent on *FLG* status in combination with atopic dermatitis (Table 2). The largest proportion was registered within *FLG* null mutation carriers with atopic dermatitis (30.0%), followed by *FLG* wild-type carriers with atopic dermatitis (17.9%), *FLG* null genotype carriers without atopic dermatitis (12.2%), and finally *FLG* wild-type carriers without atopic dermatitis (9.8%).

The relationship between nickel sensitivity and atopic dermatitis is not clear (5, 18–20), but previous studies may have been confounded by atopic dermatitis subpopulations with respect to *FLG* genotype. We previously found that the presence of *FLG* null mutations increased the risk of self-reported hand eczema among adult Danes with atopic dermatitis (21). Thus, it appears that atopic participants with null mutations have increased skin reactivity in general. Nevertheless, there was evidence in support of the association between *FLG* null mutation status and nickel dermatitis being, to a large degree, explained by increased nickel sensitivity, as nickel allergy was significantly associated with nickel dermatitis in the regression analysis (Table 1). Because of small groups, it was not possible to stratify by atopic dermatitis in the risk time analyses (Fig. 1; Table 3). Such analyses would be interesting to perform on suitable data.

In this study, an affirmative answer to the question 'Have you ever had eczema under the buckle of your watch strap, under the button of your jeans, or from wearing ear pins or earrings?' could potentially include reactions towards other metals such as cobalt or chromate, or be a marker of irritation. The validity of self-reported nickel allergy has been questioned in a Swedish study, where they found some discrepancies (22). However, nickel is the most prevalent metal allergen (23), and, in a number of earlier studies, the majority of cases of self-reported metal dermatitis were associated with nickel allergy (24–28). In our analysis, 76.5% of the participants who were found to be allergic to nickel in patch testing also reported nickel dermatitis (Table 1). We therefore consider our results to primarily reflect nickel-allergic reactions, but acknowledge that misclassification may be present to a small degree.

In conclusion, *FLG* null mutations may lower the age of onset of nickel dermatitis. The hypothesis that ear piercings obscure the effect of *FLG* null mutations on the development of nickel allergy in statistical analyses was supported. An association between the null genotype and increased nickel sensitivity was indicated by patch test reading and questionnaire data.

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MANUSCRIPT II

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The role of glutathione S-transferase and claudin-1 gene polymorphisms in contact sensitization: a cross-sectional study

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Conflicts of interest
None declared.

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Summary

Background Contact sensitization is frequent in the general population and arises from excessive or repeated skin exposure to chemicals and metals. However, little is known about its genetic susceptibility.

Objectives To determine the role of polymorphisms of glutathione S-transferase (GST) genes and the claudin-1 gene (*CLDN1*) on the risk of contact sensitization, taking common filaggrin gene (*FLG*) mutations into account.

Methods In total, 3471 adult Danes from the general population were standard patch tested and filled out a questionnaire on their general health. They were genotyped for the following polymorphisms: *GSTM1* and *GSTT1* deletion, *GSTP1* single nucleotide polymorphism (SNP) rs1695, four *CLDN1* SNPs (rs893051, rs9290927, rs9290929 and rs17501010) and the *FLG* null mutations R501X and 2282del4.

Results In individuals without ear piercings, a higher prevalence of nickel sensitization was found in those with the minor allele of *CLDN1* SNP rs9290927 (P_{trend} = 0·013). For *CLDN1* rs17501010, contact sensitization to organic compounds was associated with the major allele (P_{trend} = 0·031). The risk pattern was also identified for self-reported nickel dermatitis (P_{trend} = 0·011). The fragrance sensitization prevalence differed in a pairwise comparison of the *CLDN1* rs893051 SNP genotypes (P = 0·022), with the minor allele being associated with a higher prevalence. The associations were confirmed in logistic regression analyses.

Conclusions The *CLDN1* polymorphisms rs9290927, rs893051 and rs17501010 were associated, respectively, with nickel contact sensitization in individuals without ear piercings, contact sensitization to fragrances, and with both organic compounds and nickel contact dermatitis. We could not find associations between GST gene polymorphisms and contact sensitization. *FLG* mutations did not affect the observed associations.

Contact sensitization to chemical substances and metals is frequent in the general population.[1] While the allergen dose per unit skin area is crucial for both induction and elicitation of dermatitis,[2,3] only some individuals react when exposed to the same allergen dose, emphasizing the great interindividual differences regarding the threshold level.[4,5] A recent comprehensive review article concluded that variations in the genes coding for N-acetyltransferases, glutathione S-transferases (GSTs), angiotensin-converting enzyme, tumour necrosis factor, interleukin-16 and filaggrin (FLG) were associated with an elevated risk of contact sensitization or increased susceptibility to certain contact sensitizers.[6] Also, it has been shown that loss-of-function mutations in *FLG* increase the risk of allergic nickel dermatitis[7–9] and contact sensitization to nonmetals in individuals with episodes of dermatitis (article submitted for publication).

The outermost compartment of the epidermis, the stratum corneum, is a compact layer of flattened and rigid corneocytes

that are embedded in a lipid-rich matrix providing mechanical resistance against offending physical, chemical and microbial agents.[10] Keratin filament alignment is facilitated by FLG proteins that are released from the keratohyalin granules at the junction between the stratum corneum and stratum granulosum.[11] FLG is formed by proteolytical cleavage of a precursor profilaggrin containing 10–12 FLG monomers.[12] FLG proteins are normally further degraded into their constituent amino acids, which help to maintain stratum corneum hydration (the so-called natural moisturizing factors).[13] In the stratum granulosum, the keratinocytes are tightly bound by tight-junction proteins, which control the passage of molecules between the cells and prevent water loss.[14] The components of tight junctions include claudin (CLDN)-1. While reduced expression of the CLDN-1 gene causes epidermal barrier dysfunction and increases the risk of atopic dermatitis – at least in individuals with darkly pigmented skin[15] – no studies have yet investigated whether *CLDN1* polymorphisms also increase the risk of contact sensitization.

GSTs are enzymes involved in cell protection by conversion of reactive molecules into less reactive products. They catalyse the conjugation of reactive electrophiles with reduced glutathione, hence their family name.[16] There are seven cytosolic GST classes in humans: α, μ, π, σ, θ, ζ and ω, also designated A, M, P, S, T, Z and O, respectively.[16] In the general population, complete deletion of the M1 and T1 members is frequently found.[17] Gene polymorphism in a π-class GST, *GSTP1*, involves an amino acid substitution (Ile105 → Val105), resulting in decreased enzyme activity, at least with certain reactive groups.[18] In relation to contact sensitization, the *GSTT1*-null genotype has been associated with chromium sensitization in Taiwanese construction workers,[19] and the double deletion of *GSTT1* and *GSTM1* with thiomersal sensitization in Germans,[20] whereas *GSTM1*, *GSTP1* and *GSTT1* polymorphisms were not correlated with *p*-phenylenediamine sensitization.[21]

We investigated the association between multiple GST gene and *CLDN1* polymorphisms, respectively, and contact sensitization in an adult general population, taking *FLG* mutation status into account. Contrary to clinical cohorts, environmental allergen exposure is suspected to be weaker overall in the general population, possibly allowing the detection of genetic susceptibility that would otherwise be overruled by high exposure to contact allergens.

Patients and methods

Study population

Between 2006 and 2008, 3471 (43·8%) of 7931 randomly invited Danish adults (aged 18–69 years), born in Denmark and residing in the capital region of Copenhagen, completed a questionnaire and underwent a general health examination at Glostrup Hospital, Denmark. Hence, the population was mainly of white descent. The investigation was not restricted to skin health. More women than men, and more older than younger patients, participated (mean age 47·5 years).[22] 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.

Patch testing

In total, 3460 participants were patch tested with the standardized ready-to-apply TRUE Test® (Mekos Laboratories, Hillerød, Denmark) as previously described.[23] Readings were restricted to day 2 for practical reasons, and the reactions were read according to the recommendations from the International Contact Dermatitis Research Group.

GSTM1, GSTP1, GSTT1, FLG and CLDN1 genotyping

Blood samples were taken on the day of the health examination, and genomic DNA was purified from the leucocytes. Two multiplex real-time polymerase chain reaction (PCR)-based assays were used for the detection of *GSTM1* and *GSTT1* genomic DNA copy number, as previously described.[24,25] The assays are based on amplification and quantification of the *GSTM1* or *GSTT1* sequence in relation to a reference gene, albumin, in a multiplex PCR using the StepOnePlus™ real-time PCR system (Applied Biosystems, Foster City, CA, U.S.A.). In total, 32 (0·9%) and 52 samples (1·5%) could not be assigned to the *GSTM1* or *GSTT1* genotypes, respectively, due to poor DNA quality.

The four *CLDN1* single nucleotide polymorphisms (SNPs; rs893051, G/C; rs9290927, A/T; rs9290929, A/G; and rs17501010, G/T) were selected, as it has previously been shown that they are associated with atopic dermatitis and/or its severity, in a comprehensive association analysis including 27 *CLDN1* SNPs in a North-American population.[15] The intronic SNPs rs17501010 and rs9290927 were associated with a lower and higher risk of atopic dermatitis, respectively. The intronic rs893051 and rs9290929, situated in the promoter region, were both associated with greater disease severity.[15] The *CLDN1* SNPs and the *GSTP1* SNP (rs1695, Ile105/Val105) were genotyped by the KBiosciences allele-specific PCR, KASPar (KBiosciences, Hoddesdon, U.K.). The genotyping success rate was above 97·4%, with a mismatch rate of 0·00% for the five SNPs, in a minimum of 362 duplicates per SNP.

Overall, 3346 participants (96·4% of the total) were *FLG* genotyped for the two most common mutations in the white population: R501X and 2282del. Regions covering these two mutations were amplified by PCR, and the PCR products were subsequently hybridized to microbeads carrying either mutation-specific or wild-type-specific probes. Detection was performed on a BioPlex 200 (BioRad, Hercules, CA, U.S.A.).[26]

Questionnaire

The prevalence of dermatitis from nickel and cosmetic products, respectively, was assessed with the questions: 'Have you ever had eczema under the tightener of your watch strap, under the button of your jeans, or from wearing ear pins or

earrings?' and 'Have you ever experienced skin redness, a rash and itching caused by exposure to cosmetics, moisturizers, lotions or such related products?'. Atopic dermatitis was defined by the U.K. Working Party's diagnostic criteria as one major plus at least two of four minor criteria.[27] The major criterion was an itchy skin condition; the minor criteria were (i) a history of involvement of the skin creases; (ii) a personal history of asthma or hay fever; (iii) a history of general dry skin within the last year; and (iv) onset before 2 years of age.[27]

Statistical analyses

Contact sensitization was defined as at least one positive patch test reaction (+, ++ or +++). The genotype prevalence was compared for the following contact sensitization categories: 'overall' (at least one positive patch-test reaction), 'nickel sensitization', 'metal' (nickel, cobalt or chromium), 'fragrance sensitization' (fragrance mix I or Myroxylon pereirae) and 'organic compounds' (at least one contact sensitization excluding metal sensitization). In the case of nickel sensitization, association analyses were also performed for individuals without ear piercings, as nickel sensitization may have developed following skin piercing rather than following prolonged skin contact.[28] To test for dependency of GST and *CLDN1* genotypes in contact sensitization, univariate analyses using the χ^2-trend test (linear-by-linear association) were conducted. Additionally, P-values from pairwise comparisons were calculated. In the cases of *GSTM1* and *GSTT1*, two deleted genes were denoted 'null/null', one deleted gene and one present were denoted 'functional/null', and 'functional/functional' referred to the presence of both gene copies being functional. In the case of *GSTP1*, 'functional/functional' referred to the presence of two genes coding for Ile at site 105 in the amino acid sequence; two genes coding for Val at site 105 were denoted 'null/null', and the heterozygote genotype was denoted 'functional/null'. To evaluate the combined effect of the GST variant genotypes and the *FLG* and *CLDN1* genotypes, respectively, logistic regression analyses were performed. The abovementioned five contact sensitization categories were considered the dependent variables in five different regression analyses adjusted for sex (male or female), age (18–34, 35–49 or 50–69 years) and, in the case of nickel and metal sensitization, ear piercing (yes or no). The *FLG* genotype status was divided into wild type and null, where null denoted at least one nonfunctional copy. Associations were expressed as odds ratios with 95% confidence intervals. Hardy–Weinberg tests were performed using the Hardy–Weinberg calculator from the 'Online Encyclopedia for Genetic Epidemiology Studies'.[29] Statistical analyses were performed using SPSS v15·0 (SPSS Inc., Chicago, IL, U.S.A.) for Windows.

Results

The prevalence of contact sensitization is shown in Table 1. Overall, 10·0% of subjects were contact sensitized to at least one allergen (men 4·7% vs. women 14·2%, $P < 0·001$). The prevalence of atopic dermatitis was 10·1% (men 6·3% vs. women 13·2%, $P < 0·001$).

Table 1 Allergens in the TRUE Test panel, and the prevalence of positive patch-test reactions in 3460 patch-tested adults from the general population

Allergen categories and single allergens	Positive reactions, % (n)
Metals	
Nickel sulphate	5·9 (204)
Cobalt dichloride	0·2 (8)
Potassium dichromate	0·1 (5)
Fragrances	
Fragrance mix I	1·6 (55)
Myroxylon pereirae	0·1 (3)
Other	
Wool alcohols	0·0 (0)
Neomycin sulphate	0·1 (2)
Caine mix	0·1 (2)
Colophony	0·6 (21)
Epoxy resin	0·5 (18)
Quinoline mix	0·1 (2)
Ethylenediamine dihydrochloride	0·2 (8)
p-tert-Butylphenol formaldehyde resin	0·1 (2)
Paraben mix	0·0 (0)
Carba mix	0·1 (4)
Black rubber mix	0·1 (4)
Cl+Me-isothiazolinone	0·2 (6)
Quaternium-15	0·2 (6)
Mercaptobenzothiazole	0·0 (0)
p-Phenylenediamine	0·1 (4)
Formaldehyde	0·2 (7)
Mercapto mix	0·0 (0)
Thiomersal	0·5 (18)
Thiuram mix	0·1 (3)
Total	10·0 (345)

Glutathione S-transferases

The *GSTM1*, *GSTT1* and *GSTP1* genotype distributions did not deviate from the Hardy–Weinberg equilibrium ($P = 0·254$, $P = 0·920$ and $P = 0·671$, respectively). The outcome of patch testing is presented in Table 1. No associations with contact sensitization or contact sensitization subgroups ('metal', 'nickel', 'fragrance' or 'organic compound') were observed for *GSTM1*, *GSTT1* or *GSTP1* (Table 2). The prevalence of self-reported metal and cosmetic dermatitis was comparable between the GST variant genotypes. However, the prevalence of 'fragrance sensitization' differed between the *GSTP1* genotypes ($P = 0·045$) (Table 2). As different GST variants can have overlapping substrate specificities, and even form functional heterodimers,[16] we examined the combined effect of GST gene polymorphisms on contact sensitization using logistic regression analyses (Table 3). Besides traditional confounders such as age, sex and ear piercing, we also performed adjustment for the two most common *FLG* mutations and the

Table 2 Association between contact sensitization and the *GSTT1*, *GSTM1* and *GSTP1* genotypes

	Glutathione S-transferase genotype status			
	Functional/functional, % (N/N_{tot})	Functional/null, % (N/N_{tot})	Null/null, % (N/N_{tot})	P (linear by linear in χ^2-tests)
At least one sensitization[a]				
GSTT1	9·0 (114/1269)	10·4 (163/1574)	10·6 (52/491)	0·213
GSTM1	9·6 (26/271)	9·8 (129/1320)	10·1 (178/1763)	0·727
GSTP1[b]	10·6 (154/1455)	8·8 (130/1478)	9·7 (35/361)	0·247
Nickel sensitization[a]				
GSTT1	5·1 (65/1269)	6·2 (98/1574)	6·3 (31/491)	0·226
GSTM1	7·0 (19/271)	6·0 (79/1320)	5·7 (100/1763)	0·417
GSTP1[b]	6·2 (90/1455)	5·4 (80/1478)	5·0 (18/361)	0·280
Nickel sensitization among individuals without ear piercing[a]				
GSTT1	1·0 (6/595)	0·8 (6/712)	2·4 (6/245)	0·169
GSTM1	0·7 (1/139)	1·3 (8/622)	1·1 (9/795)	0·881
GSTP1[b]	0·7 (5/675)	1·2 (8/695)	2·5 (4/161)	0·081
Metal sensitization[a,c]				
GSTT1	5·4 (69/1269)	6·4 (101/1574)	6·7 (33/491)	0·231
GSTM1	7·0 (19/271)	6·1 (80/1320)	6·1 (108/1763)	0·729
GSTP1[b]	6·5 (95/1455)	5·6 (83/1478)	5·3 (19/361)	0·242
At least one sensitization excluding metal[a,c]				
GSTT1	4·4 (56/1269)	4·3 (68/1574)	4·5 (22/491)	0·993
GSTM1	3·0 (8/271)	4·5 (59/1320)	4·5 (79/1763)	0·423
GSTP1[b]	4·7 (69/1455)	3·7 (54/1478)	5·0 (18/361)	0·589
Fragrance sensitization[a,d]				
GSTT1	1·7 (21/1269)	1·4 (22/1574)	2·4 (12/491)	0·449
GSTM1	0·7 (2/271)	1·9 (25/1320)	1·6 (28/1763)	0·744
GSTP1[b]	1·9 (27/1455)	1·1 (16/1478)	2·8 (10/361)	0·900[e]
Nickel dermatitis[f]				
GSTT1	23·3 (294/1260)	25·0 (391/1561)	22·7 (111/489)	0·887
GSTM1	23·3 (63/270)	24·4 (320/1311)	24·0 (419/1749)	0·990
GSTP1[b]	23·9 (347/1449)	23·7 (347/1465)	25·2 (90/357)	0·770
Nickel dermatitis among individuals without ear piercing[f]				
GSTT1	9·2 (55/595)	11·8 (84/711)	11·1 (27/244)	0·261
GSTM1	7·9 (11/139)	10·5 (65/620)	11·3 (90/795)	0·260
GSTP1[b]	10·7 (72/674)	10·5 (73/696)	11·9 (19/159)	0·778
Dermatitis from a cosmetic product[g]				
GSTT1	46·3 (582/1256)	48·2 (750/1557)	48·4 (236/488)	0·514
GSTM1	47·0 (127/270)	47·5 (621/1306)	47·4 (826/1744)	0·980
GSTP1[b]	48·5 (699/1441)	46·7 (683/1461)	48·2 (173/359)	0·787

[a]Defined as positive patch-test readings (+, ++, +++) according to the International Contact Dermatitis Research Group criteria. [b]For *GSTP1*, functional/functional refers to the Ile105/Ile105 genotype, functional/null refers to the Ile105/Val105 genotype and null/null refers to the Val105/Val105 genotype. [c]Metals included in the TRUE Test are nickel, cobalt and chromium. [d]Fragrances included in the TRUE Test are fragrance mix I and Myroxylon pereirae. [e]In pairwise χ^2-test P = 0·045. [f]An affirmative answer to the question 'Have you ever had eczema under the tightener of your watch strap, under the button of your jeans, or from wearing ear pins or earrings?' [g]An affirmative answer to the question 'Have you ever experienced skin redness, a rash and itching caused by exposure to cosmetics, moisturizers, lotions or such related products?'

four *CLDN1* SNPs to evaluate the significance of GST function in subjects having an inherited skin barrier abnormality. No associations between the GST gene polymorphisms and contact sensitization were found in any of the models (data not shown).

Claudin 1

The genotype distributions of the *CLDN1* SNPs rs893051, rs9290927, rs9290929 and rs17501010 were in Hardy–Weinberg equilibrium (P = 0·699, P = 0·752, P = 1·00 and P = 0·075, respectively). The four SNPs were analysed for association with contact sensitization (Table 4). In individuals without ear piercings, we found a higher prevalence of nickel sensitization associated with the minor allele compared with the major allele of SNP rs9290927 (P_{trend} = 0·013). For rs17501010, a significant trend towards a decreasing prevalence of contact sensitization to organic compounds was observed (P_{trend} = 0·031). The rs17501010 SNP was also significantly associated with self-reported nickel dermatitis, with the highest prevalence identi-

Table 3 Logistic regression analyses on contact sensitization in the general population adjusted for *GSTT1*, *GSTM1*, *GSTP1* and *FLG* genotypes and other known risk factors

	Adjusted odds ratio (95% confidence interval)				
	At least one sensitization[a] (n = 3197)	Nickel sensitization[a] (n = 3197)	Metal sensitization[a,b] (n = 3197)	At least one sensitization other than metal[a,b] (n = 3227)	Fragrance sensitization[a,c] (n = 3227)
Age					
18–35	1·00 (ref.)	1·00 (ref.)	1·00 (ref.)	1·00 (ref.)	1·00 (ref.)
36–49	1·06 (0·78–1·44)	1·17 (0·80–1·71)	1·16 (0·79–1·68)	0·99 (0·63–1·56)	0·90 (0·45–1·78)
50–69	0·72 (0·50–1·03)	0·49 (0·30–0·81)*	0·58 (0·36–0·94)*	0·93 (0·57–1·52)	0·61 (0·28–1·36)
Sex					
Male	1·00 (ref.)	1·00 (ref.)	1·00 (ref.)	1·00 (ref.)	1·00 (ref.)
Female	2·79 (1·95–3·99)**	7·21 (3·64–14·3)**	7·88 (4·00–15·6)**	1·30 (0·92–1·84)	1·30 (0·74–2·28)
Ear piercing					
No	1·00 (ref.)	1·00 (ref.)	1·00 (ref.)	—	—
Yes	1·41 (1·01–1·98)*	2·93 (1·66–5·19)**	2·75 (1·60–4·72)**	—	—
GSTT1[d]					
Functional/functional	1·00 (ref.)	1·00 (ref.)	1·00 (ref.)	1·00 (ref.)	1·00 (ref.)
Functional/null	1·05 (0·81–1·37)	1·12 (0·80–1·58)	1·08 (0·77–1·51)	0·91 (0·63–1·32)	0·79 (0·43–1·45)
Null/null	1·19 (0·83–1·70)	1·28 (0·81–2·04)	1·29 (0·82–2·03)	0·97 (0·58–1·63)	1·38 (0·66–2·88)
GSTM1[e]					
Functional/functional	1·00 (ref.)	1·00 (ref.)	1·00 (ref.)	1·00 (ref.)	1·00 (ref.)
Functional/null	0·94 (0·59–1·51)	0·71 (0·41–1·24)	0·72 (0·41–1·26)	1·59 (0·72–3·53)	4·78 (0·64–35·5)
Null/null	0·95 (0·60–1·50)	0·64 (0·37–1·10)	0·70 (0·41–1·20)	1·65 (0·75–3·63)	4·08 (0·55–30·2)
GSTP1[f]					
Ile/Ile	1·00 (ref.)	1·00 (ref.)	1·00 (ref.)	1·00 (ref.)	1·00 (ref.)
Ile/Val	0·82 (0·64–1·06)	0·86 (0·62–1·19)	0·84 (0·61–1·16)	0·77 (0·54–1·11)	0·59 (0·32–1·10)
Val/Val	0·90 (0·60–1·33)	0·76 (0·44–1·30)	0·76 (0·45–1·28)	1·10 (0·62–1·79)	1·50 (0·72–3·13)

*P < 0·05; **P < 0·001; ref., reference value. [a] Defined as positive patch-test readings (+, ++, +++) according to the International Contact Dermatitis Research Group criteria. [b]Metals included in the TRUE Test are nickel, cobalt and chromium. [c]Fragrances included in the TRUE Test are fragrance mix I and Myroxylon pereirae. [d]*GSTT1* genotype for the deletion allele. [e]*GSTM1* genotype for the deletion allele. [f]*GSTP1* genotype for the Ile105/Val105 single nucleotide polymorphism.

fied among individuals who were homozygous for the major allele (P_{trend} = 0·011). The fragrance sensitization prevalence differed significantly when a pairwise comparison of the rs893051 SNP genotypes (P = 0·022) was performed, with the minor allele being associated with a higher prevalence of fragrance sensitization. No associations were found for the SNP rs9290929. The four SNPs and their associations with contact sensitization and contact dermatitis were also evaluated in logistic regression analyses that were adjusted for age, sex, ear piercing and atopic dermatitis. The associations between rs9290927 and nickel sensitization in individuals without ear piercing, rs893051 and fragrance sensitization, and rs17501010 and nickel dermatitis were all confirmed in the logistic regression analyses (Table 5). The association between rs17501010 and contact sensitization to the organic substances of the TRUE Test was only borderline significant (Table 5). Additional adjustment for *FLG* mutation status did not alter the outcomes for rs17501010 and rs893051. However, in individuals without ear piercings, the association between the rs9290927 minor allele and nickel sensitization became insignificant even though the increased risk pattern was still found (data not shown). None of the four *CLDN1* SNPs was associated with atopic dermatitis in this cohort. We had insufficient study power to perform interaction analyses on *FLG* and *CLDN1*, but crude risk analyses showed no indications of additive effects.

Discussion

Skin exposure to contact allergens is practically impossible to avoid due to their frequent use in consumer and occupational products. Identification of the critical determinants of interindividual variation of sensitization and elicitation threshold levels may ultimately improve prevention, as genetically susceptible individuals may be instructed to take particular precautions. GST gene polymorphisms have previously been associated with contact sensitization and other inflammatory disorders,[16,19,20] whereas *CLDN1* polymorphisms have been associated with atopic dermatitis in a case–control study.[15] We investigated the association with contact sensitization using data from a general adult population study. Moreover, common *FLG* mutations, which have been associated with nickel sensitization, were taken into account.

Some *CLDN1* SNPs were significantly associated with nickel sensitization and dermatitis, as well as contact sensitization to

Table 4 Association between contact sensitization and the claudin-1 gene (*CLDN1*) single nucleotide polymorphism (SNP) genotype

	CLDN1 SNP genotype status			
	A/A, % (N/N_{tot})	A/a, % (N/N_{tot})	a/a, % (N/N_{tot})	P (linear by linear in χ^2-tests)
At least one contact sensitization[a]				
rs893051	9·6 (110/1142)	10·2 (164/1609)	9·5 (55/580)	0·966
rs9290927	10·1 (250/2485)	9·5 (75/791)	11 (7/66)	0·750
rs9290929	9·9 (96/967)	9·9 (164/1656)	9·5 (67/708)	0·768
rs17501010	10·1 (280/2781)	9·2 (50/545)	0 (0/17)	0·289
Nickel sensitization[a]				
rs893051	6·2 (71/1142)	5·8 (94/1609)	5·7 (33/580)	0·630
rs9290927	5·7 (141/2485)	6·6 (52/791)	6 (4/66)	0·401
rs9290929	5·6 (54/967)	5·9 (98/1656)	5·9 (42/708)	0·746
rs17501010	5·8 (161/2781)	6·6 (36/545)	0 (0/17)	0·724
Nickel sensitization among individuals without ear piercing[a]				
rs893051	1·3 (7/546)	1·4 (10/732)	0·4 (1/268)	0·352
rs9290927	0·9 (10/1149)	1·6 (6/375)	8 (2/26)	0·013*
rs9290929	1·1 (5/450)	0·9 (7/756)	1·7 (6/346)	0·463
rs17501010	1·2 (16/1301)	0·8 (2/246)	0 (0/6)	0·539
Metal sensitization[a,b]				
rs893051	6·7 (76/1142)	6·0 (97/1609)	5·9 (34/580)	0·468
rs9290927	6·0 (150/2485)	6·6 (52/791)	6 (4/66)	0·648
rs9290929	5·7 (55/967)	6·3 (104/1656)	6·2 (44/708)	0·623
rs17501010	6·0 (167/2781)	7·2 (39/545)	0 (0/17)	0·542
At least one sensitization excluding metal[a,b]				
rs893051	3·9 (44/1142)	4·7 (75/1609)	4·0 (23/580)	0·717
rs9290927	4·5 (112/2485)	3·7 (29/791)	6 (4/66)	0·597
rs9290929	4·8 (46/967)	4·2 (70/1656)	4·0 (28/708)	0·411
rs17501010	4·6 (129/2781)	2·8 (15/545)	0 (0/17)	0·031*
Fragrance sensitization[a,c]				
rs893051	0·9 (10/1142)	2·2 (36/1609)	1·6 (9/580)	0·107[d]
rs9290927	1·6 (40/2485)	1·6 (13/791)	2 (1/66)	0·981
rs9290929	2·0 (19/967)	1·8 (30/1656)	0·8 (6/708)	0·092
rs17501010	1·8 (50/2781)	0·9 (5/545)	0 (0/17)	0·116
Nickel dermatitis[e]				
rs893051	23·5 (267/1134)	24·0 (383/1595)	25·4 (147/579)	0·426
rs9290927	23·6 (583/2470)	25·3 (198/784)	29 (19/65)	0·192
rs9290929	24·7 (237/958)	23·7 (390/1648)	23·5 (165/703)	0·526
rs17501010	24·9 (688/2762)	19·6 (106/542)	24 (4/17)	0·011*
Nickel dermatitis among individuals without ear piercing[e]				
rs893051	10·5 (57/545)	10·5 (77/730)	10·8 (29/269)	0·894
rs9290927	10·4 (119/1149)	11·2 (42/374)	16 (4/25)	0·410
rs9290929	12·1 (54/448)	9·4 (71/756)	11·6 (40/346)	0·717
rs17501010	10·9 (141/1299)	8·5 (21/246)	0 (0/6)	0·197
Dermatitis from a cosmetic product[f]				
rs893051	46·9 (532/1134)	48·0 (762/1589)	48·2 (277/575)	0·574
rs9290927	47·1 (1158/2458)	48·4 (380/785)	59 (39/66)	0·136
rs9290929	47·0 (448/953)	47·4 (778/1642)	47·4 (334/704)	0·853
rs17501010	47·2 (1300/2754)	49·2 (266/541)	41 (7/17)	0·540

*P < 0·05. A, major allele; a, minor allele. Actual SNP bases – rs893051: major allele G, minor allele C; rs9290927: major allele A, minor allele T; rs9290929: major allele A, minor allele G; rs17501010: major allele G, minor allele T. [a]Defined as positive patch-test readings (+, ++, +++) according to the International Contact Dermatitis Research Group criteria. [b]Metals included in the TRUE Test are nickel, cobalt and chromium. [c]Fragrances included in the TRUE Test are fragrance mix I and Myroxylon pereirae. [d]In pairwise χ^2-test P = 0·022. [e]An affirmative answer to the question 'Have you ever had eczema under the tightener of your watch strap, under the button of your jeans, or from wearing ear pins or earrings?' [f]An affirmative answer to the question 'Have you ever experienced skin redness, a rash and itching caused by exposure to cosmetics, moisturizers, lotions or such related products?'

organic compounds and fragrances (Tables 4 and 5). However, none of the *CLDN1* SNPs increased the overall risk of contact sensitization. We suspect that the study lacked sufficient statistical power to detect small effects; for example, the association between *CLDN1* SNP rs893051 and fragrance sensitization was stronger in heterozygous than homozygous individuals. Also,

Table 5 Logistic regression analyses of contact sensitization in the general population involving statistically significant claudin-1 gene single nucleotide polymorphism associations from Table 4

	Adjusted odds ratio (95% confidence interval)			
	Nickel sensitization in individuals without ear piercings[a] (n = 1550)	At least one sensitization excluding metals[a,b] (n = 3343)	Fragrance sensitization[a,c] (n = 3331)	Nickel dermatitis[d] (n = 3202)
Age				
18–35	1·00 (ref.)	1·00 (ref.)	1·00 (ref.)	1·00 (ref.)
36–49	3·17 (0·40–25·0)	1·03 (0·65–1·62)	0·90 (0·45–1·78)	1·40 (1·11–1·77)*
50–69	1·43 (0·17–12·1)	0·95 (0·58–1·54)	0·72 (0·33–1·55)	0·84 (0·64–1·10)
Sex				
Male	1·00 (ref.)	1·00 (ref.)	1·00 (ref.)	1·00 (ref.)
Female	4·81 (1·85–12·5)**	1·31 (0·93–1·85)	1·27 (0·73–2·20)	4·23 (3·27–5·47)**
Ear piercing				
No				1·00 (ref.)
Yes				1·96 (1·54–2·50)**
Atopic dermatitis[e]				
No				1·00 (ref.)
Yes				1·84 (1·42–2·39)**
rs9290927				
A/A	1·00 (ref.)			
A/T	1·63 (0·58–4·55)			
T/T	7·29 (1·44–36·8)*			
rs893051				
G/G			1·00 (ref.)	
G/C			2·56 (1·27–5·19)*	
C/C			1·77 (0·71–4·38)	
rs17501010				
G/G		1·00 (ref.)		1·00 (ref.)
G/T		0·59 (0·34–1·01)[f]		0·72 (0·56–0·92)*
T/T		0 (—)		0·62 (0·16–2·39)

*$P < 0·05$; **$P < 0·001$; ref., reference value. [a]Defined as positive patch-test readings (+, ++, +++) according to the International Contact Dermatitis Research Group criteria. [b]Metals included in the TRUE Test are nickel, cobalt and chromium. [c]Fragrances included in the TRUE Test are fragrance mix I and Myroxylon pereirae. [d]An affirmative answer to the question 'Have you ever had eczema under the tightener of your watch strap, under the button of your jeans, or from wearing ear pins or earrings?' [e]Defined by the U.K. Working Party's diagnostic criteria for atopic dermatitis (one major and at least two of four minor criteria). [f]$P = 0·053$.

we could not confirm the association between atopic dermatitis and *CLDN1* polymorphisms, which could be explained by the use of a general population cohort where morbidity is generally limited. Although our findings need to be replicated in other populations to rule out random error, they could indicate that tight junctions may indeed be involved in susceptibility to contact sensitization and allergic contact dermatitis. The results were not corrected for multiple testing (e.g. Bonferroni) as an *a priori* hypothesis was defined. We acknowledge that the probability of making type I errors is higher without correction, but on the contrary the type II error rate is not exaggerated.

It is also acknowledged that the investigated contact allergens have different chemical properties, being either water or lipid soluble and also having different valency, factors that may influence the capacity to penetrate the epidermis and induce contact sensitization. Hence, the stratum corneum works as an air–liquid barrier where keratinocytes are embedded in lipids to prevent water loss. The lipid-rich stratum corneum may also theoretically facilitate the penetration of especially lipid-soluble contact allergens, and hamper the penetration of water-soluble ones. However, barrier disruption caused by exposure to irritants will affect the quantity of lipids and therefore, at least in theory, increase the capacity of water-soluble allergens to also penetrate the stratum corneum and reach the tight junctions. Thus, the risk of allergen penetration is determined not only by dose and exposure time, but also by the chemical properties of the contact allergens in question, as well as the presence of irritants. In a recent review article, the stratum corneum, tight junctions and dendritic cells were referred to as 'the three musketeers' of the epidermal barrier due to their crucial role in skin homeostasis.[10] The tight-junction barriers exist covertly under the stratum corneum and divide two adjacent compartments to maintain different solute concentrations.[10] As the tight-junction barrier may be regarded as a second line of defence, *CLDN1* mutations could have an impact on the susceptibility to develop contact sensitization and dermatitis. Even though CLDN-1 is just one of several tight-junction proteins, it is a

key component of the epidermal protein structures. Absence of CLDN-1 is implicated in neonatal sclerosing cholangitis associated with ichthyosis, and it has been suggested that hypomorphic *CLDN1* mutations are responsible for related mild phenotypes.[30] Provided that an inherited impairment of the stratum corneum, e.g. due to *FLG* mutations, causes increased allergen penetration of 'the first line of defence', one would also expect increased effects of a concomitant leaky 'second line of defence' due to genetic variation in the tight junctions. However, we could not confirm an increased effect of *CLDN1* polymorphisms when *FLG* mutations were present. It must be underscored that the numbers in the subanalyses were often small and that the results therefore should be interpreted with caution.

Some previous studies have suggested an association between GST gene polymorphisms and contact sensitization to certain chemicals;[19,20] however, a recent meta-analysis by Pot *et al.* concluded that polymorphisms of GST genes are not major contributors to contact sensitization, and that they are disease modifying rather than causal.[21] When we examined one GST gene at a time (*GSTT1*, *GSTM1* and *GSTP1*), no association between the functional gene copy number and contact sensitization was identified. Moreover, we could not find evidence for an association in mutually adjusted regression analyses. Taking *FLG* or *CLDN1* polymorphisms into account did not change the outcome, even though a compromised skin barrier would theoretically increase the effect of GST enzyme presence or absence. The seven classes of human cytosolic GSTs comprise 16 different isoforms, which gives 16 different functional homodimers.[16] It has also been shown that some GSTs are capable of forming functional heterodimers,[31] and if one counts in the numerous gene polymorphisms giving rise to an even broader repertoire of functional dimers, the potential detoxification spectre could be enormous. Considering differential regulation of GST gene variant expression, and the tissue specificity and overlapping metabolic function of this huge enzyme network, it may be that analyses on single variant polymorphisms are too simplified to interpret at disease level.

We are aware that the contact sensitization categories that were constructed for this study grouped structurally different chemical compounds, although GST variants display a degree of substrate preference. However, as GST enzyme activities have not been evaluated for all chemical substances, and because GST variants have been shown to have overlapping substrate specificities, we chose a clinical approach. GST variant polymorphisms might have an impact on sensitization rates for specific allergens such as thiomersal or chromate, as previously reported.[19,20] Such specific allergen associations would have been missed in our study due to low prevalence rates. Similarly, the penetration efficiency of single allergens may vary with *CLDN1* SNP genotype status, depending on which route, inter- or intracellular, is the primary. Nevertheless, general population studies are typically unbiased, offering reliable estimates. Another limitation of the study includes the fact that patch-test readings were performed on day 2 only, which may introduce both false-negative and false-positive readings, depending on the allergen in question.[32–34]

In summary, the *CLDN1* SNP polymorphisms rs9290927, rs893051 and rs17501010 may be associated, respectively, with nickel contact sensitization in individuals without ear piercings, contact sensitization to fragrances, and with both organic compounds and nickel contact dermatitis. We could not find associations between GST gene polymorphisms and contact sensitization in a general population for *GSTT1*, *GSTM1* and *GSTP1* variants in combination or alone.

What's already known about this topic?

- Polymorphisms in the gene encoding the tight-junction protein claudin (CLDN)-1 were recently associated with atopic dermatitis.
- Polymorphisms in the glutathione S-transferase (GST) genes have been associated with contact sensitization, but their role remains unclear.

What does this study add?

- *CLDN1* polymorphisms may affect the propensity to develop contact sensitization to common haptens.
- Polymorphisms in the GST genes do not seem to be of importance in contact sensitization when assessed in a general population.

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MANUSCRIPT III

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Title
Filaggrin is a predominant member of the nickel binding proteome of human epidermis

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Short title
Filaggrin and epidermal nickel binding

Abbreviations
SC - stratum corneum
IMAC - immobilized metal affinity chromatography
MS - mass spectrometry
EDTA - ethylenediaminetetraacetic acid
SDS - sodium dodecyl sulphate
DTT - dithiothreitol
TCEP - tris-(2-carboxyethyl)phosphine

Abstract

Nickel is ubiquitous in the environment. It causes allergy upon excessive skin exposure, and is bound in the outermost skin layer, *stratum corneum* (SC). Filaggrin is a major epidermal protein. Its biochemical properties indicate that it contributes to the epidermal nickel binding. Therefore, we evaluated the metal binding potential of human epidermal proteins by immobilized metal affinity chromatography (IMAC) under varying conditions, and identified the proteins involved in binding by electrophoresis and high resolution tandem mass spectrometry (MS). Filaggrin derived from full epidermal extracts as well as SC samples was found to chelate nickel. The nickel binding property was strong, and maintained under denaturing and reducing conditions. Additionally, filaggrin monomers bound zinc, copper and cobalt. The most predominant epidermal proteins that may also bind nickel included keratins, β-actin, glyceraldehyde-3-phosphate dehydrogenase, histones, pyruvate kinase, haemoglobins, Aldolase A, NME1-NME2 protein, bleomycin hydrolase, elongation factor 2, lactate dehydrogenase, heat shock protein 70 kDa and high-mobility group box 1. In conclusion, filaggrin is a predominant nickel chelator of the epidermal proteome. Filaggrin gene mutations have been associated with an increased risk of allergic nickel dermatitis in European populations. This study provides a possible link between genetics, protein expression and function.

Introduction

Nickel is ubiquitous in the human environment. It is present in metal objects such as jewellery, coins, keys and even mobile phones and laptops [68-70]. Excessive nickel skin exposure can cause contact sensitization, which affects 10–15% of the general population [10]. The outermost skin layer, *stratum corneum*, consists of flattened cells. They are extremely resistant due to a heavily cross-linked protein shell, the cornified envelope, which replaces the cell membrane during

terminal differentiation [16]. The protein filaggrin is expressed just below the SC in the *stratum granulosum* as a large polyprotein, profilaggrin [45]. Each precursor molecule consists of 10-12 filaggrin units of equal size (324 amino acids) that vary by up to 40% in their amino acid composition [41, 71, 72]. The filaggrin monomers are released by enzymatic processing at the junction between the *strata* [45]. *In vitro*, filaggrin monomers aggregate keratins into macrofibrils [73]. In the SC, filaggrin is further degraded to amino acids, which are major contributors to the water binding properties [60]. Hence, filaggrin molecules constitute a heterogeneous protein population. Double allele null mutations in the filaggrin gene lead to complete lack of filaggrin in the epidermis. Approximately 10% of Northern Europeans carry at least one null mutation, which is associated with xerosis [38], atopic dermatitis [40] and allergic nickel dermatitis [35, 37, 74]. Thus, there appears to be a link between the expression of filaggrin and the propensity of becoming sensitized to nickel. In 1956, Wells coloured *ex vivo* skin and showed that nickel accumulated in the outer layers of exposed areas [22]. Subsequently, this finding was corroborated in *ex vivo* penetration studies showing high lag times associated with percutaneous nickel diffusion [24, 75]. Additionally, homogenized, freeze-dried epidermis binds nickel reversibly [27]. Since filaggrin is an abundant histidine-rich protein with an average of 30 histidines per monomer [41], we hypothesized that filaggrin proteins contribute to the nickel binding capacity of the epidermis. Consequently, lack of epidermal filaggrin could be associated with a higher rate of percutaneous nickel penetration and ultimately sensitization. We investigated whether filaggrin extracted from human epidermis could bind nickel and evaluated the binding potential of other epidermal proteins by using IMAC in combination with antibody and MS mediated protein identification.

Results

Filaggrin and other epidermal proteins are enriched by nickel IMAC

Proteins were extracted from *ex vivo* epidermal samples, incubated with Ni Sepharose under non-denaturing conditions and a column was packed with the mixture. The full spectrum of extracted proteins was visualized by SDS-PAGE (fig. 1, lane 1). In the absence of nickel, no proteins were bound by the column (data not shown). Immunoblotting with monoclonal anti-filaggrin antibodies revealed the size range of filaggrin molecules with major reactivity at a molecular weight corresponding to a heterogeneous monomer population (fig 1, right panel, arrowhead). Unbound proteins were collected in the column effluent (fig 1, lane 2 and 7). No anti-filaggrin reactive molecules were detected in this fraction (Fig. 1, lane 7). Column washing allowed for removal of loosely bound proteins. The proteins ultimately retained on the Ni Sepharose were released by ethylenediaminetetraacetic acid (EDTA). These fractions contained filaggrin monomers (fig 1, lanes 3-5 and 8-10). Thus, nickel IMAC enriches a protein population that includes filaggrin but also other proteins. To verify the specificity of the anti-filaggrin antibodies and identify the other proteins, the protein content of lane 4 (Fig. 1) was recovered by systematically cutting out gel slices, digesting with trypsin and analyzing by tandem MS. Using spectral counts as a semiquantitative measure, the 20 most abundant proteins bound by Ni Sepharose and identified by this approach are listed in table 1. The highest amount of filaggrin was found in the region of the gel corresponding to the monomer population, but filaggrin was detected across a wide molecular weight range. Thus, additional high-abundance areas included both heavier and lighter filaggrin species (asterisks, fig. 1). Like filaggrin, the assigned spectra of the keratins peaked at multiple yet coincident areas, and serum albumin was detected at heavier molecular weights apart from peaking at the expected size. The rest of the proteins were confined to gel positions corresponding to their expected sizes.

Filaggrin is a nickel binding protein under denaturing and reducing conditions

To evaluate whether filaggrin was retained on the nickel column through interaction with other proteins, nickel IMAC was performed under different column washing stringency conditions. Increasing salt concentration (0.5–2 M NaCl) did not result in filaggrin release, neither did urea gradient washing (2–8 M urea) (data not shown). Further, the 20 proteins from table 1 were all detected in the fractions collected after EDTA treatment. We next examined the nickel binding under strong denaturing and reducing conditions by washing the column with 2% sodium dodecyl sulphate (SDS) and 0.1 M dithiothreitol (DTT) (fig. 2). Whereas most proteins including a low molecular weight filaggrin species were released upon this treatment (fig. 2, lanes 5 and 13), the monomers and larger filaggrin molecules retained their nickel binding properties (fig. 2, lanes 7 and 15). The band found in all lanes at approximately 70 kDa is human serum albumin.

Filaggrin from SC chelates nickel

Since the topographical origin of filaggrin from full epidermis was unknown, we next extracted the proteins from skin scrapings to evaluate the nickel binding potential of SC derived filaggrin. The samples were boiled in SDS and DTT in order to solubilise the extractable proteins. A high molecular weight (55-60 kD) filaggrin was detected in the EDTA released fraction (fig. 3, lanes 3 and 6). This filaggrin species was not present in the effluent or wash fractions (fig. 3, lanes 1-2 and 4-5). Filaggrin presence was verified by tandem MS, which revealed additional filaggrin at a gel position corresponding to the monomers. This was verified by use of a polyclonal anti-filaggrin antibody, which reacted with the monomers (arrowhead) in addition to the high-weight filaggrin (asterisk) (fig. 3, lane 7).

Filaggrin binding of copper, zinc and cobalt

Filaggrin's capacity to bind other divalent transition metals was investigated in a series of IMAC with metal chelating Sepharose loaded with Zn^{2+}, Cu^{2+} and Co^{2+} ions, respectively (fig. 4). Filaggrin was retained by all the metals. While the Zn and Cu IMAC procedures had filaggrin release patterns similar to that of the Ni IMAC (fig. 4, lane 7, Zn^{2+} and Cu^{2+}), a proportion of filaggrin was released in the denaturing step in the Co IMAC (fig. 4, lanes 5 and 6, Co^{2+}).

Discussion

We demonstrated that filaggrin is a very strong nickel chelator and that the nickel binding resisted strong denaturing conditions. Since filaggrin is an abundant protein of the outermost skin compartments, inherited filaggrin deficiency probably reduce the nickel binding capacity of an intact skin barrier markedly. In agreement with this, null mutations in the filaggrin gene are associated with an increased risk of nickel sensitization and dermatitis compared with non-mutation carriers [35, 37, 74]. Filaggrin exists as a heterogeneous protein population in the epidermis because of its processing from a non-soluble precursor, to soluble monomers and ultimately free amino acid residues [45]. Accordingly, we detected multiple filaggrin species. In full epidermal extracts, filaggrin monomers were the primary nickel chelators; in SC extracts, an additional high-weight filaggrin species was enriched by nickel IMAC. This species may be a product of isodipeptide cross-linking by transglutaminases; a feature conferring the extreme stability of cornified envelopes [17]. Thus, the cornified envelope proteins remain insoluble even after exhaustive boiling in SDS and DTT. Whereas the major portion of filaggrin resides in the keratin-matrix interior of the cell, some filaggrin is incorporated into the cornified envelope (filaggrin is estimated to constitute 7% of the cornified envelope protein content) [76]. Only a small fraction of filaggrin from epidermis is substrate for *in vitro* transglutaminase cross-linking. This filaggrin may represent a subgroup accessible for modification or non-specific processing [77].

Whether the nickel binding, high-weight filaggrin arises from transglutaminase cross-linking remains unclear. Yet the failure of monomer recognition by monoclonal antibodies in the SC extracts, as opposed to readily recognizing monomers from full epidermal extracts, suggests that modifications of this specific population do take place. Whereas filaggrin was the only major epidermal protein to retain nickel binding under strong denaturing conditions, we identified a group of proteins that may also chelate nickel in the epidermis. Salt and urea gradient washes did not negate the binding to the Ni Sepharose, but we cannot rule out completely that some of the identified proteins may be captured on the column through protein-protein interactions. Nonetheless, many of them were previously reported to bind nickel and/or other divalent transition metals. These proteins included β-actin, serum albumin, histone H2A, haemoglobins and heat shock protein 70 kDa [78-83]. Additionally, metal binding has been shown for homologues of glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, histone H4, aldolase A and lactate dehydrogenase [84-89]. To our knowledge, there is no literature showing metal binding by the epidermal keratins, and they may be retained by protein-protein rather than nickel-protein interactions. We show that all other proteins than filaggrin lose their nickel binding property with protein unfolding. This corroborates the notion of filaggrin as a protein without a specific secondary conformation, *i.e.* a natively unfolded protein. The relevance of nickel binding depends on the proteins' topographical location in epidermis. The keratins and filaggrin are important for nickel binding in the SC as they are major constituents there. The rest of the proteins probably originate from lower epidermal layers. Unfortunately, we cannot assess the relative abundance of the nickel binding proteins in each layer. Firstly, the cornified envelope amalgam is insoluble unless reagents that also cleave peptide bonds are employed. Therefore, the protein constituents of this structure are not accessible for analysis. Secondly, since SC protein extraction must be performed under denaturing conditions, proteins that need their secondary or

tertiary structure for nickel binding cannot be evaluated. Thirdly, the study set-up did not allow for assessment of the nickel binding potential of free amino acids. Especially in the outermost SC, where filaggrin is degraded to its amino acid components, potent nickel chelators such as histidine, arginine or their derivatives may contribute. The IMAC series showed that filaggrin binds divalent metals such as zinc, copper and cobalt. In case of cobalt, a proportion of the monomers elute under reducing conditions. This may be due to a lower affinity for this metal ion. Alternatively, the partial elution results from ion leakage. The employed reducing agent, tris-(2-carboxyethyl)phosphine (TCEP), was used at a relatively low dose and is mild compared with DTT, yet cobalt ions are readily reduced. Nevertheless, metal affinity studies could point to whether retention of metal ions in epidermis has a biologically relevant potential in allergic sensitization and/or elicitation in terms of metal ion shuttling between outer and inner skin compartments. Metal sequestering at the skin surface could potentially be beneficial. Considering that 30% of all bacterial enzymes are estimated to require metal co-factors [90], metal depletion would certainly affect many metabolic processes in bacteria. One major cause of human skin infections is *Staphylococcus aureus* [91]. It exists superficially as a commensal bacterium, but given certain conditions it becomes a pathogen. In addition to the mechanical restriction by the SC, colonization is normally resisted by competitive growth of other bacteria, antimicrobial peptides, low temperature and low pH. However, once *S. aureus* breaches the epithelial barrier, a battle for essential metal nutrients such as Fe^{2+}, Mn^{2+} and Zn^{2+} begins [92]. Perhaps the battle starts at the skin surface. Since *S. aureus* is a common infection in atopic dermatitis, one may speculate if inherited lack of filaggrin and consequently reduced metal sequestering in the topmost skin layers could disturb the bacterial balance and favour virulence. In summary, we have shown that filaggrin derived from both SC and full epidermis binds Ni^{2+} and other divalent metal ions such as Cu^{2+}, Co^{2+} and Zn^{2+}. Other epidermal proteins are also

capable of nickel chelation, but filaggrin is a major chelator and the relevance of the other proteins e.g. in SC nickel accumulation remains unclear and is a topic for future experiments. Since filaggrin gene mutations have been associated with an increased risk of allergic nickel dermatitis in European populations, this study provides a possible link between genetics, protein expression and function.

Materials and Methods

Tissue samples

Skin samples from 10 different donors were obtained from plastic surgical waste at Copenhagen University Hospital Herlev. The study was conducted in accordance with the principles of the Declaration of Helsinki, it was approved by the Ethics Committee for Copenhagen (H-2-2010-070) and informed written consent was obtained before the surgeries.

Protein extraction from human epidermis and SC

The extraction procedure was modified from [93]. The surgical waste samples were incubated at 56 °C for 20 min in storage buffer (10 mM potassium phosphate, 2 mM Na_2EDTA, pH 7). The epidermal layer was peeled off using tweezers and stored at –20 °C unless processing continued immediately. The samples were ground with mortar and pestle in the presence of extraction buffer (1 M potassium phosphate, 2 mM Na_2EDTA, 0.1% sodium azide, 1 tablet Complete Mini protease inhibitor (Roche, Basel, Switzerland) per 50 mL, pH 6.9) and quartz sand (Merck, Whitehouse Station, NJ, USA) as described by Palosuo *et al.* Sonication was done for 30 sec at 60% of maximum output (Vibra-Cell 100W, Sonics, Danbury, CT, USA). For delipidation, 2-bromo-2-chloro-1,1,1-trifluoroethane (Sigma-Aldrich, St. Louis, MO, USA) was employed. The protein extracts were dialysed overnight in phosphate-buffered saline in Slide-A-Lyzer 7K

cassettes (Thermo Fischer Scientific, Waltham, MA, USA) and stored at –20 °C. SC was sampled from four donors by scraping an approximate area of 24 cm^2 with a scalpel. Scraping was superficial, cells were tapped off on a weighing paper, and the cornified keratinocyte yield per individual approximated the 100 μL mark in an Eppendorf vial. Hairs were removed with tweezers, 1 mL SDS buffer was added (0.05 M Tris, 10% glycerol, 2% SDS, 0.1 M DTT, pH 7.5) and the samples were boiled at 100 °C for 30 min. The supernatant was collected after brief centrifugation.

Immobilized nickel affinity chromatography

Ni Sepharose slurry (Ni Sepharose 6 Fast Flow, GE Healthcare, Little Chalfont, UK) was equilibrated in binding buffer (100 mM Tris, 0.15 M NaCl, pH 7.5) according to manufacturer's instructions. In case of full epidermal extracts, 1 mL sample was incubated with 3 mL Ni Sepharose slurry; for SC samples, 1 mL sample was incubated with 1 mL slurry. Incubation was for 1 h at room temperature while rotating. The protein slurry was poured onto Poly-Prep Chromatography Columns (Bio-Rad, Hercules, CA, USA), the effluent was collected and the washing procedure was initiated. Washing was done with 2 column volumes. Washing buffer was binding buffer in the non-denaturing binding studies. Salt gradient washing included one wash with binding buffer then four consecutive washes with increasing salt concentration: 0.5, 1, 1.5 and 2 M NaCl in 100 mM Tris at pH 7.5. Similarly, urea gradient washing was done with: 2, 4, 6 and 8 M urea in 25 mM Tris at pH 7.5. In the binding studies with on-column reducing and denaturing, the columns were washed twice with binding buffer then SDS buffer. For all experiments, EDTA buffer (100 mM Tris, 0.5 M NaCl, 50 mM EDTA, pH 7.5) was used for nickel elution. As a binding control, the Ni Sepharose was stripped for ions by washing six times

with EDTA buffer then six times with milli-Q H_2O. The slurry was equilibrated in binding buffer, and the experiment proceeded as for the non-denaturing binding studies.

Protein electrophoresis and western blotting

Fractions from the chromatographic experiments were separated by SDS-PAGE (4–20% Tris-Glycine gels, Novex; Life Technologies, Paisley, UK) with reducing conditions (0.05 M Tris, 10% glycerol, 2% SDS, 0.1 M DTT). Gels were stained by either Coomassie brilliant blue (GelCode Blue Stain Reagent; Thermo Fischer Scientific, Waltham, MA, USA) or silver nitrate [94]. For western blotting, the proteins were transferred to nitrocellulose (iBlot, Invitrogen; Life Technologies, Paisley, UK) according to manufacturer's instructions, and the membranes were blocked with TBS buffer (0.05 M Tris, 0.3 M NaCl, 1% Tween 20, pH 7.5) for 1 h or over-night. Membranes were incubated for 1 h at room temperature with either mouse monoclonal anti-filaggrin antibodies diluted 1:500 in PBS (FLG01; AbCam, Cambridge, UK) or rabbit polyclonal anti-filaggrin antibodies diluted 1:1000 (Prestige Antibodies; Sigma-Aldrich, St. Louis, MO, USA). After washing with TBS buffer, membranes were incubated with alkaline phosphatase conjugated goat anti-mouse or anti-rabbit (whole molecule) IgG antibodies (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:2000 in PBS for 1 h at room temperature. TBS buffer washing preceded immunoreactive protein band visualization with Sigmafast BCIP/NBT (Sigma-Aldrich, St. Louis, MO, USA). Molecular weight markers were Prestained SDS-PAGE Low Range Standard or Precision Plus All Blue Protein Standard (Bio-Rad, Hercules, CA, USA).

In-gel digestion and analysis by tandem mass spectrometry

The relevant lane from the SDS-PAGE was systematically cut into 15 approximately equally sized pieces and digested in-gel by trypsin according to Shevchenko et al. [94]. The peptides were

dried in a vacuum concentrator, reconstituted in 5% formic acid, and desalted in-line using a C18 trap-column (Dionex Acclaim PepMap100, 100 μm x 2 cm, 5 μm, C18, 100 Å). The peptides were separated using a second C18 column (Dionex Acclaim Pepmap100, 75 μm x 15 cm, 3 μm, C18, 100 Å) and analyzed by MS/MS using an Orbitrap XL mass spectrometer (Thermo Fischer Scientific, Waltham, MA, USA) equipped with a nanoelectrospray source (Proxeon, Odense, Denmark). The flow rate was 200 nL/min; the mobile phases consisted of (A) 2% v/v acetonitrile, 0.1% v/v formic acid and (B) 95% v/v acetonitrile, 0.1% v/v formic acid. The gradient was as follows: 0 min: 0% B; 5 min: 5% B; 64 min: 35% B, 68 min 100% B; 86 min: 100% B; 87 min: 0% B; 90 min: 0% B with data acquisition from 15 min to 77 min. MS data was acquired recording full scan spectra (250-1800 m/z) in the Orbitrap with 60000 resolution at 400 m/z. MS/MS data was recorded in parallel in a data-dependent mode fragmenting the five most abundant ions (charge state +2 or higher) by collision-induced dissociation in the LTQ ion trap at 35% collision energy. MS/MS spectra were recorded using dynamic exclusion (40 sec) to minimize repeated fragmentation of the same peptides.

Protein identification by database search

Peak lists were extracted from the raw-files using DTASuperCharge (v2.0a7) and searched using Mascot (ver 2.2.06) against the UniProt database ver 56.0 using the settings: Taxonomy: Human; fixed modifications: Carbamidomethylation of Cys; variable modifications: Oxidation of Met. Mass tolerances were set to 10 ppm on the peptide and 0.6 Da on the fragment masses. Results were processed using Scaffold (ver 3.6.1) requiring at least two peptides for positive identification and a 1% maximum false discovery rate.

Copper, Zinc and Cobalt IMAC

Chelating Sepharose Fast Flow was prepared with 0.2 M of the metal ion in question according to manufacturers' instructions (GE Healthcare, Little Chalfont, UK); the employed metal salts were: CuO_4S, $ZnCl_2$ and $CoCl_2 \bullet 6H_2O$. The metal Sepharose was equilibrated in binding buffer, and the experiments proceeded as for the Nickel IMAC. Due to on-column reduction and precipitation of Co^{2+} and Zn^{2+} by DTT, the reducing agent TCEP (50 μM) was employed.

Conflict of interests

The authors state no conflicts of interest.

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Table 1. Top 20 most predominant proteins retained on a nickel column based on spectral counts (accounting for 49% of assigned spectra)

Protein name	Molecular weight (kDa)	Spectral counts (Number (% of total counts))	Reported binding to divalent transition metals (homologous proteins)	References
Keratin 1	66	866 (9.4)		
β-actin	41	503 (5.5)	Ni^{2+}, Mn^{2+}, Zn^{2+}	[78]
Keratin 10	63	340 (3.7)		
Human serum albumin	67	317 (3.5)	Ni^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+}, Cd^{2+}	[79, 80]
Glyceraldehyde-3-phosphate dehydrogenase	36	240 (2.6)	(Zn^{2+}, Cu^{2+})	[84]
Keratin 9	62	237 (2.6)		
Filaggrin	36	236 (2.6)		
Histone H4	14	222 (2.4)	(Ni^{2+})	[85]
Pyruvate kinase	60	216 (2.4)	(Ni^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+})	[86]
β-chain haemoglobin	16	179 (2.0)	Ni^{2+}, Fe^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}	[81]
Aldolase A	40	153 (1.7)	(Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+})	[87]
Keratin 2	65	138 (1.5)		
Histone H2A	14	127 (1.4)	Ni^{2+}	[82]
NME1-NME2 protein	30	112 (1.2)		
Bleomycin hydrolase	53	109 (1.2)		
Elongation factor 2	95	104 (1.1)		
L-Lactate dehydrogenase	37	100 (1.1)	(Ni^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+})	[88, 89]
Heat shock protein 70 kDa	70	97 (1.1)	Ni^{2+}	[83]
α-chain haemoglobin	15	96 (1.0)	Ni^{2+}, Fe^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}	[81]
High-mobility group box 1	24	94 (1.0)		

Figures

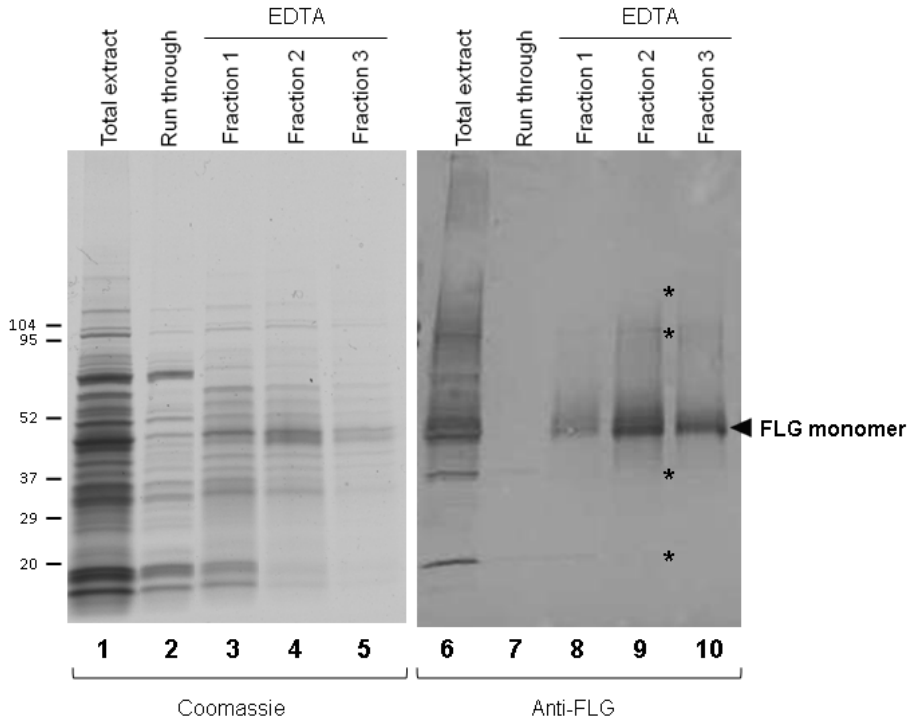

Figure 1. Filaggrin is enriched by immobilized nickel affinity chromatography. Epidermal extracts were incubated with Ni Sepharose and poured onto a column. Fractions were collected from effluent and EDTA treatment. The full protein spectrum was visualized by Coomassie stain after SDS-PAGE. Filaggrin was identified by blotting with monoclonal antibodies (anti-FLG). Asterisks denote filaggrin molecules identified by tandem mass spectrometry in addition to the monomer population (FLG monomer, arrowhead). Size (kDa) is indicated on the left.

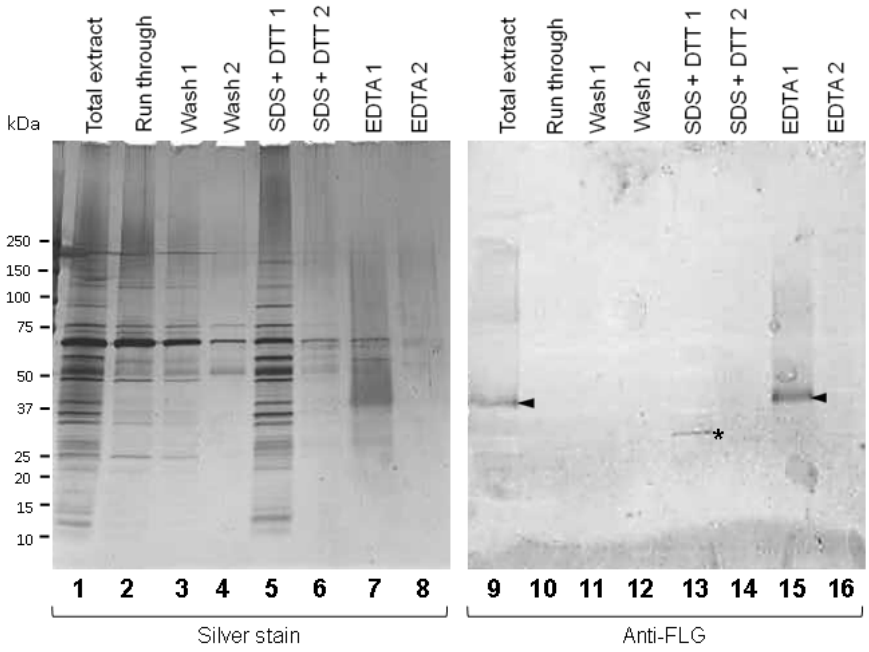

Figure 2. The filaggrin monomers resist elution from the nickel column by reducing and denaturing conditions. Epidermal extracts were incubated with Ni Sepharose and poured onto a column. Fractions were collected from run through, washings, reducing/denaturing steps and EDTA treatment. The full protein spectrum was visualized by silver staining after SDS-PAGE. Filaggrin was identified by blotting with monoclonal antibodies (anti-FLG). Arrow heads: Filaggrin monomer population. Asterisk: low-weight filaggrin species.

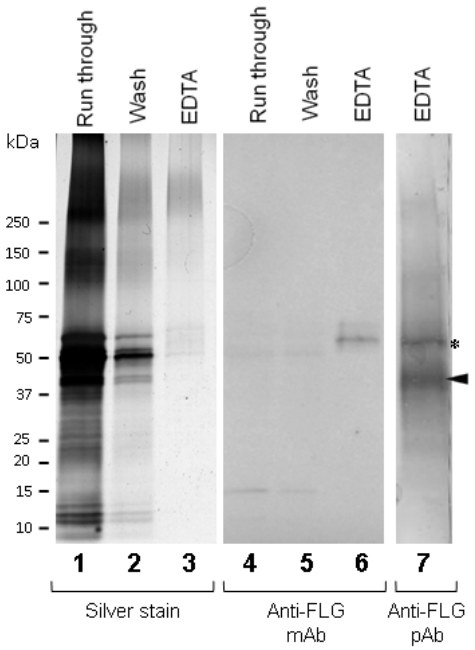

Figure 3. Filaggrin recovered from the *stratum corneum* (SC) binds nickel. The soluble proteins from SC were extracted at denaturing and reducing conditions. The extracts were incubated with Ni Sepharose and poured onto a column. Fractions were collected from run through, washing and EDTA treatment. The full protein spectrum was visualized by silver stain after SDS-PAGE. Filaggrin was identified by blotting with either mono- or polyclonal antibodies (anti-FLG mAb and pAb, respectively). Arrow head: Filaggrin monomer population. Asterisk: high molecular weight filaggrin species.

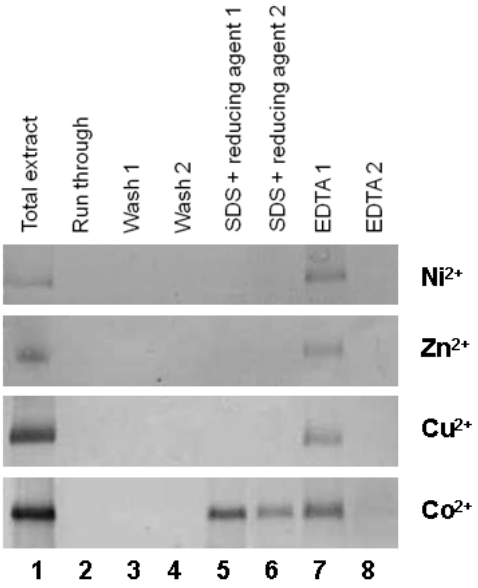

Figure 4. The filaggrin monomer binds other divalent transition metals than nickel. Epidermal extracts were incubated with either Ni, Zn, Cu or Co Sepharose and poured onto a column. Fractions were collected from run through, washings, reducing/denaturing steps (Ni: SDS + DTT; Zn, Cu and Co: SDS + TCEP) and EDTA treatment. Samples were separated by SDS-PAGE and filaggrin was identified by blotting with monoclonal anti-filaggrin antibodies.

COMMENTS AND CONSIDERATIONS ON METHODOLOGY AND VALIDITY

In this section, additional comments and considerations are included on methodology and validity that are either not presented or were only briefly touched upon in the manuscripts.

Epidemiological studies

The epidemiological studies (Manuscript I and II) are based on cross-sectional population data sampled by Thyssen *et al.* between 2006 and 2008 [9, 37]. When analysing population data, the first thing to consider is how well the participants represent the population you wish to describe. The participation rate of 43.8% (3471 participants of 7931 invited individuals) was low, which potentially introduces selection bias. For example, diseased individuals could more likely to participate than healthy individuals. Originally, this study was designed to describe the general health in the Danish adult population. Thus, the questionnaire included questions on present or previous disease status, various symptoms, exercise, dietary habits, alcohol consumption, smoking, general state of mind, housing conditions, social status, job and family disease history. Since skin symptoms and contact allergy were only a smaller part of the main study, the likelihood of overrepresentation with respect to skin disease is small. In fact, the prevalence estimate of contact allergy in this population is relatively low compared with other general population estimates [3]. The low prevalence may owe to the use of day 2 patch test readings only, which ideally should be performed at day 3 or 4 at the earliest [7]. For nickel, neomycin sulphate and formaldehyde, the day 2 reading would tend to underestimate true sensitization rates, as late positive patch test reactions to these particular allergens are common [95-97]. For other allergens such as quinoline mix, day 2 readings may prompt false-positive readings [97].

The definitions of atopic dermatitis, nickel dermatitis and dermatitis from a cosmetic product relied on questionnaire data, which potentially introduce recall 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 [98]. Hypothetically, *FLG* null mutation carriers could have a clearer recollection of previous eczema events than non-mutation carriers due to more severe eczema cases or increased focus on skin symptoms. Nonetheless, since the participant's nickel reactivity differed in Kaplan-Meier event history analyses which was based on self-reported age of onset, and there was no significant difference in the proportion of mutation versus non-mutation carriers remembering their age of onset (97.1% (66/68) vs.

94.4% (689/730), $P_{Fischer's\ exact}$=0.571), such recall bias is probably of minor or no importance. The participants were only genotyped for the two most common *FLG* null mutations, R501X and 2282del4. Although these mutations account for approximately 80% of the null mutations, incomplete mutation coverage probably skews the results to some degree.

In epidemiology, it is often debated which risk estimate is the most appropriate to use: relative risk (RR) or odds ratio (OR). The RR is intuitively easier to comprehend than the OR, because it is a measure of the risk of having a disease in one group in proportion to the risk in another group [99]. In an example from manuscript I, the RR of developing nickel dermatitis in women vs. men would be: RR = 0.369/0.083 = 4.45, meaning that you are 4.45 times more likely to develop nickel dermatitis if you are a woman than if you are a man. The OR is 6.50, because this risk ratio estimate reflects the probability of developing nickel dermatitis in relation to the probability of not developing nickel dermatitis within each sex and then dividing the two. Assuming that the disease or the event you are describing is rare, the OR approximates the RR, but the OR always overestimates the RR [99]. This is also the case for nickel dermatitis, which is not a rare event in the Danish population (24.1% reported nickel dermatitis at some point in their lives) [Manuscript I]. Nevertheless, as long as the OR is interpreted correctly and is not presented as an absolute risk difference, there is no problem in employing this effect measurement.

When multiple statistical tests are performed, which is the case in Manuscript II, obtaining a statistically significant result merely by chance is high. Actually, 1 out of 20 of tests will yield a statistically significant result by chance, if the significance level is set to 5% (p=0.05). Therefore, it is always extremely important to specify the biological rationale for doing the tests before scrutinizing the data; having so-called defined *a prori* hypotheses. Bonferroni correction is a procedure which is often put forward by manuscript reviewers to minimize the risk of rejecting a null hypothesis, which is actually true. That is, reporting that there is a true genetic effect, when there is actually not. The principle is to lower the significance level according to how many tests you perform. Hence, if 20 tests are performed you reduce the significance level to 0.05/20=0.0025, e.g. the result is now statistically significant if p=2.5%. In doing so, you optimize the risk of accepting a null hypothesis, which is actually not true. That is, reporting that there is no genetic effect, when there actually is. Consequently, correction for multiple testing is a trade-off, and as long as you are aware of the pitfalls and report your results on that basis,

reporting what you find can never be misleading. It is paramount to bear in mind that statistics are just tools that estimate the likelihood of whether the observed result has happened by chance or not. Thus, it is equally important to report on statistically significant and insignificant results with humbleness. Ultimately, genetic associations with diseases should without exception be demonstrated in multiple cohorts and preferably by different methods.

Biochemical studies

The literature on filaggrin protein characterization varies as regards the molecular weight of epidermally derived filaggrin [56, 73, 77, 93, 100]. The inconsistency may arise from the evident heterogeneity of filaggrin molecules depending on their topographical origin in epidermis and/or the processing stage. However, the major differences can probably be attributed to differences in laboratory procedures. These include the mode of protein extraction, general sample handling and the specificity of the utilised anti-filaggrin antibodies. In this study (Manuscript III), the employed protein extraction procedure was slightly modified from Palosuo *et al.* (1998), who purified filaggrin from skin samples by reversed-phase high-performance liquid chromatography [93]. The method was chosen because it was a relatively new and seemingly efficient purification method. Whereas the protein extraction procedure was readily replicated, our attempts to purify filaggrin by ultra-performance liquid chromatography (UPLC) were fruitless. Nevertheless, the SDS-Page visualization (coomassie and silver staining) of the epidermal protein extracts from 10 different donors verified that the extraction method was highly reproducible. Additionally, immunoblot detection and tandem mass spectrometry (MS) confirmed the existence of profilaggrin and filaggrin peptide sequences in the SDS gel pieces corresponding to the immune-reactive areas of the immunoblots.

In the technique of immobilized metal-affinity chromatography (IMAC) proteins are separated based on their affinity for metal ions. The high-affinity binding between nickel ions and the amino acid histidine is utilized in both small scale laboratory experiments and large scale industrial settings, and recombinant proteins with a tail of six histidine residues are readily purified on nickel columns [101]. Some proteins can be purified without a histidine tag due to a very high content of histidines [83], whereas others can be purified because of few exposed histidine residues constituting high-affinity nickel binding complexes [102]. In contrast to our attempts to separate epidermal proteins by UPLC, which caused protein aggregation that clogged

the system, the table-based IMAC procedure was very robust and reproducible with protein extracts from various donors both from full epidermis and *stratum corneum* samples. The results presented in Manuscript III strongly imply that filaggrin molecules of varying sizes and the monomers in particular are strong nickel chelators due to their many histidine residues and unfolded protein conformation [53].

DISCUSSION

This section includes additional discussion of the results presented in Manuscript I-III as well as a more general discussion.

Following the coupling of *FLG* null mutations with an increased risk of developing AD [40], the general perception has been that a leaky skin barrier allows increased allergen ingress, which causes skin immune modulation towards a disease mediating cytokine milieu [103-105]. Therefore, it was also reasonable to consider the potential of a leaky skin barrier in the development of contact allergy [35, 36, manuscript I-II]. Past reports on overall associations between AD and contact allergy are opposing [106]. However, it may be that AD should be evaluated in terms of disease subtypes, and that contact allergy should be evaluated in terms of the likely route of sensitization, to determine a true causality relation [106, manuscript I-II]. The newest approach to mapping out heritability factors in complex diseases like AD is a genome wide association study (GWAS) [107]. With this method, the genomes of cases and controls are "scanned" for allele frequencies of single nucleotide polymorphisms (SNPs), meaning a one-base change at a particular location in the DNA. As the sample size is quite large, the statistics highly sophisticated and the data amount massive, a GWAS is typically hypothesis generating and requires follow-up studies [108]. The major gain, on the other hand, is that novel disease mechanisms can be revealed [108]. Multiple potential susceptibility loci have now been identified for AD. Some are related to skin barrier integrity, some to immunological mechanisms [107, 109-112]. We did not confirm the GWAS-derived association between *CLDN1* polymorphisms and AD, but we did find trends indicating that a leaky skin barrier due to tight junction alterations could cause increased risk of contact sensitization [Manuscript II]. Hence, AD candidate gene mapping by GWAS seems to be a feasible starting point in the study of genetic predisposition to contact allergy, at least for the SNPs related to barrier integrity. Nonetheless, it truly remains a complicated task to discriminate between the effects of hereditary disposition versus environmental exposure load.

In proportion to the many association studies on filaggrin null mutations and eczema disease as well as their effect in indirect measures of barrier integrity, only few studies concern the structural changes in epidermis associated with filaggrin deficiency [51, 103, 105]. Such studies are particularly important for appreciating the percutaneous penetration potential of chemically

different allergens. Flaky-tail mice with highly reduced murine filaggrin expression exhibit increased paracellular permeability of the water soluble lanthanum tracer into the *stratum corneum*. In addition, abnormal lamellar body secretion was reported [103]. A similar phenomenon has been observed in human *ichthyosis vulgaris* skin associated with *FLG* null mutations [105]. This study also reports disorganized lamellar bilayer architecture in homozygous mutation carriers and a paracellular barrier defect. Last-mentioned was shown by lanthanum tracer absorption in biopsies derived from both homo- and heterozygous *FLG* null mutation carriers, which was immersed in tracer solution. The tracer extended into the *stratum corneum* by the paracellular route [105]. Likewise, paracellular tracer perfusion halted at the *stratum granulosum-stratum corneum* interface in organotypic skin culture samples that were transfected with scrambled *FLG* siRNA. In contrast, the tracer moved into the *stratum corneum* following siRNA knockdown of filaggrin expression [105]. Hence, *FLG* null mutations may generally allow for easier ingress of water-soluble contact allergens through *stratum corneum*. Yet, filaggrin deficiency has also been reported to cause alterations in the general epidermal homeostasis. Both in *FLG* null mutation carriers with *ichthyosis vulgaris* and filaggrin knockdown skin models, there was an increase in the number of proliferating cells in the basal stem cell layer and consequently a thickening of the epidermal layers including the *stratum corneum* [51, 105]. This could be a compensatory mechanism for the compromised water-barrier, which would likely also counteract increased allergen ingress to some degree.

Interestingly, tight junction protein expression seems to be affected with filaggrin deficiency, which has been demonstrated by immunofluorescent staining of epidermal cross-sections [105]. Thus, the staining of the tight junction proteins occludin and ZO-1, which is clear and distinct at the border between the *strata granulosum* and *corneum* in wild type skin, fades away in a dose-dependent manner in skin hetero- and homozygous for *FLG* null mutations, respectively [105]. Such interdependence of filaggrin and tight junction protein expression has also been established the other way around. In organotypic skin cultures where tight junction function was disrupted, both lipid lamellae and filaggrin processing was affected [113]. This relation may explain why there is no additional effect of *FLG* null mutations on the association between *CLDN1* SNP polymorphisms and the risk of developing contact dermatitis and allergy [Manuscript II].

The lanthanum tracer employed in the aforementioned skin penetration studies comprises colloidal lanthanum particles, which are on average 40 Å in size [114]. The lanthanum tracer was originally used as an indicator of alteration in cell membrane permeability, as colloidal lanthanum salts do not penetrate intact cell membranes [114]. In case of nickel skin penetration, size probably matters. Hence, nickel ions are approximately 30 times smaller than the lanthanum tracer particles, and they likely penetrate transcellularly in addition. Which penetration route is the most predominant possibly relies on the exposure type, e.g. whether it is occluded or not. Thus, filaggrin deficient skin potentially facilitates both para- and transcellular nickel ion penetration through the *stratum corneum*. The free amino acids, the NMFs, of the *stratum corneum* may also impact nickel skin penetration. Free histidines bind nickel ions. Yet part of the histidines is converted to UCA in the outermost skin layers, and whether this modification interferes with nickel binding is unknown. PCA, the most predominant amino acid NMF, is derived from glutamic acid [59]. Glutamic acid, like histidine, often resides in nickel coordination sites of metalloproteins [115], and may also bind nickel in its free form. Other amino acid NMFs exist, which are all potential contributors to nickel binding. Considering that they can make up as much as 10% of the corneocyte dry weight [59], the impact on overall *stratum corneum* nickel binding capacity could be substantial. At any rate, increasing evidence suggests that the skin barrier abnormality caused by *FLG* null mutations predisposes to nickel contact allergy when the sensitization route is percutaneous (topical exposure) as opposed to intracutaneous (by piercing) [35, 37, Manuscript I]. Additionally, the strong nickel binding properties shown for epidermally derived filaggrin molecules indicate that they contribute to the nickel retention in *stratum corneum* [Manuscript III], which has been described previously [22-25].

Nickel is a quite remarkable allergen because it is able to interact with the human skin in many ways. Not only is it bound by *stratum corneum* molecules [22], it can be chelated by several proteins residing in the underlying epidermal layers [Manuscript III, 116], and finally it can activate components of the human immune apparatus by direct interaction [117-119]. The immunological basis for contact allergy is allergen-specific T cells, but T cell activation requires additional proinflammatory signals [120]. In humans, nickel induces an innate immune signal [121], which was elegantly demonstrated by Schmidt *et al.* to arise from direct binding to the toll-like receptor 4 (TLR4) [119]. TLR4 is expressed on various cell types, e.g. dendritic cells, and is usually activated on binding bacterial lipopolysaccharides [122]. Nickel is able to crosslink TLR4 receptors through

two non-conserved histidine residues, which is the initial step for receptor activation [119, 123]. Classically, the T cells are activated by T cell receptor (TCR)-mediated recognition of antigens that are embedded in the major histocompatibility complex (MHC) molecules of the presenting cells. However, the mode of nickel recognition is non-classical and diverse [124], and the two types of TCR nickel recognition that are molecularly characterized are quite different [117, 118]. In one, the TCR ligand was a nickel ion complexed by both the MHC and a B cell-derived peptide presented therein [118]. The other involved superantigen-like bridging of the TCR and MHC molecules through nickel ions, which was independent of peptide presentation [117]. Yet in both cases, nickel recognition depended on the same conserved histidine in the MHC molecule [117, 118]. The concept of free nickel ions in a protein matrix such as the skin is probably limited due to the ions' ability to bind proteins reversibly. The decisive step for whether nickel will elicit an effect is probably affinity. Hence, the nickel ions are available for shuttling between proteins if the binding is week [125]. Considering filaggrin's amino acid composition it potentially chelates nickel avidly. This corresponds well with the found filaggrin-nickel interaction that resists very harsh chemical conditions in IMAC [Manuscript III]. Shuttling of nickel ions from the *stratum corneum* cells seems improbable, but for the nickel ions that do cross this outer epidermal layer human serum albumin has been put forward as a candidate nickel transfer protein [116]. Indeed, nickel ions complexed to human serum albumine activate nickel specific T cells *in vitro* [116]; the nickel binding motive of human serum albumine also includes a histidine residue (Asp-Ala-His-Lys). Apparently, histidine is a versatile player in nickel contact allergy. This amino acid probably both protect us by restricting nickel passage through the *stratum corneum*, while its presence in immune receptors disposes to both innate and adaptive reactions in the skin.

As reviewed in Manuscript III, it was not possible to assess the nickel binding properties of the proteins comprising the cornified envelope in the *stratum corneum*. This is unfortunate since certain protein constituents therein also contain fair amounts of histidine. These include involucrin and hornerin [17, 126]. Nonetheless, the amount of filaggrin is considerably higher than the amount of the aforementioned proteins [17]. Secondly, the histidines of filaggrin are probably more accessible due to the protein's configuration, and the fact that it for the most part is not confined by isodipeptide bonds to other proteins [17, 53, Manuscript III].

The metal chelating property of filaggrin, which was demonstrated in Manuscript III, was not confined to nickel but included other divalent metal ions such as cobalt, zinc and copper. Of these, only cobalt is problematic in terms of contact allergy [10]. Cobalt and nickel allergies are often concomitant, which is thought to occur because they often coexist in the exposure sources [10]. Cobalt and nickel ions share many chemical characteristics, and in addition to filaggrin binding, cobalt, in a similar fashion to nickel, can induce innate proinflammatory signalling through TLR4 [121, 123]. The discussion of cobalt interaction with skin residing molecules will probably mimic the nickel story in the sense that cobalt too is bound by histidines, and often they share protein binding sites. However, they differ according to affinity and reducing propensity [Manuscript III]. The third major metal allergen is chromium [10]. It chemically differs from the divalent metal ions and is not as binding specific in its allergen forms: hexa- and trivalent chromium. Whereas hexavalent chromium is rather inert, trivalent chromium binds proteins avidly and fairly promiscuous [127, 128]. In turn, hexavalent chromium can be metabolized to the protein interacting trivalent form within the skin [127]. No epidemiological data point to an association between *FLG* null mutations and an increased risk of developing chromium allergy. It may be that the binding properties of chromium ions make them less responsive to the absence of filaggrin in the outermost skin layers. Moreover, chromium's mode of immune activation probably differs remarkably from the mode of nickel due to their dissimilar electron configuration. Lastly, chromium allergy has historically mainly originated in occupational exposure, which could be associated with a substantial chromium exposure load [10]. In general, extensive metal ion exposure potentially saturates the binding capacity of the outermost skin layers, causes net ion influx to the viable skin layers and prompts immune activation.

CONCLUSION

This thesis concerns genetic risk factors and their effect on contact allergy development, especially the association between nickel allergy and *FLG* null mutations. It contributes to the research area with the following observations:

- *FLG* null mutations seemingly lower the age of onset of nickel dermatitis. This was suggested by Kaplan-Meier event history analyses of adult Danes, in which the contribution of sensitization by ear piercing was regarded (Manuscript I)

- The patch test reactivity patterns of *FLG* null mutation carriers and non-mutation carriers, respectively, indicate increased nickel sensitivity among the former group (Manuscript I)

- No associations were found between *GST* gene polymorphisms (T1, M1 or P1 variants) and contact allergy in the Danish, adult population (Manuscript II)

- Danish population data suggests that *CLDN1* SNP polymorphisms may affect the propensity to develop contact allergy by the following associations: rs9290927 minor allele and increased risk of nickel allergy in individuals without ear piercings, rs893051 minor allele and increased risk of fragrance allergy, rs17501010 major allele and increased risk of both contact allergy to organic substances and nickel contact dermatitis (Manuscript II)

- *FLG* genotype status did not affect the risk potential of *GST* nor *CLDN1* polymorphisms on contact allergy development in the Danish population (Manuscript II)

- Epidermally derived human filaggrin is able to bind nickel and additional transition metals such as zinc, cobalt and copper. The filaggrin monomer is a major chelator in both full epidermal protein and *stratum corneum* extracts. Other proteins that may be part of the epidermal nickel binding proteome include keratins, β-actin, human serum albumin, glyceraldehyde-3-phosphate dehydrogenase, histones, pyruvate kinase, haemoglobin, aldolase A, NME1-NME2 protein, bleomycin hydrolase, elongation factor 2, lactate

dehydrogenase, heat shock protein 70 kDa and high mobility group box 1 (Manuscript III)

PERSPECTIVES AND FUTURE STUDIES

Just as the causes of contact allergy are versatile, so are the perspectives of how to administer the new knowledge on the conditional gene-environment interactions. Foremost, restricted allergen skin exposure will inevitably diminish both sensitization and elicitation rates. The legislations on nickel and chromium use are good examples of effective prevention strategies that limit contact allergy prevalence at the population level. Yet consumer habits dictate that most chemical substances remain in our environment to some extent. Secondly, as the exposure pattern for a given allergen is highly individual, for example due to repeated exposure to the same allergen from multiple product sources, single product limitations will never yield absolute protection. Further, it is probably at the low to moderate allergen exposure doses that genetic predisposition impacts contact allergy incidence rates. For prevention purposes, the clinical significance of candidate genes should be sought determined by examining the dose-response relationship with the given allergen or allergen type. However, the genetic impact may not be measurable at single gene level but rather as a predisposing unit of multiple 'unfortunate' genetic variants.

The *FLG* null mutations cause *ichthyosis vulgaris* by a single locus inheritance pattern, and their association to nickel contact allergy may be of a similar nature. Although we have provided a possible link between *FLG* null mutation status and increased risk of nickel allergy by showing that epidermally derived filaggrin binds nickel, the dose-dependency between filaggrin amount and *stratum corneum* nickel binding capacity remains unproven. Experimentally, the percutaneous penetration of nickel through *stratum corneum* could be evaluated by *in vivo* topical nickel application on the skin of *FLG* mutation and non-mutation carriers, respectively. Examining then the amount of nickel in successive *stratum corneum* cell layers by adhesive tape strip sampling with concomitant quantification of filaggrin protein and/or filaggrin breakdown products in the same tape strips would provide a more direct linkage analysis. Considering that *FLG* null mutation carriers are probably more sensitive to nickel skin contact, they may not be sufficiently protected by the nickel legislation. Therefore, future epidemiological analyses of the general population, where the cohort effect from previous times massive nickel exposures will be

gone, should be conducted. At future low level nickel exposure rates, the true hereditary effect of *FLG* null mutations on the risk of developing nickel contact allergy might be quantifiable.

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SUMMARIES

SUMMARY IN ENGLISH

The modern way of living entails a substantial load of chemicals in our surroundings. Repeated or prolonged skin contact with such chemical substances can cause contact allergy, which is a frequent condition in the general population. In Denmark, 10–15% of the adult population is contact sensitized. Nickel is the most frequent sensitizer accounting for as much as 5.9%. Even though allergen exposure is a prerequisite for contact allergy, many studies point to hereditary components as contributing risk factors. One approach to examine genetic predisposition is to focus on candidate genes, which have a known biological function and divergence. In this way, polymorphisms in the genes encoding cellular detoxification enzymes glutathione S-transferases (GSTs) were found associated with thiomersal and chromate sensitization. Moreover, null mutations in the filaggrin gene (*FLG*) have proven to cause perturbations in the skin barrier, which associate with increased risk of nickel contact sensitization. *FLG* encodes a major epidermal protein, which affects many homeostatic processes in epidermis. Recently, polymorphisms in the gene *CLDN1* coding for another structural epidermal protein, the tight junction component claudin-1, were also found associated with barrier deficiency.

This thesis aimed to evaluate the effect of the aforementioned potential genetic risk factors in contact allergy development. In particular, we examined the association between *FLG* null mutations and nickel allergy. The studies were based on epidemiological data from the general population in Denmark and biochemical analyses of proteins extracted from skin samples.

In the Danish population, *FLG* null mutations lowered the age of onset of nickel dermatitis as suggested by Kaplan-Meier event history analyses where the effect of sensitization by ear piercing was regarded. Moreover, the reactivity pattern in patch tests indicated increased nickel sensitivity in mutation carriers, since relatively more mutation carriers had stronger reactions (+3 and +2) than did non-mutation carriers of which a larger fraction had moderate reactions (+1).

The *GST* gene polymorphisms (the T1, M1 or P1 variants) did not associate with contact allergy in the adult Danish population. Contrarily, *CLDN1* small nuclear polymorphisms may affect the propensity to develop contact allergy as indicated by the statistically significant associations: rs9290927 minor allele and increased risk of nickel allergy in individuals without piercings,

rs893051 minor allele and increased risk of fragrance allergy, rs17501010 major allele and increased risk of contact allergy to organic substances and nickel contact dermatitis, respectively. Neither the risk potential of the *GST* nor *CLDN1* variants was affected by *FLG* genotype status.

Biochemical analyses on skin proteins revealed that human filaggrin binds nickel, which may provide the link between the epidemiologically shown association between *FLG* null mutations and the increased risk of developing nickel allergy. Hence, lack of filaggrin may cause increased epidermal nickel penetration. Additionally, filaggrin binds other divalent transition metals such as zinc, cobalt and copper. Immobilized nickel affinity chromatography with epidermal extracts suggested that other proteins could be included in the epidermal nickel binding proteome. Nevertheless, since filaggrin is a major protein in the *stratum corneum*, it potentially is a predominant nickel chelator there.

There is no doubt that restricted skin exposure to chemical substances would diminish contact sensitization rates. However, consumer habits dictate that a certain level of chemical exposure load will remain in our environment. The effect of genetic predisposition in contact allergy should be sought quantified for prevention purposes, which may in the future involve analyses of various genes altogether comprising genetic predisposing units.

SUMMARY IN DANISH

Vores moderne livsstil medfører en massiv mængde af kemikalier i vores omgivelser. Gentagen eller længerevarende hudkontakt med sådanne kemiske substanser kan forårsage kontaktallergi, hvilket forekommer hyppigt i den almene befolkning. I Danmark har 10 – 15 % af den voksne befolkning kontaktallergi. Nikkel er den hyppigste kontaktallergi, som tegner sig for hele 5.9 %. Selvom allergen eksponering er en forudsætning for udvikling af kontaktallergi, peger resultaterne fra flere studier på, at der også findes arvelige risikofaktorer. Evalueringen af eventuel genetisk disponering kan tilgås ved at undersøge sammenhængen til specifikke genvarianter, der har kendt biologisk funktion. På den måde har man fundet en sammenhæng mellem henholdsvis thiomersal- og kromallergi og variation i generne, der koder for glutathion S-transferaser (GST'er). GST'er er enzymer, der fungerer i cellulær detoksificering. Ydermere har mutationer i genet, der koder for filaggrin (*FLG*), vist sig at medføre uregelmæssigheder i hudbarrieren, som er fundet associeret til en forhøjet risiko for at udvikle nikkelallergi. *FLG* koder for et epidermalt protein, som har indflydelse på flere parametre i epidermal homeostase. For nyligt blev genetisk variation i *CLDN1* også fundet associeret til barrieredefekt. Dette gen koder for et andet strukturelt protein i epidermis, claudin-1, der indgår i de såkaldte 'tight junctions'.

Formålet med denne afhandling var at evaluere effekten af de ovenstående potentielle genetiske risikofaktorer på udviklingen af kontaktallergi. Vi havde særligt fokus på den påviste association mellem *FLG*-mutationer og nikkelallergi. Studierne tog udgangspunkt i epidemiologiske data fra den almene befolkning i Danmark samt i analyser af proteiner, der blev udvundet fra hudprøver.

Kaplan-Meier overlevelsesanalyser af sammenhængen mellem nikkeleksem og *FLG* genotypestatus indikerede at bærere af *FLG*-mutationer har en lavere debutalder end vildtyper. Effekten af sensibiliseringen, der opstår når folk får huller i ørerne modsat eksponering på en intakt barriere, blev taget i betragtning i analyserne. Derudover antyder reaktionsmønstrene fra lappetestning at bærere af *FLG*-mutationer modsat vildtyper er mere reaktive ved nikkelkontakt. Der var nemlig relativt flere stærke reaktioner (+3 og +2) blandt mutations-bærerne, hvorimod vildtyperne havde en større andel af svagere reaktioner (+1).

Vi fandt ingen sammenhæng mellem variationerne i *GST* generne (type T1, M1 og P1) og kontaktallergi i den danske voksenbefolkning. Derimod tyder analyserne af *CLDN1* genvarianter på, at der kan være forøget tilbøjelighed til at udvikle kontaktallergi for visse genotyper. Følgende sammenhænge blev fundet statistisk signifikante: Den mindst hyppige allel af rs9290927 og forøget risiko for nikkelallergi i individer uden huller i ørerne, den mindst hyppige allel af rs893051 og forøget risiko for parfumeallergi, den mest hyppige allel af rs17501010 og henholdsvis forøget risiko for kontaktallergi overfor organiske forbindelser og nikkeleksem. *FLG*-mutationerne havde ikke indflydelse på risikopotentialet for de genetiske varianter af hverken *GST* eller *CLDN1*.

De biokemiske analyser af hudproteiner afslørede, at humant filaggrin kan kelere nikkel. Det kunne være årsagen til den forøgede risiko for nikkelallergi, der er fundet for bærere af *FLG* mutationer i befolkningsanalyser. Mangel på filaggrin kunne altså potentielt medføre øget nikkelgennemtrængelighed af de yderste hudlag. Det blev også vist, at filaggrin kunne binde andre transitionsmetaller såsom zink, kobolt og kobber. Immobiliseret nikkel affinitetskromatografi med epidermale ekstrakter antydede, at der kan være andre proteiner, der har betydning for den overordnede nikkelbindingskapacitet i epidermis. Siden filaggrin er et fremherskende protein i *stratum corneum*, er det dog sandsynligt, at det har stor betydning for nikkelkeleringen der.

Der er ingen tvivl om, at hvis mængden af kemikaler, der kommer på huden, begrænses, vil prævalensen af kontaktallergi følgeligt falde. Vores forbrugsvaner dikterer dog, at der altid vil være et vist niveau af kemikalieeksponering på huden. I fremtiden bør man forsøge at kvantificere effekten af genetiske risikofaktorer i forebyggelsesøjemed. Det kommer muligvis til at involvere analyser af flere gener på en gang, såkaldte genetiske risikoenheder, der tilsammen har stor effekt på risikoen for at udvikle kontaktallergi.