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1 Introduction, aim and outline of this thesis
1. Function and structure of the skin

The function of the skin is to act as an effective barrier against unwanted environmental influences as well as to prevent excessive water loss from the body. The skin consists of the epidermis and dermis as well as the subcutaneous fat tissue (Figure 1).

![Figure 1](http://www.dermnetnz.org)

Figure 1. Schematic overview of human skin and its different layers. Adapted from: http://www.dermnetnz.org.

1.1 The epidermis

The epidermis is the outermost layer of the skin and can be divided into four distinctive layers, characterized by different stages of differentiation. These include the stratum corneum (SC), stratum granulosum (SG), stratum spinosum (SS), and stratum basale (SB). The SG, SS and SB are part of the viable epidermis (thickness 50-100 μm), while the SC (thickness 10-20 μm) is part of the non-viable epidermis.

The SB is the innermost layer of the viable epidermis and consists of the proliferating cells of the skin. All the keratinocytes (the most prevalent cell type in the epidermis) are derived from these cells. The keratinocytes transiently migrate and differentiate from the SB towards the SC, after which they are finally released from the skin surface, a process called desquamation. During the differentiation, which starts in the SS, the keratinocytes flatten out and begin to assume the dimensions which are characteristic for the dead cells of the SC, the corneocytes. As the cells flatten they become filled with keratin. In the SG enzymes are activated that start to degrade the viable cell components such as the nuclei and organelles. The SG also contains many membrane-coating granules called lamellar bodies (LBs), in which glucosylceramides, sphingomyelin and phospholipids are stored. These are precursors of the SC ceramides (CERs) and free fatty acids (FFAs).

The lipid and hydrolytic enzyme content of the LB is released via exocytose at the SG/SC interface. The SC consists of corneocytes which are embedded in a lipid matrix and acts as the main barrier for diffusion of substances through the skin.
1.2 Structure of the stratum corneum

SC contains 10 to 25 corneocyte layers which are embedded in lipid bilayers and are oriented parallel to the skin surface. The corneocytes are filled with water and microfibrillar keratin. The corneocytes are surrounded by a cornified cell envelope which consists of a densely crosslinked layer of proteins. A monolayer of non-polar lipids is esterified to the cornified envelope. This monolayer forms the matrix for the intercellular lipid bilayers. This envelope minimizes the uptake of most substances into the corneocytes. The structure of the SC is often referred to as a “bricks in mortar” structure, in which the corneocytes are the bricks and the lipids are the mortar.

There are two possible penetration routes for a compound diffusing into intact human skin: the transappendageal route and the transepidermal route. The first route involves transport via the sweat glands, sebaceous glands and hair follicles. This route circumvents penetration through the SC and is considered to be a route less contributing to the transport of the compounds compared to the transepidermal route. This is based on its relatively small surface area of 0.1% of the total skin area. The transepidermal route can occur either via the transcellular (intracellular) or intercellular pathway. The latter has been suggested to be the most important route for drugs since the above described cornified envelope is highly impermeable for compounds. Therefore, the intercellular pathway has been suggested to be the preferred route for most compounds. Several studies report that the transport of compounds is mainly directed along the intercellular SC lipids. For that reason the lipids play an important role in the skin barrier function, and alterations in lipid composition and/or organization may play a role in a decreased skin barrier function.

1.3 Stratum corneum lipid composition

The intercellular lipid matrix mainly consists of CERs, cholesterol (CHOL) and FFAs in an approximately equimolar ratio. CERs consist of a sphingoid base to which a fatty acid is linked. To date, 12 CER subclasses have been identified in human SC. They differ from each other by their head-group architecture and acyl chain length. The sphingoid moiety can be dihydrosphingosine (dS), sphingosine (S), phytosphingosine (P) or 6-hydroxysphingosine (H). The fatty acid moiety is non-hydroxylated (N) or α-hydroxylated (A) with chain lengths of predominantly 24 to 26 carbon atoms. Furthermore, the acyl-CERs possess a unique molecular architecture: a very long ω-hydroxy fatty acid (EO) with a chain length of 30-34 hydrocarbon atoms to which linoleic acid (C18:2) is esterified. Figure 2 shows the molecular structure of the 12 CER subclasses present in human SC.
Figure 2. Structure and nomenclature of CERs present in human SC. All CERs consist of a polar head group and two long carbon chains. The polar head group varies in molecular architecture (at the carbon positions marked in red), resulting in 12 different subclasses in human SC. Both chains in every CER subclass show varying carbon chain lengths (marked by red arrows). Each CER subclass is denoted by its sphingoid base (blue) and fatty acid chain (gray) resulting in 12 CER subclasses. The abbreviations are as follows: For the sphingoid base: dihydrosphingosine (dS), sphingosine (S), phytosphingosine (P), 6-hydroxysphingosine (H). The various acyl chains are denoted by: non-hydroxy fatty acid (N), α-hydroxy fatty acid (A) and esterified ω-hydroxy fatty acid (EO). This results in the 12 CER subclasses notations: [NdS], [AdS], [EOdS], [NS], [AS], [EOS], [NP], [AP], [EOP], [NH], [AH], [EOH].

The CER synthesis takes place in 4 steps. The variety in CER subclasses is generated in the final 2 steps. These last 2 steps start with the lipid dihydrosphingosine, which is acylated by one of the 6 CER synthases (CERS1-6). Each CERS has a specificity for the fatty acid chain length and degree of saturation that it attaches to the dihydrosphingosine. In the last step the dihydrosphingosine based CER is converted to either sphingosine, phytosphingosine or 6-hydroxysphingosine. The de novo synthesis occurs at the cytosolic leaflet of the endoplasmatic reticulum. After the final synthesis step the CERs are transferred to the Golgi apparatus. There they are transferred into either glucosylceramides by linkage of glucose to the primary hydroxyl group of the CERs or to sphingomyelin by linkage of a phosphorylcholine group to this hydroxyl group. During movement of the cells into the direction of the skin surface the glucosylceramides and sphingomyelin are stored into the cells mainly in lamellar bodies. At the viable epidermis-SC interface sphingomyelin and glucosylceramides are converted back to CERs. The enzyme acid sphingomyelinase catalyzes the
hydrolysis of the phosphorylcholine from sphingomyelin. Cleavage of the glucose group from glucosylceramides is catalyzed by the enzyme $\beta$-glucocerebrosidase. Glucosylceramides are precursors of all 12 CER subclasses in human SC while sphingomyelin is the precursor of CER [AS] and CER [NS]$^{18-21}$.

FFAs mainly consist of saturated carbon-chains with lengths varying between 14 and 32 carbon atoms. The most abundant chain lengths are 24 and 26 carbon atoms$^{22,23}$. FFAs are generated by the catabolism of phospholipids that are extruded from the lamellar bodies by phospholipases$^{24}$. After synthesis of FFAs with a chain length of 16 carbon atoms by fatty acid synthase, elongation is performed by 7 elongases (ELOVLs1-7)$^{25-27}$. Each ELOVL has its own fatty acid chain length specificity. Stearyl-CoA desaturase catalyzes saturated FFAs to mono-unsaturated FFAs or poly-unsaturated FFAs$^{28}$.

1.4 Stratum corneum lipid organization
Human SC consists of a unique lamellar arrangement of the intercellular lipids (Figure 3). Small angle X-ray diffraction (SAXD) studies on human SC revealed the presence of a 13 nm lamellar phase, referred to as the long periodicity phase (LPP). Besides a 13 nm lamellar phase, a second lamellar phase has also been identified. The periodicity of this phase is approximately 6 nm and is therefore indicated as the short periodicity phase (SPP)$^{29-31}$. In contrast to the SPP, the LPP is only identified in the SC and not in other biological membranes, which suggests that the LPP phase plays an important role in the barrier function of the skin$^{32}$. Because of their unusual long esterified fatty acid chain, the acyl-CERs are able to span a layer and extend into another adjacent layer. These long-chain CERs are therefore thought to link the different lipid layers in the LPP. The long $\omega$-hydroxy chain also induces strong Van der Waals interactions. Both aspects contribute to the stability of the 13 nm lamellar phase. CER [EOS] even has shown to be crucial for the formation of the LPP$^{33}$.

The packing of the lipids within the lamellae is referred to as the lateral lipid organization (Figure 3). With increasing packing density this is either liquid, hexagonal or orthorhombic. In healthy human SC, the lipids form mainly an orthorhombic lipid organization at skin temperature, although also domains with a hexagonal or liquid organization exist$^{22,34}$.

2. SC hydration
The hydration level of the SC is regulated by the natural moisturizing factor (NMF)$^{35}$. NMF is composed of filaggrin-derived amino acids (e.g. pyrrolidone carboxylic acid and urocanic acid), specific sugars and salts. The amino acid derived components of NMF are produced by hydrolysis of the SC protein
Figure 3. Overview of lamellar and lateral organization in human stratum corneum. (1) The outermost layer of the epidermis, the stratum corneum (SC), consists of dead cells (corneocytes) embedded in a lipid matrix, also referred to as the bricks (corneocytes) and mortar (lipids) structure (2). The intercellular lipids are arranged in layers (lamellae) (3), with two coexisting lamellar phases (4). These lamellar phases have a repeat distance of 6 nm (referred to as the short periodicity phase (SPP)) or 13 nm (referred to as the long periodicity phase (LPP)). The lateral organization (5) is the plane perpendicular to the direction of the lamellar organization. There are three possible arrangements of the lipids: a very dense, ordered orthorhombic organization, a less dense, ordered hexagonal organization, or a disordered liquid organization.

Filaggrin36-38. This degradation process of filaggrin results in production of most of the NMF and depends on the SC water level, which in turn is partially dependent on the presence of NMF39, leading to a vicious cycle. The presence of NMF in the SC is not only important for hydration, but also maintenance of pH40. Changes in pH can have an effect on hydrolytic enzymes and effect enzymes that are involved in the lipid synthesis, as further described below41.
3. Atopic eczema

Atopic eczema (AE, also called atopic dermatitis) is a chronic relapsing inflammatory skin disease that is characterized by a broad spectrum of clinical manifestations such as dryness, erythema and pruritus. The diagnosis of AE is based on a constellation of clinical findings, there is no pathognomonic biomarker for diagnosis.

AE almost always starts in infancy and tends to resolve or improve remarkably by the age of 5 and it persists into adult life in approximately 15% of the cases. AE can have a considerable effect on the quality of life; it can interfere with sleep due to itching and can therefore affect mental development and physical growth. As the disease is visible to the environment (Figure 4), patients can also have problems in their social life.

![Figure 4. Patient with atopic eczema. Lesional (red) as well as non-lesional (normal appearing) skin can be observed.](image)

The past 20 years the prevalence of AE is increasing and is now almost affecting 20% of children in developed countries, while in adults this is 5-10%. A high percentage of AE patients develop other atopic diseases such as asthma and peanut allergy, referred to as the atopic march.

AE is triggered by a multifactorial setting including environmental factors (e.g. stress and infections) as well as genetic factors. AE patients have several barrier abnormalities, including an increased transepidermal water loss (TEWL), increased surface pH and reduced SC hydration. Even the non-lesional ('clinically unaffected') skin is not normal: it is frequently dry and has a high irritant skin response. Furthermore, an increased TEWL was observed in these skin regions indicating a decreased skin barrier function.

Two different subtypes of AE exist, the intrinsic and extrinsic form of AE. In the extrinsic subtype (~80% of all patients) allergic sensitization to an external antigen with subsequent allergen specific IgE production occurs. The other subgroup of patients has low serum IgE levels. These patients have the intrinsic variant of AE.
The etiology of AE is complex, it is a multifactorial disease, an interplay between genetics, environmental factors and immunological dysfunction.

There are two hypotheses proposed concerning the mechanism of AE:

i) The so called ‘inside-to-outside’ hypothesis in which AE is considered to be an immunological disorder. This view holds that the primary defect resides in a Th2-driven immunologic disturbance with epithelial barrier dysfunction as a consequence of this inflammation. The keratinocytes play a crucial role in the inflammatory response as they are immunologically active cells producing cytokines and chemokines. Features of a Th2-driven immune response are local production of cytokines like IL-4, IL-5, IL-13, IL-17 and IL-22 (all Th2 cytokines), production of allergen-specific IgE, and activation of eosinophils and mast cells. The secreted cytokines induce the production of thymic stromal lymphopoietin (TSLP), which can activate dendritic cells and stimulate the production of high levels of Th2 cytokines by human mast cells. In chronic lesions there is a shift from solely a Th2 cell cytokine milieu to an environment of both Th2 and Th1 cell type cytokines like IFN-\(\gamma\).

ii) The other hypothesis is the ‘outside-to-inside’ view in which the barrier abnormality is not a secondary phenomenon, but the ‘driver’ of the disease activity in AE. The immunological aspects are secondary to the epithelial barrier dysfunction. In 2006 an important finding was reported, namely that AE is associated with filaggrin mutations. As filaggrin is a barrier protein heavily involved in SC formation this is a major breakthrough in the validation of the outside-inside hypothesis as will be explained in the next paragraph. The extent of the permeability barrier abnormality correlates with disease severity and clinically non-lesional skin sites display impaired barrier function. A defect skin barrier may therefore facilitate the transport of allergens and irritants into the skin resulting in activation of both innate and adaptive immune system and inflammation. This in turn may exacerbate the barrier defects which leads to a vicious circle.

Nowadays, accumulating evidence shows that the mechanisms in both hypotheses interplay and/or may reflect the heterogeneity of AE.

### 3.1 Filaggrin loss-of-function mutations and AE

In 2006, Palmer et al. reported that two common polymorphisms (R501X and 2282del4) in the filaggrin gene (FLG) are strong predisposing factors for AE. Since then, more than 45 FLG mutations have been identified, including many European-specific and Asian-specific mutations. FLG mutations have been
associated with asthma, contact dermatitis, allergies (e.g. peanut, nickel) and are causal for ichthyosis vulgaris, a disorder of keratinization that is characterized by dry and scaly skin, decreased skin barrier function and often also by features of AE\textsuperscript{74-79}. Between 8% and 48% of AE patients are carriers of FLG mutations\textsuperscript{80}.

FLG is located in the epidermal differentiation complex, a cluster of approximately 60 genes involved in epithelial differentiation, on chromosome 1q21. This group of genes is involved in the terminal differentiation of keratinocytes\textsuperscript{81}. FLG encodes profilaggrin, a large insoluble polypeptide that is the major constituent of keratohyalin granules\textsuperscript{35,82,83}. Profilaggrin is cleaved into 10-12 copies of the filaggrin peptide\textsuperscript{84}. Filaggrin is a component of the cornified envelope in SC. It associates with keratin filaments and contributes to their alignment, facilitating compaction of cells into squames\textsuperscript{82}. Filaggrin is metabolized into a pool of free amino acids including histidine and glutamine which are further converted to urocanic acid (UCA) and 2-pyrrolidone-5-carboxylic acid (PCA), respectively\textsuperscript{38}. Together with specific salts and sugars these free amino acids are referred to as NMF, which is important for SC hydration\textsuperscript{35}. NMF acts as a very efficient humectant, by absorbing atmospheric water and dissolving it in the SC thereby allowing this layer to remain hydrated\textsuperscript{85}. Expression of FLG and subsequent hydrolysis of filaggrin peptides into NMF are influenced by the properties of the microenvironment, including local pH, external humidity and transepidermal water loss\textsuperscript{86}.

Several studies have shown the correlation between FLG mutation status and NMF levels: AE patients that were carriers of FLG mutations had significantly lower NMF levels compared to non-carriers\textsuperscript{87,88}. In addition, within the subgroup of FLG mutation carriers it is possible to distinguish between AE patients with homozygous (i.e. double null alleles) and heterozygous (1 null allele) FLG mutations by studying the NMF levels\textsuperscript{88}.

Howell et al. found that inflammation has an effect on filaggrin: Th2 cytokines down-regulate filaggrin expression\textsuperscript{89}. They report that lesional skin of AE patients exhibit lower levels of filaggrin expression compared to non-lesional skin in the same patient.

Various studies report on the skin barrier function as assessed by TEWL and the presence of FLG mutations, however the role of FLG mutations in the barrier dysfunction as measured with TEWL is yet inconclusive; several studies report that TEWL did not associate with FLG genotype subgroups\textsuperscript{88,90-92}. However, Gruber et al. report filaggrin dose dependent alterations in skin barrier function in ichthyosis vulgaris patients: TEWL did not differ significantly between controls without FLG mutations and carriers of heterozygous mutations, but was significantly increased in carriers of homozygous FLG mutations compared to controls\textsuperscript{93}. These findings imply that the role of filaggrin in the impaired skin barrier function remains indistinct and that also other factors, like the
SC lipids, may play a role in the barrier dysfunction of AE patients.

3.2 SC lipids in AE

3.2.1 Lipid composition

The total amount of SC lipids was found to be decreased in non-lesional as well as lesional skin. There is contradicting evidence about the lipid ratios in the literature: Di Nardo et al. observed that the CER/CHOL ratio is reduced in non-lesional AE skin. However, other studies do not report a decrease in CER content in non-lesional AE skin.

The first study on the CER composition in skin of AE was performed by Imokawa et al. They observed that CER [EOS] was reduced in SC of both non-lesional and lesional AE compared to control SC. A significant decrease in CER [NP] and CER [EOS] levels in SC of AE patients in the presence and absence of lesional skin lesions have also been reported. In addition, an increased level of free CHOL was detected in this study. The level of CER [NP] significantly correlated with skin barrier function. Other studies also report reductions in CER [EOS], CER [EOP], CER [EOH], CER [NH] or CER [NP] in non-lesional and lesional AE SC. In addition, it was found that CER [AS] is increased in lesional AE SC. These changes in lipid composition all strongly correlated with skin barrier function assessed with TEWL but did not show a clear relationship with the presence of FLG mutations.

Until now, most papers report on the CER subclass composition, not on the chain length distribution within the CER subclasses. Only one study focused on the chain length distribution. An increased level of CERs with very short chains (with a total chain length of 34 carbon atoms, referred to as C34) in the CER [NS] subclass in lesional AE skin was found. The increased level in CER [NS] C34 correlated significantly with the skin barrier function as measured by TEWL: a higher level of short chain CER [NS] C34 correlated with a higher TEWL. The same study reports that the presence of long chain acyl-CERs correlates negatively with TEWL. These findings indicate that not only CER subclasses, but also the lipid chain length may play an important role in the skin barrier function in AE patients.

Compared to the composition of CERs, hardly no information about the FFA composition in AE skin is available. It was reported that the level of very long chain fatty acids (more than 24 carbon atoms) was reduced in non-lesional as well as lesional skin of AE patients.

3.2.2 Effect of inflammation on lipid composition in AE

It has been previously reported that production of cytokines can affect the lipid synthesis and therefore the skin barrier in AE. TNF-α and several cytokines downregulate filaggrin expression. Downregulation of filaggrin expression may result in lower NMF levels and therefore the local pH may be affected. An
increase in pH can lead to altered catalytic activities of lipid processing enzymes sphingomyelinase and acid β-Glucocerebrosidase that require an acidic pH for their optimal catalytic activity\textsuperscript{106}. Furthermore, the enzyme sphingomyelin deacylase has an abnormal expression in skin of AE patients\textsuperscript{107}. This enzyme hydrolyzes sphingomyelin into sphingosylphosphorylcholine and FFA precluding the formation of CERs, therefore lowering CER levels in the SC of AE patients. Finally, peroxisome proliferator-activated receptors (PPARs), which are closely related to the skin lipid metabolic pathway show a relation to AE\textsuperscript{108}: In AE the expression of PPAR-\(\alpha\) and PPAR-\(\gamma\) are decreased whereas expression of PPAR-\(\beta/\delta\) is upregulated\textsuperscript{109,110}. These nuclear receptors promote SC barrier formation by stimulation of keratinocyte differentiation, lipid synthesis, lamellar body formation and secretion, and increased activity of enzymes required for the extracellular processing of lipids. Furthermore, PPARs are suggested to have anti-inflammatory properties in skin\textsuperscript{111-114}.

### 3.2.3 Lipid organization

As a result of an altered SC lipid composition, the lipid organization in SC of AE patients may be altered. To date, only little is known about the lipid organization in SC of AE patients. Pilgram et al. used ED to study the lateral lipid organization in SC of 3 controls and non-lesional SC of 3 AE patients\textsuperscript{115}. They report an increased percentage of hexagonal organized lipids in non-lesional AE SC compared to SC of control subjects. Furthermore, they did not compare the lipid organization between carriers and non-carriers of \(FLG\) mutations.

Electron microscopic analysis of biopsies of AE patients using ruthenium tetroxide (RuO\(_4\)) postfixation revealed a delayed and probably incomplete extrusion of lamellar bodies, suggesting that retention of lipids at their synthesis site occurs which makes them unavailable to the intercellular lipid matrix of the SC\textsuperscript{116}. Abnormalities in lamellar architecture have been found in \(FLG\) deficient SC of ichthyosis vulgaris patients\textsuperscript{93}. As this finding was \(FLG\) dependent, this may indicate similar alterations in AE SC.

Until now, no studies have been carried out in which both the lipid composition as well as the lipid organization have been determined in AE patients and controls \textit{in vivo}. From \textit{in vitro} studies it is known that an altered lipid composition results in an altered lipid organization. Long-chain acyl-CERs are important for the formation of the LPP in human SC\textsuperscript{117}. In addition, the presence of long-chain FFAs is crucial for the formation of an orthorhombic lateral lipid organization\textsuperscript{118}. \textit{In vitro} diffusion studies with lipid mixtures varying in lipid composition reveal the importance a proper lipid organization for the barrier function\textsuperscript{118-120}. Therefore, it is expected that changes in lipid composition in SC of AE patients lead to an altered lipid organization, resulting in a decreased barrier function of the skin.
4. This Thesis

As described above, the skin barrier function of AE patients is impaired in non-lesional as well as in lesional skin. Although AE is associated with $FLG$ mutations, the precise causes of AE-associated barrier dysfunction are not fully understood.

4.1 Objectives of this thesis

The main objective of the research described in this thesis is to address the role of the SC lipids in the impaired skin barrier function of AE patients. In order to achieve this we investigated (illustrated in Figure 5):

i) The SC lipid composition in non-lesional and lesional skin of AE patients and in SC of control subjects. The levels of the lipid subclasses as well as the chain length distribution were examined.

ii) The lamellar organization and lateral organization in SC of AE patients and of control subjects.

iii) The relation between lipid composition, lipid organization, skin barrier function and disease severity.

iv) The lipid/protein ratio in lesional and non-lesional skin of AE patients and control subjects and how this is related to the skin barrier function.

v) The effect of $FLG$ mutations as well as NMF levels on the SC lipid composition, organization and lipid/protein ratio.

Role of stratum corneum lipids in atopic eczema

![Diagram](Figure 5. Schematic overview of the parameters that are studied throughout this thesis.)
4.2 Outline of this thesis

In chapter 2 of this thesis the lamellar lipid organization and CER subclass composition in SC of non-lesional skin are reported in a small group of AE patients and control subjects. The relation between CER subclass composition and lipid organization is examined.

In chapter 3 a detailed analysis of lipid organization (lamellar as well as lateral) and a comprehensive analysis of CER composition in non-lesional SC of a large group of AE patients are described. The relation between lipid composition, organization and skin barrier function are assessed. In addition, we examine whether the lipid properties correlate with NMF, disease severity (Scoring Atopic Dermatitis, SCORAD) and presence of FLG mutations.

In chapter 4 the use of electron diffraction to study the lateral lipid organization in non-lesional SC of AE patients and control subjects is reported. The occurrence of either a hexagonal or an orthorhombic lateral organization is examined and it is studied whether a correlation exists between the lateral organization and skin barrier function or presence of FLG mutations.

In chapter 5 the lipid organization and total lipid composition (including FFAs) is provided in non-lesional and in lesional SC of patients with AE. In particular, the influence of the lipid chain length on the skin barrier function and lipid organization is described. Correlations between the lipid parameters and barrier function, disease severity and presence of FLG mutations are investigated.

In chapter 6 studies are described using confocal Raman spectroscopy to determine the lipid/protein ratio in control subjects, non-lesional and lesional SC of patients with AE. In addition, the relation between lipid/protein ratio and skin barrier function is examined.

In chapter 7 the lipid organization as well as the lipid composition in another inflammatory skin disease, Netherton syndrome, are described. The findings are compared to those obtained for AE.

In chapter 8 the results of this thesis are summarized and suggestions for future research are provided.
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