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Summary and perspectives
Summary

The primary function of the skin is to form an effective barrier of the human body against allergens, irritants and microorganisms and to prevent excessive water loss from the body. The skin barrier function strongly relies on the outermost layer of the skin, the stratum corneum (SC), which consists of dead corneocytes embedded in a highly organized extracellular lipid matrix. The main penetration pathway of most substances through the SC is suggested to take place via the intercellular lipid matrix. Therefore, the lipids are thought to play a crucial role in the skin barrier function. This lipid matrix consists mainly of ceramides (CERs), cholesterol (CHOL) and free fatty acids (FFAs) in an approximately equimolar ratio. CERs consist of a sphingoid base linked to a fatty acid. 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 acyl-CERs have a unique structure as they contain a very long acyl chain linked to linoleic acid. FFAs mainly consist of saturated carbon-chains with lengths varying between 14 and 32 carbon atoms, of which the most abundant chain lengths are 24 and 26 carbon atoms.

SC lipids are arranged in layers, referred to as lamellae. These lamellae are stacked on top of each other and are oriented approximately parallel to the skin surface. In human SC, two lamellar phases are formed with repeat distances of 6 and 13 nm, referred to as the short periodicity phase (SPP) and long periodicity phase (LPP), respectively. Within these layers the lipids form a very dense, highly ordered packing – the so-called orthorhombic organization – but a subpopulation of lipids forms also a less dense (hexagonal) and even a liquid organization. In healthy human SC the lipids are mainly arranged in a dense orthorhombic packing.

In diseased skin, like atopic eczema (AE), the SC lipid composition and organization may be altered. AE is a chronic relapsing inflammatory skin disease that is characterized by dryness, erythema and pruritus. It affects almost 20% of Caucasian children and 2-10% of adults, and its prevalence is increasing rapidly, especially in developed countries. The exact cause of AE is unknown, but in previous studies it has been shown that AE is strongly associated with mutations in the filaggrin gene (FLG). Filaggrin is a skin barrier protein that plays a role in the alignment of keratin filaments. Furthermore, filaggrin is the precursor of the amino acid derived components of the natural moisturising factor (NMF), which is important for SC hydration.

AE patients have a decreased skin barrier function in non-lesional as well as lesional skin, as monitored with transepidermal water loss (TEWL). However, the role of FLG mutations in the reduced skin barrier function is yet inconclusive. Therefore, other factors such as an altered SC lipid composition and organization may play a role in the decreased skin barrier of AE patients.
The main objective of this thesis is to determine the SC lipid composition, lipid organization and lipid/protein ratio in AE patients and control subjects and to determine how these changes in lipid properties are associated with the impaired skin barrier function and disease severity of AE patients.

The main questions to be answered in this thesis are:

i) Are the lipid composition and lipid organization altered in non-lesional and lesional SC of AE patients compared to control subjects?

ii) Is there a correlation between an altered SC lipid organization and lipid composition in AE patients and control subjects?

iii) Are the changes in the SC lipid properties associated with the impaired skin barrier function and disease severity?

iv) Are the changes in SC lipid properties associated with the presence of FLG mutations and NMF levels?

v) Is the SC lipid/protein level altered in AE and does this correlate with skin barrier function?

vi) Are the SC lipid properties altered in the related skin disease Netherton syndrome?

In chapter 2 the lamellar lipid organization and the level of CER subclasses of control SC and non-lesional SC of six AE patients is described. In two AE patients the lamellar lipid organization was altered: shorter periodicities of the lamellar phases in the SC were noticed compared to controls. The level of long chain acyl-CERs was also reduced in these two patients. These results indicate that the presence of long chain acyl-CERs may play a role in shortening of the repeat distances of the lipid lamellar phases in AE patients. The association of the level of long chain acyl-CERs and presence of the LPP was previously reported for in vitro SC lipid mixtures\textsuperscript{32}. These results showed that a more detailed analysis of the lipid organization and composition in patients with AE is warranted.

In the study described in chapter 3 a comprehensive analysis of CER composition and lipid organization in non-lesional SC of AE patients and control subjects was performed. SC was harvested by tape-stripping in order to determine the lipid composition. A biopsy was taken in order to obtain information on the lamellar lipid organization. The lateral organization of the lipids and the NMF levels were examined non-invasively. In addition, the skin barrier function and clinical state of the disease were examined. The results show that the levels of several CER subclasses were altered in non-lesional SC of AE patients compared to controls. The level of acyl-CERs was reduced while the level of CERs with an extreme short chain length (total chain length 34 carbon atoms, referred to as C\textsubscript{34} CERs) was drastically increased in
SC of AE patients. These changes in CER composition contributed to a reduced average CER chain length in AE SC compared to control SC.

Not only the CER composition was modified, but also changes in the lipid organization were observed: the lamellar lipid organization was altered in a subclass of AE patients, indicating lamellar phases with a shorter repeat distance. Within these lamellae the lipids were organized in a less dense structure in AE patients compared to controls. The reduced average chain length of the CERs was associated with these changes in lipid organization. The reduced CER chain length as well as the modified lipid organization correlated with the impaired skin barrier function and disease severity, but were independent of the presence of FLG mutations. However, the changes in lipid composition and organization correlated with the levels of NMF, the degradation product of filaggrin. These results indicate that SC lipids play a role in the skin barrier dysfunction in non-lesional SC of patients with AE.

Chapter 4 describes a study in which electron diffraction (ED) is used to examine the lateral lipid organization of non-lesional SC of AE patients and controls. This method is used in combination with tape-stripping. With ED it is possible to sample the local lateral packing (an area of \( \sim 1 \mu m^2 \) is exposed to the electron beam) and obtain information on the relative occurrence of orthorhombic (dense) and hexagonal (less dense) lipid domains as well as lipid domains containing a combination of both types of lipid organization. The results showed an increased presence of the hexagonal lipid organization in non-lesional SC of AE patients at the expense of the orthorhombic lipid organization. These changes correlated with a reduced skin barrier function, but are independent of the presence of FLG mutations.

In chapter 5 a detailed analysis of the FFA composition as well as the lipid organization in non-lesional as well as lesional SC of AE patients and control SC is reported. We focused particularly on the chain length distribution of the FFAs as the results described in chapter 3 indicate that CER chain length is important for a proper lipid organization and skin barrier function. We observed that the level of very long chain FFAs are strongly reduced while the level of shorter FFAs are increased in SC of AE patients compared to that in SC of control subjects. These alterations were more pronounced in lesional skin sites but already present in non-lesional AE skin sites. These changes resulted in a significant reduction of the average FFA chain length in SC in lesional and non-lesional skin of AE patients compared to controls. Not only the FFA chain length was altered, but also an increased level of unsaturated FFAs and a reduced level of hydroxy-FFAs were observed in SC of AE patients. Again, this was more pronounced in lesional skin sites compared to non-lesional sites. In addition, we analyzed whether the
FFA chain length distribution was associated with the chain length distribution of CERs. We observed that changes in FFA chain length composition strongly correlated with changes in CER chain length. Furthermore, the reduced levels of acyl-CERs and increased levels of C34 CERs were more prominent in SC of lesional skin than in non-lesional skin sites of patients with AE.

The changes in lipid composition resulted in an altered lateral lipid organization; an increased level of lipids formed a hexagonal (less dense) organization and the lipid chains were more disordered in SC of AE patients compared to controls. Also here, these alterations were more pronounced in lesional AE SC compared to non-lesional SC. Both, the chain length distribution of the lipids and the lipid organization correlated excellently with the impaired skin barrier function, but no association with FLG mutations was observed.

The results described in this chapter clearly demonstrate that not only the CERs, but also changes in the chain length distribution of FFAs contribute to changes in lipid organization and impaired skin barrier function in AE patients.

In *chapter 6* the use of Raman spectroscopy in order to determine the lipid/protein level in SC is reported. The lipid/protein ratio was determined by calculating the ratio of the integrated signal intensity from 2866 to 2900 cm\(^{-1}\) (lipid signal) and from 2910 to 2966 cm\(^{-1}\) (protein signal). In addition, the dry SC mass per surface area of non-lesional and lesional AE skin sites was determined and compared to control SC. The results show that the dry SC mass per skin area is altered to some extent in lesional skin sites in AE patients compared to control subjects. In contrast, we observed a reduction in lipid/protein ratio already in non-lesional skin and this reduction was more pronounced in lesional SC of AE patients compared to controls. The lipid/protein ratio showed a very strong association with the skin barrier function. This demonstrates that besides the lipid composition and organization, the lipid/protein ratio is another important determinant of the skin barrier function.

*Chapter 7* describes the lipid composition and organization in 8 patients suffering from Netherton syndrome (NeS), an inflammatory skin disease related to AE.

The disease is caused by mutations in the serine protease inhibitor Kazal-type 5 (SPINK5) gene, which encodes the protease inhibitor lympho-epithelial Kazal-type–related inhibitor (LEKTI). Lack of LEKTI causes epidermal proteases hyperactivity which results in severe SC detachment. As hardly any information is available on the SC lipid properties of these patients, our aim was to investigate whether the SC lipid composition and organization are altered in NeS patients. The results show that in SC of NeS patients there are drastic changes in the composition of CER subclasses, such as a reduction in the level of acyl-CERs. In addition we noticed a strong increase in C34 CERs, a shift to shorter FFA chain
lengths and a strong increase in the level of unsaturated FFAs. Not only the lipid composition changed, but also changes in the lipid organization were detected: the lipid chains were more disordered. A subgroup of patients showed no lamellar ordering, and these patients also had the most drastic reduction of acyl-CERs in their SC.

This study showed changes in the SC lipid profiles in NeS that are similar, but much more pronounced than in patients with AE. These changes in SC lipids are expected to contribute to the barrier dysfunction in NeS, as observed for patients with AE.

**Conclusions**

The studies described in this thesis demonstrate that there is an altered lipid composition in non-lesional as well as lesional SC of AE patients. Not only the levels of CER and FFA subclasses are altered in AE, also the levels of long-chain CERs and long-chain FFAs are decreased, while the level of short-chain CERs and short-chain FFAs are increased. These coordinated changes in lipid composition result in an altered lipid organization that is associated with an impaired skin barrier function. However, besides the altered lipid composition, the lipid/protein ratio is decreased in SC of AE patients. The reduced lipid/protein level also correlates strongly with the impaired skin barrier function. Then the question arises whether the changes in lipid composition correlate with the decreased lipid level. Preliminary results indicate a correlation coefficient of r=0.79 between the average lipid chain length and lipid/protein ratio, indicative for a common factor underlying the altered lipid composition and the lipid/protein level. This factor is yet unknown and may be subject of future studies.

A direct intra-subject comparison between lesional and non-lesional skin revealed that the alterations in SC lipid properties are more pronounced in lesional SC in most cases. This suggests that the lipid biosynthesis is most affected in lesional skin which may be a result of inflammation, altered pH or increased levels of cutaneous microbes.

Changes in SC lipid properties of NeS patients were comparable to those found in AE but were more pronounced, indicating similarities between these two atopic skin diseases.

Our results show that the SC lipid composition, lipid organization and lipid/protein ratio are important determinants of the impaired skin barrier function in AE patients and are also related to the severity of the disease. A possible future treatment aiming to repair the skin barrier in patients with AE may focus on normalizing the lipid composition. This can be achieved in two fundamentally different ways; by supplementation of those lipids that
are present in reduced levels in SC or by affecting the lipid biosynthesis in the epidermis. This may improve the lipid organization and skin barrier function of these patients.

**Perspectives**

The studies described in this thesis demonstrate the importance of the SC lipids in the impaired skin barrier function of AE; the altered SC lipid composition results in an altered lipid organization which results in a decreased skin barrier function.

**Barrier repair in AE**

**Use of in vitro lipid mixtures to study the effect of individual lipids on the lipid organization and permeability**

In order to obtain more insight in the effect of an altered lipid composition on the lipid organization, *in vitro* lipid mixtures can be used to study in more detail the effect of individual lipid classes on the lipid organization. Of special interest is an increase in the level of C34 CERs, a reduction in the level of acyl-CERs, an increased level of short chain FFAs and an increased level of unsaturated FFAs. Not only the modulation in lipid organization, but also changes in the permeability across the lipid matrix are of interest to examine. Such studies can be carried out with *in vitro* lipid mixtures sprayed on a porous membrane. It may be possible to monitor the lipid barrier function by transepidermal water loss also used *in vivo* to monitor the skin barrier function. In addition, it is of interest to measure the diffusion of compounds across the same lipid membrane model. Then even the question can be tackled whether the skin barrier as measured by transepidermal water loss (inside-out) is correlated with the penetration of substances inducing allergic response (outside-in). Currently hardly any information is available on these issues.

**Future treatments to normalize the lipid composition in AE patients**

This can be achieved in two ways; that is by supplementation of those lipids that are present in reduced levels in SC or by affecting the lipid biosynthesis in the epidermis.

a. **Topical supplementation of lipids.** Based on the results obtained in this thesis formulations can be designed to supplement the SC of AE patients with those lipids that are present in reduced levels. As the reduced chain length may be an
important determinant for skin barrier modulation, formulations should include lipids that increase the average lipid chain length. To determine whether this approach is successful, fundamental studies are required in order to determine whether the applied lipids indeed diffuse into the SC and subsequently modulate the lipid organization. This can be achieved by an extensive assessment of the lipid composition and organization before and after such a treatment. This should answer the question whether the formulations form an additional layer onto the skin surface or that the formulations diffuse into the skin provoking changes in the lipid properties within the SC.

b. Lipid metabolism as an important target in AE. The altered lipid composition observed in SC of AE patients demonstrates changes in the lipid metabolism in the skin of AE patients. Two major changes in lipid composition were observed: changes in the levels of CER subclasses, and a reduction in the chain lengths of CERs and FFAs. Regarding the changes in CER subclasses, we found increased levels of those CERs that not only have glucosylceramides, but also sphingomyelin as precursors. This suggests a misbalance in activity between acid sphingomyelinase that catalyses the hydrolysis of the phosphorylcholine from sphingomyelin, and β-glucocerebrosidase that cleaves the glucose group from glucosylceramides. Therefore, it is of great interest to study the activity and level of the enzymes acid sphingomyelinase and β-glucocerebrosidase.

The reduced chain lengths of CERs and FFAs may indicate reduced expression or activity of enzymes that elongate the lipid chains. As our studies showed that the changes in chain lengths of FFAs and CERs were highly correlated, normalization of elongation of the FFAs will most probably also normalize the chain lengths of the CERs. The elongation of FFAs from C14-C16 to longer chain lengths occurs by the elongase family. There are 7 elongases (ELOVL1-7) identified, all having a specific preference for a certain chain length of the FFA. ELOVL4 is observed in the epidermis on a protein level and plays a crucial role in the elongation of FFAs ≥C24.33,34 This has been reported by two studies in which a strong reduction in very long chain FFAs was observed in ELOVL4 knockout mice.33,35 However, also other members of this ELOVL family may be of interest. In particular ELOVL1 and ELOVL6 that may be responsible for the elongation of fatty acids with chain lengths between 16 to 20 carbons. Future studies may therefore focus on the expression level and activity of ELOVLs in AE patients.

A less pronounced change in lipid composition is the increased level of unsaturated FFAs in SC of AE patients. It would be very desirable to examine the expression and activity of enzymes involved in desaturation of fatty acids, the stearoyl-coenzyme A desaturases (SCDs). As mono-unsaturated fatty acids with chain lengths of 16 carbon and 18 carbon atoms are most abundantly present in SC of AE patients, it is interesting to study in particular the level and activity of SCD-1.
Effect of FLG mutations on skin barrier properties

To date, the presence of FLG mutations is a well-known predisposing factor for AE, however we did not observe correlations between alterations in the SC lipids and presence of these mutations.

In our studies 4 subjects were included that had a homozygous mutation and 1 subject was included that had 2 heterozygous (compound heterozygous) mutations. Therefore, we were not able to compare the SC lipid properties of AE patients that had heterozygous, homozygous and compound heterozygous mutations. This may be an interesting target for future studies. Another approach to study in more detail whether FLG mutations do affect SC lipid composition and organization is to examine subjects with and without these mutations with any history of inflammation. In this way solely the effect of filaggrin mutations in lipid composition could be assessed without the interference of inflammation. Candidate subjects are control subjects carrying FLG mutations or infants with AE, without inflammation carrying FLG mutations.

Netherton syndrome

The study with NeS patients was carried out with a small group of patients. The results, however, demonstrate an altered SC lipid composition and organization. In the present study no TEWL was measured. Therefore, no relation between lipid organization, composition and skin barrier function could be assessed. It would be desirable to carry out such a study in a larger group of patients. Not only TEWL, but also expression levels of various proteases including LEKTI as well as lipid processing enzymes are of interest to examine. This might give more insight in the effect of proteases on lipid processing enzymes, lipid composition and organization in SC of patients with NeS. Studying this more severe atopic skin disease in greater detail may give more insights that can be of great help in understanding the disease mechanisms of AE.
References


