# A breached barrier : analysis of stratum corneum lipids and their role in eczematous patients

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# PART V

SUMMARY AND APPENDICES

SUMMARY AND PERSPECTIVES

## Rationale

Atopic eczema (AE) is one of the most common types of inflammatory skin diseases. It is characterized by eczematous lesions (lesional skin), whereas other areas of the body may visually appear normal (non-lesional skin). The diagnosis of AE is based on a constellation of clinical findings, as there is currently no biomarker characteristic for diagnosis of AE. The disease is often referred to as atopic dermatitis, neurodermatitis, and endogenous eczema. Because up to 60 percent of patients with the clinical phenotype do not have elevation of total or allergen-specific IgE levels in serum, there is still a controversy in the terminology of 'atopic'. The inflammation is primarily related to penetration of allergens into the skin that provoke an immune response, resulting in clinical symptoms like erythema (red skin) and pruritus (itch)[1-3]. Also xerosis (dry skin) is one of the main symptoms of AE, indicating a dysfunction in skin hydration, regulated by the skin's natural moisturizing factors (NMF)[4-6]. The NMF are breakdown products of the filaggrin protein. Mutations in the filaggrin gene (*FLG*) are to date the single most predisposing factor for developing AE[7], although it can only explain 20-50% of all occurrences[8,9]. This subscribes the heterogeneity of AE and implies the role of other genetic and environmental factors in the pathophysiology of AE. One of these is the importance of a reduced barrier function that facilitates the penetration of exogenous compounds into the skin[10-15].

The main barrier of the skin is located in the outermost layer of the skin, the stratum corneum (SC)[16-18]. It consists of corneocytes embedded in a highly ordered lipid matrix, and prevents the penetration of environmental substances into the

skin[19]. In addition, it protects the body from excessive transepidermal water loss (TEWL).

In AE, the SC barrier is impaired, and allergens can penetrate through the extracellular lipids into the lower layers of the epidermis[2,11,13,20]. This stresses the importance of the SC lipids for a proper skin barrier function. SC lipids consist of free fatty acids (FFAs), ceramides (CERs) and cholesterol (CHOL)[21,22]. Both FFAs and CERs contain respectively 1 and 2 long carbon chains. These may differ in their molecular structure: FFAs are predominantly saturated, although mono-unsaturated FFAs (MUFAs) are present as well[23,24]. CERs consist of a sphingoid base and a fatty acid (acyl) chain. Variations in both carbon chains (chain length and head group structure) lead to 12 CER subclasses[25,26]. In addition to these structural variations, both FFAs and CERs show a wide distribution in their carbon chain lengths. The variation in carbon chain length and chemical structure is unique for SC lipids and results in a distinctive lipid organization: the lipid lamellae[27,28]. These stacked lipid layers have a repeat distance of approximately 6 nm and 13 nm, referred to as the short periodicity phase (SPP) and long periodicity phase (LPP)[29-31]. Besides this lamellar lipid organization, SC lipids are packed in a lateral lipid organization: Human SC lipids are mainly present in a very dense – orthorhombic – organization, although a subpopulation is packed in a less dense – hexagonal – organization[32-36].

Analytical methods to study the lipid composition are limited, and no methods are currently available to study the chain length of the SC lipids. This is one of the reasons that the role of the SC lipids in AE remains inconclusive: Reports on the CER composition give conflicting information[37-45]; hardly any information is available on the composition of the FFAs[46,47]; and only one study in a limited number of AE patients is present that studies the lipid organization, only mentioning the lateral packing[48]. The lack of knowledge about the SC barrier lipids in relation to the impaired skin barrier function of AE was the basis for the aims of this thesis.

**Aims**

The primary aim of this thesis is to study in detail the SC lipid composition and organization as well as their role in the skin barrier function in eczematous patients. This is achieved by pursuing the following challenges:

1. Developing robust methods that enables quantitative analysis of all main SC lipid classes in detail.
2. Determine the comprehensive SC lipid composition of both lesional and non-lesional skin in AE patients and compare the lipid profile to healthy controls.
3. Establish how changes in the SC lipid composition of AE patients are related to changes observed in the lipid organization, and how those affect the skin barrier function.

4. Determine the relationship between SC lipid composition and organization with respect to other SC sources that show a reduced skin barrier function (i.e. Netherton syndrome (NTS) and human skin equivalents (HSEs)).

**Part I: Method development for detailed stratum corneum lipid analysis**

Part I addresses the first aim in which a novel method was developed that would permit detailed analysis on all main SC lipids (CERs, CHOL and FFAs) in a single setup. To achieve this, liquid chromatography coupled to mass spectrometry (LC/MS) was preferred, as it can analyze the SC lipid classes and subclasses as well as the chain length of each individual lipid.

The development of such a method was started with the analysis of CERs, reported in **Chapter 3**. It describes a quick and robust LC/MS method that enables quantitative analysis of all human SC CER subclasses using only limited sample preparation. The combination of normal phase LC in combination with atmospheric pressure chemical ionization (APCI) MS is uncommon. Nevertheless, it proved to be an excellent choice for the analysis of SC CERs, as all subclasses and chain lengths were adequately separated and could easily be identified. Human SC shows the presence of 11 known CER subclasses. In addition, unidentified lipid subclasses were discovered. Using high mass accuracy MS as well as by acquiring fragmentation data (MS/MS), one of these lipid subclasses was identified as the 12th (missing) CER subclass: the ester-linked ω-hydroxy acyl chain linked to a dihydrosphingosine base (CER [EODS]). The method appeared successful in analyzing all 12 CER subclasses from both *ex-vivo* skin, SC obtained by tape stripping and SC from human skin equivalents (HSEs).

The development of the second major SC lipid class, FFAs is described in **Chapter 4**. The first challenge was to develop and subsequently validate an LC/MS method for quantitative analysis of SC FFAs. Such a method has not been reported before for SC FFAs, as gas chromatography is the most common method. Besides, LC/MS of underivatized FFAs results usually in unstable ions or is not sensitive. Using reverse phase LC in combination with APCI-MS in negative ion mode as well as the addition of chloroform as an ionization enhancer contributed to a large extend to the formation of stable chloride adducts. A quantitative validation protocol demonstrated that the method is robust, reproducible, sensitive, fast, whereas ion suppression was negligible. This method was therefore suitable for analysis of human SC FFAs and could be combined with the analysis of CERs, as the same ionization source (APCI) was used. The second challenge was to enable quantitative analysis of CHOL, which was achieved by optimizing the LC-gradient for the CER method. The combined methods enable (semi-)quantitative analysis of all major SC lipid classes

in a single setup. The last part of the chapter describes the application on *ex vivo* human SC, human SC obtained from tape stripping and human skin substitutes (porcine SC and human skin equivalents). In conjunction with CER profiles, clear differences in FFA profiles were observed between these different SC sources.

Now that the developed LC/MS methods enable the analysis of all major SC lipids, this method was used in several studies in which the role of the SC lipids was investigated with respect to the skin barrier function. These studies are described in Part II and III.

**Part II: Stratum corneum lipid composition in atopic eczema and its role in the skin barrier function**

Using the LC/MS methods developed in part I, the second aim of the study was tackled, in which the SC lipid composition of AE patients was examined and compared to healthy subjects (controls). Part II describes 4 chapters that investigate different aspects of the SC lipid composition. These chapters also cover the third aim of the thesis, in which the relation between the lipid composition and the lipid organization was studied, as well as their involvement in the reduced skin barrier function of AE patients.

The first, explorative, study in AE patients is described in **Chapter 5**. The composition of the CER subclasses as well as the lamellar lipid organization of SC from six AE patients was compared to that of 6 control subjects. Only non-lesional skin of the ventral forearms was investigated. The CER composition was examined by tape stripping and successively analyzed by the newly developed LC/MS method. Skin biopsies were harvested in order to examine the lamellar lipid organization by small-angle X-ray diffraction studies. Regarding the CER composition, all 12 CER subclasses were observed in AE patients. However, a significant decrease in the level of CER [EO] subclasses (those with an ester-linked ω-hydroxy acyl chain linked to a sphingoid base, also referred to as acyl-CERs) and CER subclass [NP] (CERs with a non-hydroxy acyl chain linked to a phytosphingosine base) was observed. Interestingly, subjects with the strongest reduction in acyl-CERs also show the most pronounced deviations in their SAXD-profiles. This suggests that changes in CER composition are related to an altered lamellar lipid organization in AE patients.

These findings were the starting point for a comprehensive study in 28 AE patients and 14 control subjects, described in **Chapter 6**. Both the CER composition and lipid organization in non-lesional SC was determined by means of LC/MS, Fourier transform infrared spectroscopy (FTIR) and small angle X-ray diffraction. In addition, the skin barrier and clinical state of the disease as well as the effect of *FLG* mutations were examined. SC of AE patients showed an increased level of CERs with a very short total chain length of 34 carbon atoms (fatty acid + sphingoid base, C34 CERs), whereas the level of very long acyl-

CERs was decreased. These changes reduced the overall CER chain length significantly, and induced an altered lipid organization that correlated to a decreased skin barrier function in these AE patients. Moreover, the changes in SC CERs also correlated with disease severity. The CERs were the first of the main lipid classes analyzed in SC of AE. Because the results show that the lipids seem to play an important role for the barrier function in AE, other main SC lipid classes were analyzed as well.

A consecutive study in AE patients focused on the second main SC lipid class, the FFAs. As no studies have been reported so far that studied the FFA composition and its chain length in SC of AE patients, **Chapter 7** presents a study that focuses on the extracellular SC FFA composition in both lesional and non-lesional AE skin, and describes how changes in FFA composition are related to the observed changes in the CER composition. In addition, it was studied how the changes in lipid composition affect the lipid organization as well as the skin barrier function. An increased abundance of MUFAs at the expense of hydroxy-FFAs was observed in SC of AE patients. Moreover, the FFA chain length distribution is shifted towards shorter chain lengths. These findings were already observed in non-lesional skin, but much more pronounced in lesional skin. The reduced FFA chain length matches the shorter chain length observed for the CERs, which is a strong indication that the CERs and FFAs share a common synthetic pathway. The chain length of the lipids was strongly associated with the lateral lipid organization: An increase in lipids with shorter chain length results in the increased presence of a hexagonal lipid organization, while longer chain length lipids contribute to the orthorhombic organization. The increased presence of a less densely packed lipid organization also correlated to the reduced skin barrier function, as monitored by TEWL.

Both Chapters 6 and 7 described the effect of FLG mutations on the lipid parameters. These mutations are a major predisposing factor for development of AE, and are therefore of interest to study regarding their relation to the SC lipids. Also the NMF – degradation products of filaggrin – are taken into account, as these play an important role in the hydration and establishing a pH gradient of the SC. The lipid composition and organization show a clear correlation with NMF levels, but not with FLG mutations. This discrepancy illustrates that between FLG genotype and filaggrin metabolites, other factors may play an important role (like filaggrin expression), and demonstrates the importance of studying filaggrin at the phenotype level in addition to profiling FLG genotype.

The studies described in the previous chapters make clear that the lipid composition and its organization play an important role in the reduced skin barrier function of AE patients. **Chapter 8** describes a study in AE patients in which the importance of the lipid to protein level was studied. This was achieved by determining for each AE patient and

control subject the dry SC mass per surface area as well as the ratio between lipid and protein bands in the Raman spectra, which is a measure for the relative lipid/protein ratio. The results demonstrate that the dry SC mass per skin area is to a minor extent changed in AE patients compared to control subjects. In contrast, the lipid/protein ratio is much more reduced in SC of AE patients, both in non-lesional skin and even more pronounced at lesional skin sites. These changes correlated with the skin barrier function and indicate that, besides lipid composition, also the lipid/protein ratio plays an important role in the reduced skin barrier function in AE.

Overall, the studies described in Part II illustrate the importance of the altered lipid composition and organization for the impaired barrier function of the skin in AE. The findings obtained in these studies formed the basis to investigate other SC sources in which a reduced barrier function is present, which are described in Part III.

**Part III: Studies on SC lipids from other skin sources showing a barrier dysfunction**

In part II the relevance of the SC lipids for the skin barrier function and its biological relevance to AE has been demonstrated. In part III, we expanded the scope beyond AE to pursue the fourth aim of this thesis. Two additional studies were performed on the SC lipids from different skin sources in which a reduced skin barrier is present: patients with Netherton syndrome (NTS) and human skin equivalents (HSEs).

**Chapter 9** describes a study in 8 NTS patients. NTS is an exceptional form of dermatitis and share similarities with AE. NTS is caused by rare specific genetic mutations in the SPINK 5 gene, which encodes for a specific protease inhibitor (LEKTI). In NTS, lack of this inhibitor results in increased activity of epidermal proteases, leading to severe SC detachment. The aim of the study was to determine the SC lipid composition and organization in NTS patients, as hardly any information on these matters is currently available. NTS patients showed a decrease in their SC FFA chain length as well as an increased amount of MUFAs compared to controls. In addition, the level of C34 CERs was increased in NTS patients whereas a subgroup of patients showed a strong reduction in long-chain CER levels. NTS patients also showed the presence of high levels of unsaturated CERs, which had not been observed in any other SC source studied so far. These changes in the lipid composition increased the disordering of the lipid packing: both the lamellar and lateral lipid organization showed significant changes. The results demonstrated that acyl-CERs are crucial for the long periodicity phase in the lamellar lipid organization. The changes in lipid composition and organization are expected to contribute to the barrier dysfunction in NTS.

**Chapter 10** describes a study on the SC lipids of human skin HSEs. These are

bioengineered skin models that mimic many features for native human SC, but they show an impaired skin barrier and a different SC lipid organization. To elucidate, in detail, the cause of the altered SC lipid organization, analysis by LC/MS was performed to determine the FFA and CER composition as well as the chain length distribution in detail. The results reveal that all HSEs *i)* show the presence of 12 CER subclasses (similar to native human SC), although CER species with short total carbon chains were significantly increased compared to human SC. *ii)* display the presence of CER species with a monounsaturated acyl chain, which are not detected in human SC (except for NTS patients); *iii)* have an increased presence of MUFAs compared with human SC, whereas the total amount of FFAs was drastically decreased; *iv)* exhibit an altered expression of stearoyl-CoA desaturase, the enzyme that converts saturated FFAs to MUFAs.

## Conclusions

The studies described in this thesis reveal that SC lipids play a crucial role for a proper skin barrier function in humans. *In vivo* studies in AE and NTS patients reveal that changes in the composition of the lipids negatively affect the lamellar and lateral lipid organization. In particular the lipid chain length excellently correlated to the organization of the lipids: shorter FFA and CER chain lengths contribute to a more hexagonal lipid organization, while longer lipid carbon chains will results in a more densely packed, orthorhombic organization. From the results in AE and NTS patients, it can also be concluded that the acyl-CERs are crucial for the long periodicity phase in the lamellar lipid organization. The use of LC/MS proved to be essential for these findings, as no other method to date can analyze the SC lipid chain lengths in all its detail. Besides, the sensitivity of MS reduced the amount of sample necessary for analysis, which permitted harvesting SC lipids in a non-invasive way by means of tape-stripping.

In addition, the level of SC lipids may be important for a proper skin barrier function, as the reduced lipid/protein ratio in AE subjects correlate to a high extent with a reduced skin barrier function. Chapters 5-7 report that TEWL correlates with the lipid composition (particularly lipid chain length), whereas chapter 8 reports a strong correlation between TEWL and the lipid/protein ratio. This raises the question whether changes in lipid composition correlate with the changes observed in lipid/protein ratio, in AE. Indeed, a high correlation is observed between the lipid chain length and the lipid/protein ratio ($r_{spearman}$=0.79). This could indicate that the changes observed in the lipid chain length and lipid/protein ratio may share a common factor in lipid metabolism that is altered in AE (discussed below).

The SC barrier lipids in AE, NTS and HSEs showed similar changes in their composition:

a reduced lipid chain length and increase in unsaturated lipids. Comparing SC from non-lesional AE with lesional AE skin and NTS reveals that more severe changes in the composition of the lipids result in more severe changes in the lipid organization. This may explain why previous studies on NTS patients show a drastic reduction in the SC barrier function. The relation between the composition of the FFAs and CERs is remarkable: the chain length and the degree of unsaturation of the FFAs complement the composition of the CERs. This is of fundamental relevance as it supports the hypothesis that human SC CERs and FFAs share a common synthetic pathway[49,50]. It is also of clinical importance, as it implies that normalizing the FFA chain length distribution in AE skin may also contribute to normalization of the CER chain length.

These studies give relevant information regarding possible changes in the epidermal lipid metabolism: *i)* The reduced FFA chain length observed in AE, NTS and HSEs suggest that FFA elongation is impaired[51]; *ii)* the changes in the relative abundances of the CER subclasses indicate that the enzymes involved in synthesis of one particular subclass may either be up- or downregulated. Two enzymes that are likely to be involved in the altered epidermal lipid metabolism are β-glucocerebrosidase and acid sphingomyelinase, which convert CER precursors into their final CER structure[52-55]. Both enzymes, however, do not have the same affinity to all CER precursors, and changes in the activity of one of these enzymes may partly explain the increase in certain CER subclasses at the expense of others[56]; *iii)* the correlation between lipid chain length and lipid/protein ratio may indicate that the changes share a common factor that is altered in AE. It goes beyond the current knowledge to speculate about these factors, but enzymes involved in de novo synthesis of the lipids are definitely a target of interest for future studies.

Moreover, the studies in AE patients contributed to a better understanding of the role of FLG and NMF with respect to the SC lipids and barrier function. Filaggrin at the phenotype and metabolic (NMF) level seems more closely related than at the genotype level, which supports the hypothesis that changes in filaggrin expression may be an important factor in AE[57,58].

Overall, the studies performed throughout this thesis increased the knowledge of the SC barrier function from both a biophysical perspective (the relation between lipid composition, lipid organization, and barrier function) as well as a clinical perspective (the essence of the SC barrier lipids in relation to AE and NTS).

## Prospects

Despite the fact that the aims set at the very beginning of this thesis may have been accomplished, the studies have raised additional questions and may serve as a basis for future studies in multiple directions. The most prominent ones will be discussed below.

### Exploiting the possibilities of LC/MS for analysis of SC lipids

Although an adequate method was developed for qualitative and quantitative analysis of all major SC lipid classes, many possibilities remain unexploited. The main advantages of the current LC/MS method compared to other methods are that it is quick (<20 minutes analysis time to acquire data on all main SC lipids), robust, sensitive, and gives detailed information on the lipid chain length. However, the main bottleneck for high-throughput analysis is currently the lipid extraction protocol (taking several hours) and manual data-processing (several hours per sample). Regarding an alternative extraction protocol, experiments that replace the current 3-step Bligh and Dyer extraction protocol with a 1-step heptane/isopropanol extraction of 20 minutes provided similar results on the lipid composition. Future validation of such an improved extraction protocol may therefore significantly reduce the amount of time spend on sample preparation. With respect to data-processing, currently automatic data-processing results in proper quantification in ~90% of the assigned peaks. With a number of >250 designated peaks, this needs to be improved for reliable automatic quantification. One way to do this is by analyzing lipids in MRM mode. Two additional advantages are that sensitivity is (usually) increased, and it may also provide more structural information, for example on the individual chains of the sphingoid base and acyl chain of the CERs. The sensitivity may also be greatly enhanced when changing from the current HPLC method to UPLC. Last but not least, it is preferable to use only a single injection for analysis of all lipid classes at once. Currently, CERs and CHOL are analyzed in positive ion mode and NPLC, whereas FFAs are analyzed in negative ion mode and RPLC. Because CERs are also observed when analyzing FFAs in negative ion mode, it may be worthwhile to analyze all SC lipids in negative ion mode, using NPLC for adequate separation of all CER subclasses and optimize it to include proper analysis of FFAs and CHOL.

Another aspect for future studies concerns the identification of additional lipid peaks that were noticed in Chapters 3 and 4. Among these are probably the glucosyl-CERs, di- and triglycerides, CHOL esters, and probably others which are yet unknown. In particular the unknown lipid species that elute in between the different CER subclasses (e.g. between CER [AP] and CER [AH]) may be of interest. Their mass and hydrophobicity may indicate that they have comparable molecular structures, but fragmentation studies (MS/MS) may

fully elucidate the molecular structure of these lipids. It is likely that some of these ions are isomers of the alpha-hydroxy fatty acid CER subclasses ([AS], [ADS], [AP], [AH]), but that the hydroxyl group in the acyl chain is located at the ω-position. This would slightly increase the polarity but not the mass, something which matches the unidentified CER located between CER [AP] and [AH]. These so-called ω-hydroxy fatty acid CERs are already known to be present in the SC as bound lipids[59], and it is therefore plausible that these could also be observed with the current method as free lipids.

**Elucidating the epidermal lipid metabolism**

It was concluded from the *in vivo* studies performed in patients with AE and NTS, but also the *in vitro* studies with HSEs, that changes in the synthesis of epidermal lipids are the underlying cause of the altered SC lipid composition. Future studies may therefore focus on the enzymes that elongate the FFAs (ELOVLs), as these may be a probable reason for the reduced lipid chain length. Since a lower level of FFAs with a chain length of 24 carbon atoms and higher was observed in AE and NTS, ELOVL4 activity may be reduced, as this enzyme is involved in elongation of FFAs ≥24 carbon atoms. Recent murine studies support this hypothesis, demonstrating that SC lipids of ELOVL knockout mice show reduced levels of long chain FFAs[60-62]. The increased amount of MUFAs suggests that expression of stearoyl-CoA desaturase (the enzyme that converts saturated FFAs to MUFAs) may be increased in AE[63].

In general, studying the enzymes in eczematous patients will give valuable information about the altered epidermal lipid pathways and may lead to more fundamental knowledge about epidermal lipid synthesis and the relation to AE. Also the role of inflammation on these epidermal lipid enzymes needs to be elucidated, as it is known that inflammatory cytokines affect the epidermal lipid synthesis in several ways. Studies related to the inflammatory aspects of AE may therefore focus on *i)* metabolic enzymes involved in CER synthesis (like β-glucocerebrosidase and acid sphingomyelinase)[52-55]; *ii)* cytokines like TNF-α as well as several interleukings (IL-17, IL-22, IL-25, IL-31) that are related to downregulation of filaggrin expression[64-68]; and *iii)* nuclear receptors (peroxisome proliferator-activated receptors) that are involved in SC barrier formation and have anti-inflammatory capabilities[69-75].

**SC barrier repair**

Restoring the SC barrier function has become one of the main challenges in AE. Two fundamentally different approaches can be pursued to achieve this: *i)* one can compensate for the reduced barrier function by application of topical formulations; *ii)* Another

approach is to restore the epidermal lipid synthesis.

With respect to the former approach, our studies give new insights in possible topical formulations which may be beneficial to the SC barrier. In particular the finding of the reduced lipid chain length in AE is something that offers a new perspective. An interesting study in AE subjects would therefore be to compensate for the reduced lipid chain length by topical application of a formulation containing long carbon chain CERs and/or FFAs for at least several weeks, and subsequently analyze the lipid composition and organization of these patients and investigate whether these lipids will be incorporated in the native SC lipid matrix and improve the lipid organization and skin barrier function. One can improve such formulations even further by analyzing the SC lipid composition at forehand. There is a large individual variation in the CER and FFA composition of AE subjects, and one specific formulation may not be suitable as a treatment for all patients. Based on deteriorations in the lipid profile, it can be decided which formulation and dose should lead to an optimal barrier recovery. This approach of individual dose/formulation regimen is something that has increased attention for pharmaceutical companies as it proves to be more beneficial to the patient.

The second approach, to restore the epidermal lipid synthesis, may be much more difficult to achieve, and needs much more knowledge on the fundamentals of AE. In order to target the enzymes that may lead to normalization of the lipid synthesis, one need to normalize the metabolic processes that take place in the cytosol and endoplasmatic reticulum of the keratinocytes, where de novo syntheses of respectively FFAs and CERs primarily take place. A proper skin hydration, pH gradient, and keratinocyte differentiation are factors that should be taken into account for proper SC barrier repair using this approach.

It should not be overlooked that AE is mainly related to the western society, and relatively simple, non-clinical/pharmaceutical adaptations can already significantly improve the barrier. For example, a proper level of the environmental humidity, or the moderate use of soap, preferably with a skin-neutral to acid pH, may already reduce the prevalence of AE.

**Additional studies in AE may elucidate fundamental questions on the disease.**

One of the most frequently asked questions with respect to AE and the barrier function, is whether the inflammatory response (inside-to-outside model) or the reduced SC barrier function (outside-to-inside model) is the primary cause of the disease[20,76-78]. Although a definite answer cannot be given at the current time, it should be noted that future studies can lead to small progressions that may stepwise unravel this question. For example, studies in newborns with a high risk of developing AE (e.g. carriers of *FLG* mutations; parents with AE) but without any clinical appearance of the disease may be monitored

for a longer period of time and regularly checked on markers for skin inflammation and SC barrier function. If the onset of AE is imminent in such a newborn, one can study changes in some of these biomarkers that led to the onset of AE. This will lead to a better understanding of the disease. However, it should be mentioned that AE is a multifactorial disease and that this 'chicken-or-egg' question may well prove to be a 'chicken-and-egg question', as discussed by Elias *et al*., suggesting the 'outside-to-inside and back-to-outside' model[11,20].

The role of *FLG* mutations, filaggrin protein, and filaggrin breakdown products (i.e. the NMF) for the SC barrier function of AE may be another aspect for future studies. Studying the SC lipids in patients with ichthyosis vulgaris may therefore be of interest, as these patients carry loss-of-function mutations in the *FLG* gene. In addition, studies in subjects that carry a *FLG* mutation and have low NMF levels but normal SC barrier properties may give a better insight in the role of *FLG* and the SC barrier function in a diseased state.

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