CHAPTER 8

LIPID TO PROTEIN RATIO PLAYS AN IMPORTANT ROLE IN THE SKIN BARRIER FUNCTION OF PATIENTS WITH ATOPIC ECZEMA

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Abstract

The barrier function of the skin is primarily provided by the stratum corneum (SC), the outermost layer of the skin. Skin barrier impairment is thought to be a primary factor in the pathogenesis of atopic eczema (AE). Filaggrin is an epidermal barrier protein and common mutations in the filaggrin gene strongly predispose for AE. However, there is no strong evidence that filaggrin mutations are related to the reduced skin barrier in AE. It was recently shown that the SC lipid composition and organization play a role in the reduced skin barrier in AE.

We determined the dry SC mass per surface area of controls and AE patients as well as the ratio between lipid and protein bands in the Raman spectra being a measure for the lipid/protein ratio in SC. The results show that the dry SC mass per skin area is altered only in lesional SC of AE patients compared to control subjects. Much more pronounced was the observed reduction in the lipid/protein ratio in SC of AE patients, both in lesional and non-lesional SC. These changes correlate with the barrier function and may indicate that the lipid/protein ratio plays a role in the reduced skin barrier function in AE.

Introduction

Atopic eczema (AE) is a chronic relapsing, inflammatory skin disease characterized by erythema, pruritus, and xerosis. AE is currently affecting over 15% of Caucasian children and 2-10% of adults, and its prevalence is increasing[1-4]. Eczematic lesions have a red, flaky appearance and cause severe itching. There is increasing evidence that AE is related to an impaired skin barrier function[5,6]. AE is strongly associated to mutations in the filaggrin gene (*FLG*), encoding the barrier protein filaggrin[7]. Filaggrin has

a role in the alignment of keratin filaments, facilitating the flattening of keratinocytes during terminal cornification[8]. In addition, filaggrin is metabolized into hygroscopic molecules, together with specific salts and sugars referred to as the natural moisturizing factor (NMF), which is important for SC hydration[9]. Despite the fact that an impaired barrier function has been demonstrated in adult AE patients, the precise role of filaggrin for the skin barrier function is not completely understood and compelling evidence that *FLG* mutations are the cause of the reduced barrier function in AE is missing[10-12].

The primary function of the skin is to reduce epidermal water loss and to prevent the penetration of harmful agents by acting as a permeability barrier to the environment. This barrier is provided by the stratum corneum (SC), the outermost layer of the skin. It contains nonviable corneocytes oriented parallel to the skin surface. The corneocytes are surrounded by a cornified cell envelope, consisting of a densely cross-linked layer of proteins. This envelope reduces the uptake of most substances into the corneocytes and therefore redirects the transport of most substances along the intercellular regions[13]. The intercellular regions are filled with highly organized lipids with a characteristic composition and organization. For this reason, lipid composition and organization are considered to be crucial for an adequate skin barrier function. The major lipid classes in the SC are ceramides (CERs), free fatty acids, and cholesterol.

In previous studies, changes have been observed in the CER composition of non-lesional as well as lesional SC of AE patients[5,14-18]. Changes in several of the CER subclasses correlate with a reduced skin barrier function as measured by transepidermal water loss (TEWL). More recently, strong evidence was observed that besides changes in CER subclasses, the CER chain length plays a prominent role in the impaired barrier function of AE skin: in non-lesional SC of AE patients both CER chain length and skin barrier function were significantly reduced[19].

Besides the lipid composition and organization, the lipid/protein ratio in the SC as well as the SC thickness may affect the skin barrier function. The aim of the present study was to determine the lipid/protein ratio in SC of control subjects and AE patients. In addition, the dry SC mass per surface area was determined, being a measure for the SC thickness. Subsequently, these parameters were related to the barrier function of the skin, as measured by TEWL. The results show that the dry SC mass per surface area is altered in lesional AE SC but not in non-lesional AE SC, as compared to controls. The lipid/protein ratio is lower in both non-lesional and lesional SC in AE compared to that in SC of control subjects. The lipid/protein ratio correlates strongly with TEWL values. Therefore, besides lipid composition and organization, the lipid/protein ratio may be an important factor in the impaired skin barrier function in AE patients.

Materials and Methods

Study population and study setup

The study was approved by the Medical Ethics Committee of the Leiden University Medical Center and conducted in accordance with the Declaration of Helsinki Principles. All subjects gave written informed consent. Twenty-eight Caucasian AE patients (25.6 ± 5.6 years; 11 males) and fifteen Caucasian control subjects without (history of) dermatological disorders (25.0 ± 5.2 years; 5 males) were included. In the AE group, 14 patients were carrier of at least one prevalent *FLG* null allele. At the time of the study, 11 out of 28 AE patients suffered from eczematic lesions on at least one of the ventral forearms. The subjects did not apply any dermatological products to their forearms for at least one week prior to the study. Measurements were performed in a temperature and humidity controlled room, in which the subjects were acclimatized for 45 minutes prior to the measurements. All measurements were performed on a single day. A dermatologist marked an area (~4.5 cm^2) of non-lesional skin and, if applicable, an area (~4.5 cm^2) of lesional skin on the forearm of each AE patient at the start of the study day. The Raman spectroscopy measurements were performed on these marked areas. After the Raman measurements, the same skin areas were tape stripped while TEWL was monitored after each two tape strips. At the end of the study day, buccal mucosa cells were collected with a cotton swab for *FLG* genotyping.

FLG mutation analysis

All subjects were screened for any of the four most prevalent mutations found in the European Caucasian population (*2282del4, R501X, R2447X, S3247X*), as described previously[19].

Skin barrier function assessment

TEWL was monitored using a Tewameter TM 210 (Courage+Khazaka, Köln, Germany). The marked area on the subjects' ventral forearm was placed in an open chamber, and TEWL values were recorded for a period of at least two minutes after which an average reading was calculated from the last 10 seconds of the measurement. Recordings were performed prior to tape stripping (baseline TEWL) and after each second tape strip, to assure proper monitoring of the changes in skin barrier function.

Confocal Raman microspectroscopy

Confocal Raman microspectroscopy (3510 Skin Composition Analyzer, River Diagnostics, Rotterdam, The Netherlands) was used to determine the lipid/protein ratio

of the ventral forearm. The principles of Raman spectroscopy and the procedures have been described elsewhere[20,21].

Spectra were recorded in the high wavenumber spectral region (2500-4000 cm^{-1}) using a 671 nm laser. The spatial resolution was 5 cm^{-1}. Laser power on the skin was 20 mW. Spectra were obtained from the skin surface to a depth of 40 µm with 2 µm steps. This was repeated on 15 different locations within the marked area. In order to avoid interfering Raman signals from skin surface contamination, such as sebum, and to avoid influences of washout effects and desquamation near the skin surface, as well as to avoid Raman signals from the viable epidermis, we determined the lipid/protein ratio between depths of 4-10 µm in the SC. This was performed by calculating the ratio of the integrated signal intensity from 2866 to 2900 cm^{-1} (CH_2 asymmetric stretching of lipids) and from 2910 to 2966 cm^{-1} (CH_3 symmetric stretching of proteins, the same region as taken for the protein band for determination of the water concentration in skin[20]) and taking into account a linear baseline between the integration boundaries. The calculated signal ratio between the lipid and protein bands is referred to as the lipid/protein ratio. Calculations were performed using SkinTools 2.0 (River Diagnostics, Rotterdam, The Netherlands).

SC isolation and lipid extraction

In order to obtain Raman spectra of human SC lipids and extracted human SC, human SC was isolated by trypsin digestion as described elsewhere[22]. The SC lipids were extracted according to a modified Bligh and Dyer procedure[23] with the addition of 0.25M KCl to extract polar lipids.

Tape stripping procedure

Multiple poly(phenylene sulfide) tape strips (Nichiban, Tokyo, Japan) were successively applied at the same area (4.5 cm^2) on the marked area of the ventral forearm. All tapes were applied on the targeted skin and pressed for 5 seconds at 450 g/cm^2 using a D-Squame pressure instrument (Cuderm Corp., Dallas, TX). Tweezers were used to remove the tape in a fluent stroke, using alternating directions for each subsequent tape strip. The Squamescan 850A (Heiland electronic, Wetzlar, Germany) was used to determine the dry SC mass removed by each tape strip.

The calibration procedure of the Squamescan was as follows: Immediately after measuring the absorption of the tape strip with the Squamescan, the tapes were incubated and shaken overnight in 1 mL 1M KOH, after which the extracts were neutralized with 79 µl 12M HCl. The final pH of the extract was set between 7.2-7.4 with 1M HCl and/or 1M KOH. Subsequently, 50 µl of the extract was added to 200 µl bicinchoninic acid (BCA, Pierce

BCA, Protein Assay, Perbio Science BV, Etten-Leur, The Netherlands), followed by heating for 2 hours at 37°C and determination of the absorption at 562 nm (OD562). In this way, we obtained corresponding OD562 values of the tape extracts with known Squamescan values. Sheets of isolated human SC with known weight (between 10-1000 µg, size ~0.05-1 cm²) were incubated overnight and treated as described above, resulting in a calibration curve of dry SC mass against OD562. With this curve, we converted Squamescan values of the tapes via their OD562 values into dry SC mass, which resulted in a calibration curve of Squamescan values against dry SC mass. In addition, 1/TEWL was plotted against the cumulative amount of tape stripped SC. Here, the intercept with the x-axis obtained by extrapolation is a measure for the dry SC mass per surface area[24]. This extrapolation was only performed with curves having a $R^2 \geq 0.9$.

Statistical analysis

Statistical analysis was performed using SPSS Statistics version 17.0. As the data show a non-normal distribution, non-parametric Mann-Whitney tests were performed for comparison of different groups with a significance level of $P<0.05$. For the paired comparison of non-lesional and lesional SC, a Wilcoxon signed rank test was used. Bivariate analysis and Spearman's ρ correlation coefficient were used to analyze correlations between parameters.

Results

From the TEWL values and the cumulative amount of stripped SC, the dry SC mass per skin area was calculated. Figure 1 shows typical examples of 1/TEWL against the cumulative amount of tape-stripped SC of three AE patients. The abscissa intercept by extrapolation is indicative for the dry SC mass per cm². In the examples in Figure 1, the dry SC mass varies between 1195.2 µg/cm² and 1802.0 µg/cm².

Figure 2a shows the dry SC mass per surface area in control SC (1619 ± 400 µg/cm²), non-lesional SC of AE patients (1407 ± 340 µg/cm²), and lesional SC of AE patients (1200±168 µg/cm²). There was neither a significant difference between controls and non-lesional skin of AE, nor between non-lesional and lesional AE skin ($P>0.08$). However, there was a difference between controls and lesional skin of AE ($P=0.01$). Open and filled circles indicate non-carriers and carriers of *FLG* mutations, respectively. There was no difference in dry SC mass in AE patients that were carriers and non-carriers of a *FLG* mutation ($P>0.1$). In Figure 2b, 1/TEWL values are plotted as function of the dry SC mass per surface area (µg/cm²). The correlation coefficient between these parameters is $r = 0.67$.

Raman spectroscopy was used to examine the lipid/protein ratio in the SC. Figure 3a

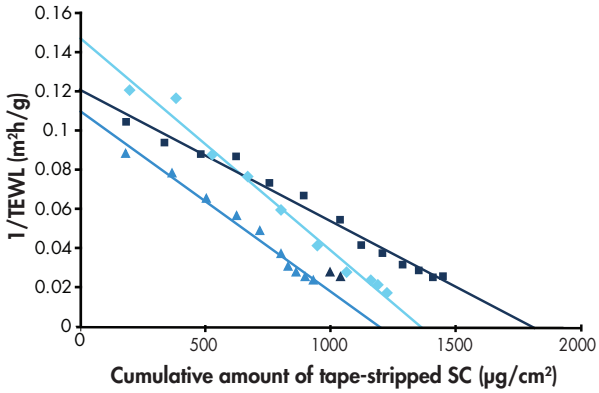

Figure 1: *Three examples of 1/TEWL plotted against the cumulative amount of tape-stripped SC. The intercept of the line with the x-axis is indicative for the dry SC mass per cm².*

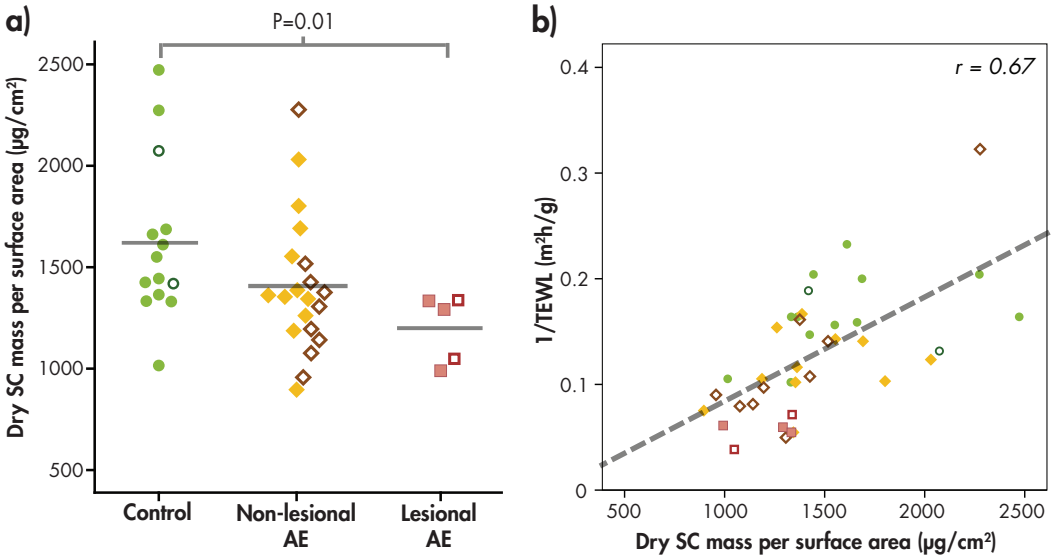

Figure 2: ***a****) Dry SC mass per surface area (µg/cm²) in control subjects, non-lesional SC of AE patients, and lesional SC of AE patients.* ***b****) Correlation between 1/TEWL and dry SC mass per cm² in control subjects and AE patients. Green, orange and red data points correspond to control SC, non-lesional AE SC and lesional AE SC, respectively. Open data points indicate carriers of FLG mutations, filled data points indicate non-carriers of FLG mutations. Control subjects are indicated by ●/○. Non-lesional skin and lesional skin of AE patients are indicated by ◆/◇ and ■/□, respectively.*

shows high wavenumber (2500-4000 cm^{-1}) Raman spectra of SC (upper), extracted SC (containing no lipids, mainly protein; middle), and extracted SC lipids (lower), respectively. The spectrum of SC exhibits peaks at 2850 cm^{-1}, 2880 cm^{-1} and 2930 cm^{-1}. The spectrum of the extracted lipids contains two characteristic peaks located at 2850 cm^{-1} and 2880 cm^{-1}. In contrast, the extracted SC spectrum consists of only one peak located at 2930 cm^{-1}. Figure 3b shows a typical example of a high wavenumber Raman spectrum of a control subject at a skin depth of 4 µm. The peaks that were used to calculate the lipid/protein ratio are shaded in red.

Figure 4a shows the lipid/protein ratio as a function of depth (between 0 and 40 µm), with a maximum between 0 and 4 µm and a gradual decrease deeper than 8 µm. Figure 4b shows the average lipid/protein ratio between 4 and 10 µm in the SC in control SC, non-lesional AE SC, and lesional AE SC. Non-lesional skin of AE patients shows a significantly lower lipid/protein ratio than the skin of control subjects (0.24 ± 0.05 and 0.28 ± 0.03, respectively, $P<0.005$). In lesional skin, the lipid/protein ratio is even further reduced (0.19 ± 0.03, $P<0.001$).

Figure 4c shows the intra-subject comparison between lesional and non-lesional skin from the same patient. In 9 out of 11 AE patients, the lipid/protein ratio is lower in lesional skin compared to non-lesional skin, which was significant ($P<0.01$).

The lipid/protein ratio in AE patients showed no difference between carriers and non-carriers of *FLG* mutations (0.24 ± 0.04 in non-carriers and 0.23 ± 0.06 in carriers $P>0.1$ for non-lesional AE SC; and 0.19 ± 0.02 in non-carriers and 0.18 ± 0.04 in carriers, $P>0.1$ for lesional AE SC).

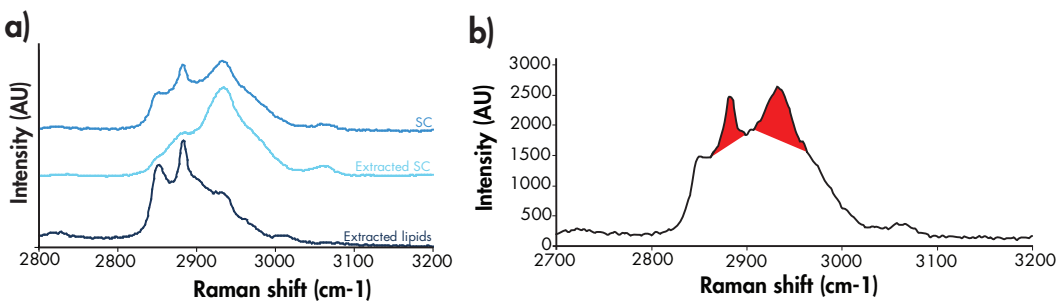

Figure 3: ***a****) High wavenumber Raman spectrum of SC (upper), extracted sc (middle) and extracted SC lipids (lower).* ***b****) Raman spectrum between 2500-4000 cm^{-1} of control SC at a depth of 4 µm. Peaks at 2880 cm^{-1} and 2930 cm^{-1} (both colored red) belong to the lipids and proteins, respectively.*

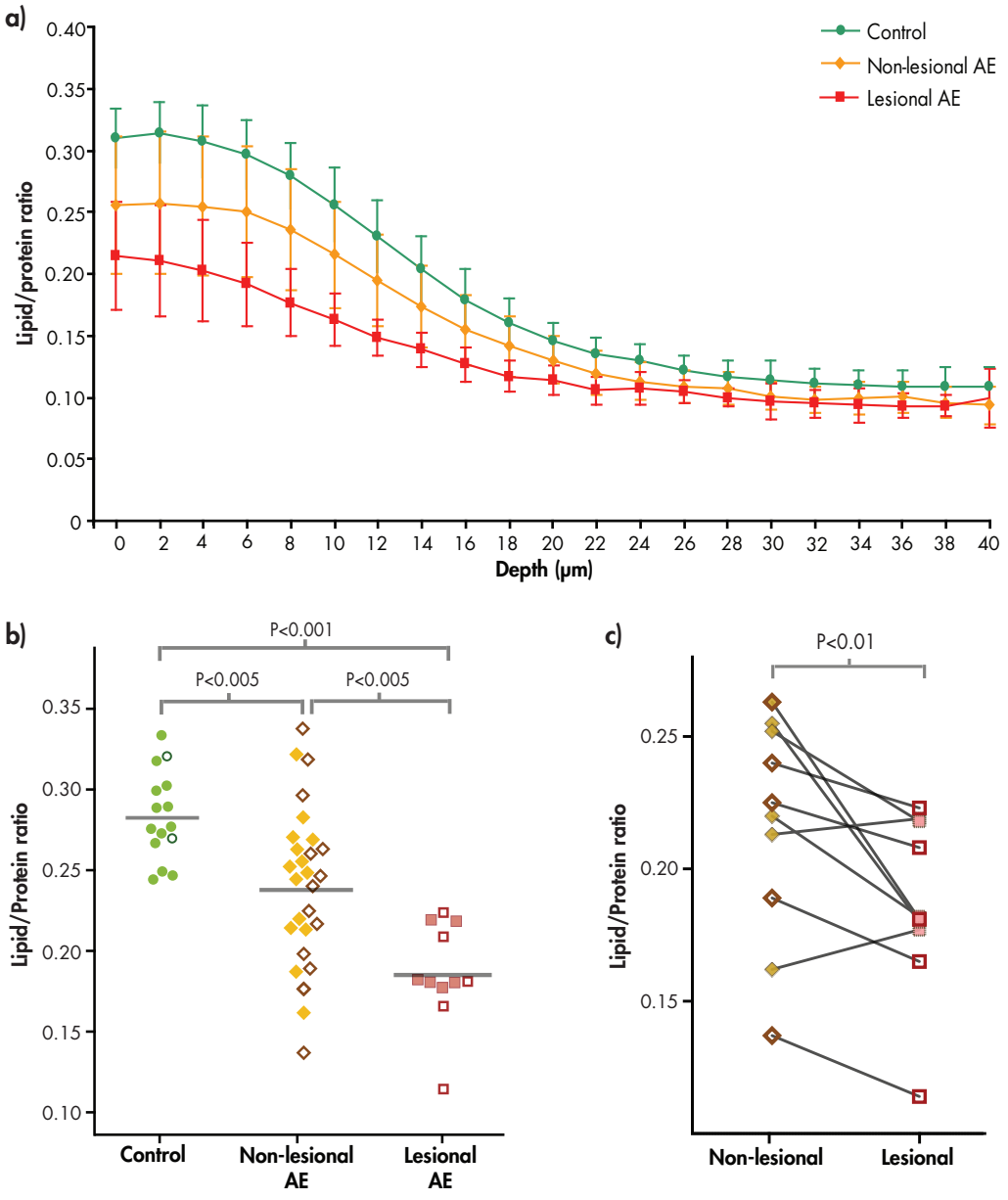

Figure 4: *Lipid/protein ratio in control subjects and AE patients.* ***a****) Lipid/protein ratio in the range of 0-40 µm. The green, orange and red lines indicate the average (±SD) of control SC, non-lesional AE SC and lesional AE SC, respectively.* ***b****) Average lipid/protein ratio between 4-10 µm in the SC of the individual patients and controls.* ***c****) Paired lipid/protein data of non-lesional and lesional AE skin. Non-carriers and carriers of a FLG mutation are represented by filled and open data points, respectively. Control subjects are indicated by ●/○. Non-lesional skin and lesional skin of AE patients are indicated by ◆/◇ and ■/□, respectively.*

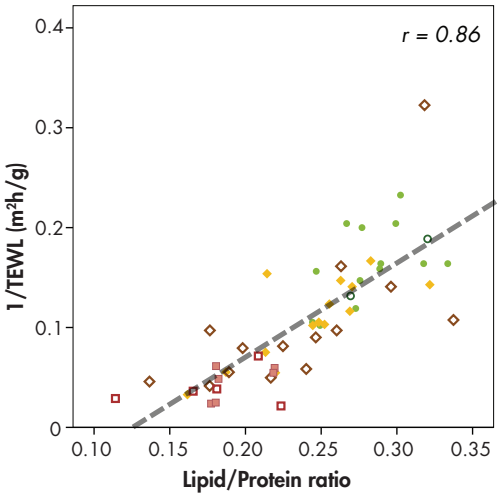

Figure 5: *Correlation between lipid/protein ratio and 1/TEWL in control subjects and AE patients. Non-carriers and carriers of a FLG mutation are represented by filled and open data points, respectively.Control subjects are indicated by ●/○. Non-lesional skin and lesional skin of AE patients are indicated by ◆/◇ and ■/□, respectively.*

The relationship between skin barrier properties and lipid/protein ratios is presented in Figure 5, showing the correlation between 1/TEWL and the lipid/protein ratio in control subjects and in non-lesional and lesional SC of AE patients. The correlation coefficient between 1/TEWL and lipid/protein ratio was 0.86 ($P<0.001$).

Discussion

In this study, we determined the lipid/protein ratio in SC of control subjects and in non-lesional as well as lesional SC of AE patients. In addition, the dry SC mass per surface area was determined in control subjects and in non-lesional skin of AE patients. Finally, we studied the correlations of these parameters with the skin barrier function as assessed by TEWL.

The Raman spectra of extracted SC lipids and extracted SC demonstrate that the ratio between the peaks located at 2880 cm^{-1} and 2930 cm^{-1} is an excellent measure for the lipid/protein ratio in SC: the contribution of the lipids to the 2930 cm^{-1} peak as well as the contribution of the proteins to the 2880 cm^{-1} peak is negligible. The selected peak boundaries of the 2930 cm^{-1} peak were based on those used for the protein band for determination of the water concentration in the skin[20]. A change of the baseline by broadening the peak boundaries did not have an effect on the lipid/protein ratio (not shown).

We observed that the lipid/protein ratio is significantly lower in non-lesional as well as lesional SC of AE patients, compared to SC of control subjects. This may be partly explained by changes in the SC lipid composition: previous studies have shown that the lipid composition is altered in AE patients as compared to controls[5,14-19,25]. Some studies

revealed that the level of long-chain CERs is reduced in non-lesional AE skin and the level of very short chain CERs (with a total chain length of 34 carbon atoms, referred to as C34 CERs) is increased[16,19]. However, the level of C34 CERs and long-chain CERs in non-lesional AE skin are together accounting for about 10 mol% of the total CER content in non-lesional skin[19]. Assuming an equimolar CER:CHOL:FFA ratio, this is only 3 mol% of the total lipids, whereas the lipid/protein level has decreased by ~14% in non-lesional AE and ~32% in lesional AE. Hence the changes in C34 CERs, long-chain CERs and total CER level may not account for the complete reduction in lipid/protein ratio, which indicates a third, yet unknown factor. The reduced lipid/protein ratio may be partly explained by a delayed or incomplete lamellar body extrusion process caused by an altered peroxisome proliferator-activated receptor (PPAR) activation, which has been reported for AE[26-28]. PPARs can be activated by lipids, and these nuclear hormone receptors may possibly be a link for the observed changes in both the lipid chain length and lipid/keratin ratio[29,30].

We could perform an intra-subject comparison of non-lesional and lesional skin as eleven subjects had both skin types on their ventral forearms. In most cases the lipid/protein ratio was lower in lesional compared to non-lesional skin. These differences within a subject may primarily be attributed to inflammation, the presence of microbes and/or an altered pH. These factors may affect the lipid synthesis[31,32] and therefore result in a lower lipid/protein ratio in lesional AE skin.

A measure of SC thickness is the total dry SC mass per surface area, which was determined by extrapolation of 1/TEWL versus the cumulative mass of tape-stripped SC. Between non-lesional AE skin and control skin no significant difference in the dry SC mass per surface area was observed, which indicates that SC thickness was comparable for both groups. In lesional AE SC the total dry SC mass per surface area was lower compared to controls. In line with our results, Voegeli *et al*.[33] have reported a decreased SC thickness in lesional AE SC. They suggested increased serine protease activity in lesional SC of AE patients as a possible explanation for the observed differences. We noticed that SC thickness does affect the TEWL values and thus the skin barrier function, as observed by the correlation between the total dry SC mass per surface area and TEWL.

An alternative method to determine SC thickness is based on the water concentration profile in the SC measured by confocal Raman spectroscopy[34-37]. The advantage of Raman spectroscopy is that it enables the direct monitoring *in vivo* without damaging the SC. The method determines the actual SC thickness, which includes the effects of swelling of the SC as a result of water uptake. In this study, we were interested in variations in the SC thickness as a result of changes in dry SC mass per surface area, independent of variations in water content of the SC. As the total amount of tape-stripped dry SC mass

and subsequent extrapolation does not change upon swelling, we used this method to determine the dry SC mass per surface area. In order to obtain a calibration curve we extracted small pieces of SC. During extraction of the SC, it is possible that not all proteins were extracted. Therefore, the amount of SC per tape-strip may be slightly overestimated. Both SC thickness and lipid/protein ratio are factors that affect the TEWL. However, the correlation between lipid/protein ratio and TEWL was much stronger than the correlation between dry SC mass per surface area (as a measure of SC thickness) and TEWL. This may suggest that the level of lipids in SC plays a more prominent role in the skin barrier function than the dry SC mass. In the same line, our previous study shows a strong correlation between CER chain length and skin barrier function[19].

FLG mutations are the largest risk factor known to date for the development of AE[7,38]. However, in this study the presence of *FLG* mutations was not associated with lipid/protein ratio or dry SC mass per surface area in AE patients. In our previous study it was concluded that *FLG* mutations do not influence CER composition and lipid organization[19]. Although *FLG* mutations are a strong risk factor in the pathogenesis of AE, the results of this study indicate that in adult AE, changes in the SC lipids seem to be the dominant factor in the reduced skin barrier.

Acknowledgements

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