CHAPTER 7

THE ESSENCE OF FREE FATTY ACIDS AND LIPID CHAIN LENGTH FOR THE SKIN BARRIER FUNCTION IN PATIENTS WITH ATOPIC ECZEMA

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Abstract

One of the features of atopic eczema (AE) is a decreased skin barrier function. The stratum corneum (SC) lipids – comprised of ceramides (CERs), free fatty acids (FFAs), and cholesterol – form a highly ordered lipid organization that fulfill a predominant role in this barrier function. Recently, the detailed CER composition in non-lesional skin has been investigated in AE patients. In the present study, we report for the first time *i)* the extracellular SC FFA composition, including chain length and degree of unsaturation, in both lesional and non-lesional skin; and *ii)* how these factors associate with the examined CER composition and the impaired skin barrier in AE.

The results show a clearly distinguishable lipid profile between control skin, non-lesional and lesional AE skin. In particular the FFA chain lengths are clearly reduced in AE SC, and the changes are much more pronounced in lesional skin than in non-lesional skin. The CER composition and lipid organization were also examined in lesional skin and compared to data on non-lesional skin, previously reported. We noticed a strong reduction in CER chain length and a reduced density in the lipid organization. Changes in the chain length distribution of FFAs and CERs were strongly associated. In addition, we encountered a strong association between a reduction in chain length of both lipid classes and the increased presence of a less dense lipid organization. The changes in lipid organization did also associate with a reduced skin barrier function and were more pronounced in lesional skin.

These changes in composition, organization and barrier function were strongly observed in lesional skin, but already present in non-lesional skin, and demonstrate the importance of the FFAs and the

lipid chain length for a proper lipid organization and skin barrier function.

Introduction

Atopic eczema (AE) is a very common skin disease with a current prevalence of 10-15% in developed countries[1-5]. AE has multiple manifestations, such as erythema, skin dryness, and pruritic skin lesions. The etiology of AE is complex: it is an multifactorial disease in which genetics, environmental factors, and an immune dysregulation all interplay. Although the immunological dysregulation is characteristic for AE patients, the skin barrier dysfunction also plays an important role[6-10]. A reduced skin barrier may result in an increased penetration of allergens and irritants, thereby provoking an immune response[11]. A commonly used parameter to indicate a (reduced) skin barrier function is transepidermal water loss (TEWL). Patients show increased TEWL values at both lesional and non-lesional sites, indicating that the skin barrier is affected in both inflamed and non-inflamed regions[8,12,13].

In 2006 it was reported – and since then replicated in many studies – that loss-of-function mutations in the filaggrin gene (*FLG*) are a major predisposing factor for developing AE[14-17]. Filaggrin is a well-known skin barrier protein responsible for proper keratin filament alignment[18]. In addition, filaggrin degradation products – i.e. the natural moisturizing factor (NMF) – are important to retain the moist and acidic nature of the stratum corneum (SC), which is the uppermost layer of the skin where the main barrier of the skin is located[19]. However, the reduced skin barrier in AE cannot fully be explained by *FLG* mutations, as i) 50-70% of all AE patients are non-carriers of such a mutation[19] and *ii)* in a number of studies no relation was observed between *FLG* mutations and the impaired skin barrier in AE[20-23].

Because the lipids play an important role in the skin barrier – also with respect to AE[7,24-27] – the aim of this study is to determine the lipid chain length distribution and in particular free fatty acid composition in AE, and their relation with the skin barrier function. The SC consists of corneocytes with a lipid matrix in the intercellular regions. The lipids are composed of an approximately equimolar ratio of cholesterol, free fatty acids (FFAs) and ceramides (CERs, molecular architecture is explained in Figure 1)[28-31]. These lipids are organized in regularly stacked lipid layers[24,25,32,33]. Within these layers, the lipids form a very dense, highly ordered packing – the so-called orthorhombic organization – but a subpopulation is also present in a less dense – hexagonal – or even liquid organization (Figure 2)[34-36]. In a recent study we examined the CER composition and lipid organization in non-lesional skin of AE patients, from which it was concluded that a reduction in the average chain length of SC CERs coincided with an increase in

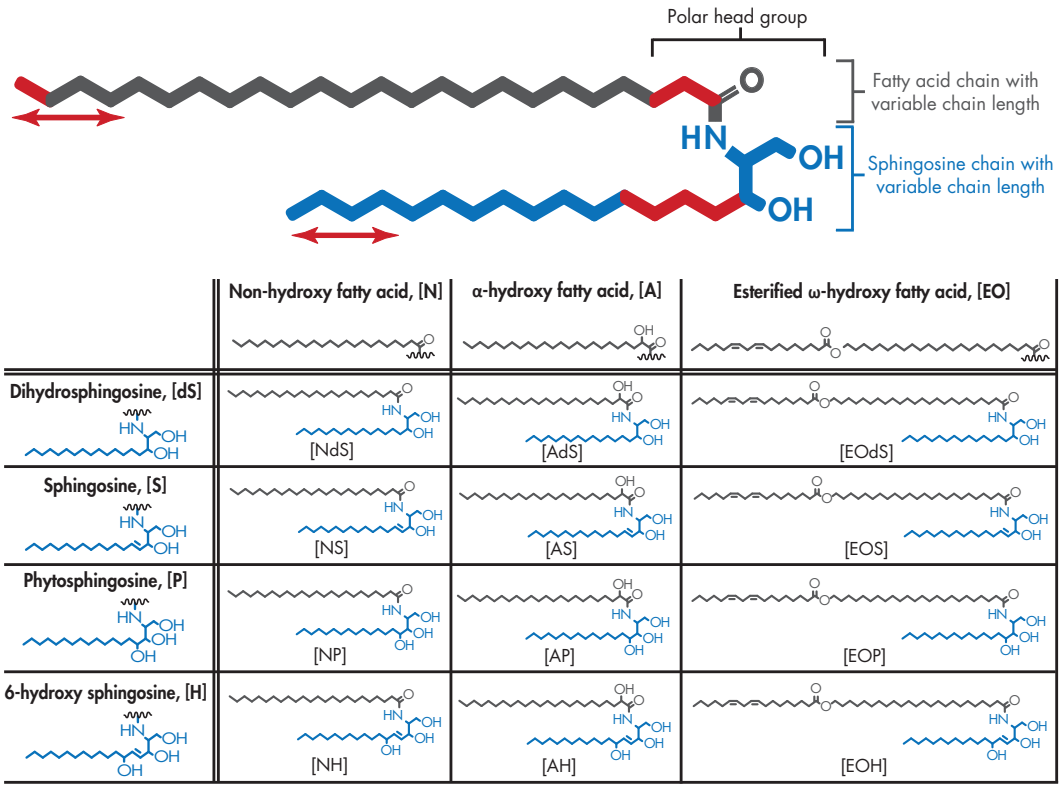

Figure 1: *Explanation of CER subclasses and CER chain length. CERs are composed of a fatty acid chain linked to a sphingosine base. Both chains show a wide distribution in their carbon chains length (indicated by the arrows, the numbers represent the general distribution). This results in a wide range of the total carbon chain length of CERs, that is, the carbon atoms of the two chains combined. In addition, CERs can have an additional functional group at the carbon positions labeled in red, which results in the presence of 12 subclasses, denoted as: [NdS], [AdS], [EOdS], [NS], [AS], [EOS], [NP], [AP], [EOP], [NH], [AH], [EOH].*

the hexagonal organization. These changes in CER composition and lipid organization strongly correlated to the degree of skin barrier dysfunction, suggesting that CER chain length may be a key factor for a proper lipid organization and impaired skin barrier function in non-lesional AE skin[37].

Building on these recent findings in non-lesional skin, this study presents for the first time a detailed analysis of the FFAs chain length distribution in lesional as well as in non-lesional AE skin. We focused on the chain length distribution as well as on the presence of mono-unsaturated FFAs (MUFAs) and the very sparsely studied hydroxy-FFAs. Previously, a wide chain length distribution has been observed in healthy subjects ranging from mainly 16 to 36 carbon atoms[38-40]. To the best of our knowledge, only one study reports on the FFA chain length in AE patients, mentioning the very long chain fatty acids only.

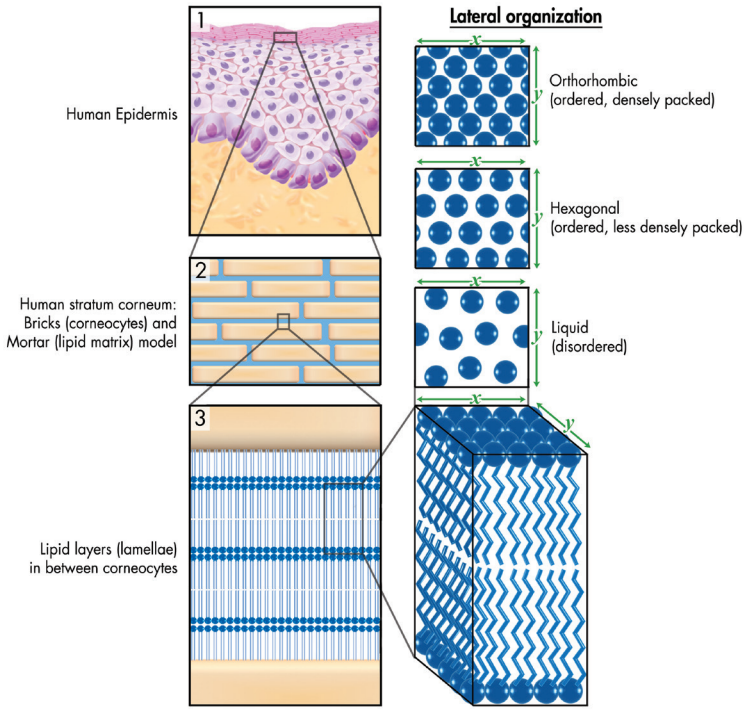

Figure 2: *Lateral organization in human stratum corneum. (**1**) The outermost layer of the epidermis, the stratum corneum (SC), consists of dead cells (corneocytes) embedded in a lipid matrix, also referred to as the brick (corneocytes) and mortar (lipids) structure (**2**). The intercellular lipids are arranged in layers (lamellae). The lateral organization 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.*

They observed a decreased presence of FFAs longer than 24 carbon atoms[41]. Based on our detailed FFA analysis, we studied the association of changes in chain length distribution of the FFAs with the chain length distribution of the CERs, as literature reports a common synthetic pathway for both lipid classes[42,43]. It was found that the average FFA chain length in AE patients is shortened, which is in conjunction with a reduced CER chain length. In addition, we examined possible correlations between changes in chain length profiles of the FFAs (and CERs) versus *i)* a modulation in lipid organization, *ii)* a reduction in skin barrier function monitored by TEWL and *iii) FLG* mutations.

Materials and Methods

General study setup

The study is in accordance to declaration of Helsinki and consists of 15 Caucasian control subjects (25.0±5.2 years; 5 males) and 28 Caucasian AE patients (25.6±5.6 years; 11 males), of which 11 did show lesional skin sites at the day in which all measurements were performed. Patients were screened by the four most prevalent mutations found in the European population (*2282del4, R501X, S3247X* and *R2447X*): 14 patients and 2 control subjects appeared as carriers for a *FLG* mutation. No dermatologic products were

applied on the forearms of any of the subjects at least one week prior to the study. Various techniques to study the lipid composition and organization are described in more detail below. All measurements were performed on both non-lesional skin and lesional skin (if present) of the ventral forearms. A dermatologist marked an area of 4.5 cm^2 on the ventral forearm of the control subjects and AE patients. On this area, the TEWL was measured using an open chamber Tewameter TM 210 (Courage+Khazaka, Köln, Germany) device. Values were recorded for a period of two minutes after which an average reading during the last 10 seconds of the measurement was calculated. This procedure was performed before tape stripping (baseline TEWL) and after each two tape strips.

SC lipids were obtained by tape stripping. In between each 2 tape strips, FTIR measurements were performed to study the lipid organization. In order to determine *FLG* mutations, buccal mucosa cells were collected by rubbing the inside of the cheeks with a cotton swab on a plastic stick after rinsing the mouth with water. Mutations were determined by genotyping after DNA extraction[15].

Tape stripping procedure and LC/MS analysis

SC lipids were harvested using multiple poly(phenylene sulfide) tape strips (Nichiban, Tokyo, Japan). Successive tapes were pressed on the marked area using a pressure device, and were removed in one fluent stroke using tweezers. To avoid contamination of surface lipids (e.g. sebum), tape strips 1-5 were discarded and only tapes 6 to 9 were used for lipid analysis of non-lesional skin and – if possible – from lesional skin. The tapes were punched to an area of 2 cm^2 and stored in chloroform/methanol/water (1:2:½) at a dark, cold (-20°C), and dry environment, under argon atmosphere. Afterwards, lipid extraction of all samples was performed using an adapted procedure of the Bligh and Dyer method described by Thakoersing *et al.*[44] Lipids were reconstituted in chloroform/methanol/heptane (2½:2½:95) and ready for analysis by LC/MS using a recently developed method for analysis of both CERs and FFAs[45,46] (van Smeden, unpublished data). Figure 1 explains the nomenclature (according to Motta *et al.*[47]) and variation in chemical structure for these CER subclasses.

Prior to the analysis of the FFA composition, a particular problem for the quantification of FFA C16:0, C17:0, C18:0 and C18:1 was encountered. These endogenous lipids are also present in the tape-strips used to harvest the SC, thereby interfering with the analysis of SC FFAs (van Smeden, unpublished data). However, it was possible to correct for the amount of these lipids on the tape. This procedure is explained in detail in the supplement (Supplementary Figure 1 and Supplementary Table I).

Lateral lipid organization by FTIR and its principles

Fourier transform infrared spectroscopy (FTIR) spectra were recorded after each two tape-strips in order to obtain information on the lateral organization and conformational ordering of the lipids. A Varian 670-IR spectrometer (Varian Inc., Santa Clara, CA) equipped with a broad band mercury-cadmium-telluride (MCT) detector and an external sample compartment containing a GladiATR (Pike, Madison, WI) attenuated total reflection (ATR) accessory with a single reflection diamond was used. The spectral resolution was 2 cm^{-1}. The instrument was continuously purged with dry N_2. Each spectrum was an average of 150 scans. For data treatment, the instrument software Resolutions Pro 4.1 (Varian Inc.) was used. Positions of the CH_2 symmetric stretching vibrations and scissoring bandwidth were calculated as described previously[48,49]. Shortly, the second derivative was calculated and it was baseline-corrected between the endpoints of the scissoring region (~1460-1480 cm^{-1}). We calculated the bandwidth at 50% of the peak height (full width half maximum, FWHM) and determined CH_2 symmetric stretching vibration positions of spectra recorded between the removal of 2 to 10 tape strips. The width of the peak is indicative for the size and amount of orthorhombic lipid domains[50]. The stretching peak position indicates whether lipids are present in either a highly ordered organization (low peak position at ~2848 cm^{-1}) or a disordered/liquid phase (high peak position ~2853 cm^{-1}).

Data analysis

All statistical outcomes were determined using SPSS Statistics 17.0. In general, data was not Gaussian distributed and therefore non-parametric Mann-Whitney tests are more appropriate when 2 groups were compared, and Spearman's ρ correlation coefficients are shown. Jonckheere trend tests were performed to assess significance of trends between control, non-lesional and lesional SC. Differences were considered significant when $P<0.05$. For some subjects in some of the parameters, data could not be determined and therefore missing. These data points were not replaced but are left blank. For lesional skin it was in a few cases not possible to obtain 4 tape strips. In that case we used merely 2 or 3 tapes. This is one of the reasons why we do not report absolute data on the lipid composition, but rather relative values. Another important reason for presenting relative data is because the lipid composition is compared to the lipid organization. The latter is depending on the relative amounts of the former, rather than absolute amounts. We therefore chose for presenting relative data.

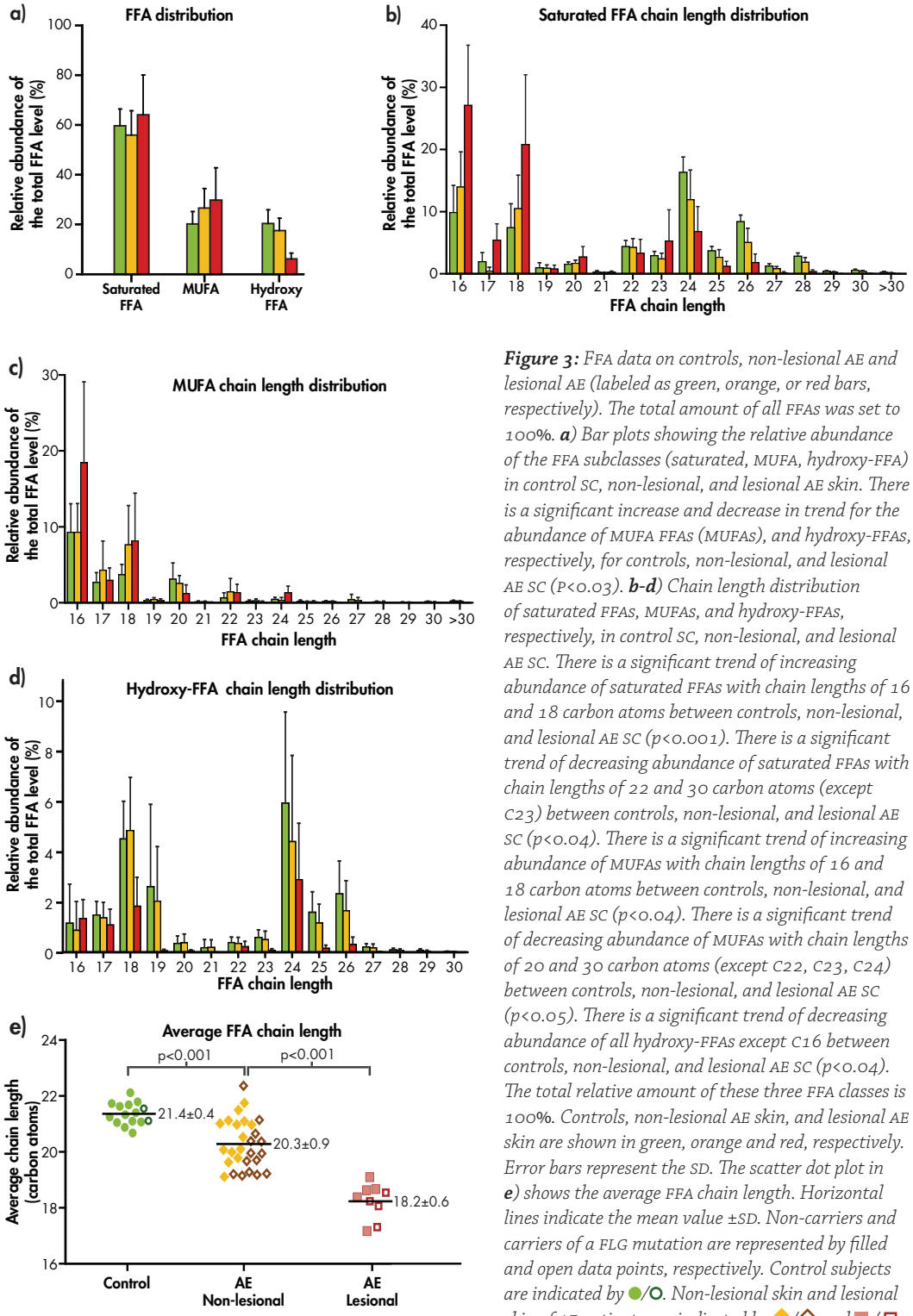

Figure 3: *FFA data on controls, non-lesional AE and lesional AE (labeled as green, orange, or red bars, respectively). The total amount of all FFAs was set to 100%.* ***a****) Bar plots showing the relative abundance of the FFA subclasses (saturated, MUFA, hydroxy-FFA) in control SC, non-lesional, and lesional AE skin. There is a significant increase and decrease in trend for the abundance of MUFA FFAs (MUFAs), and hydroxy-FFAs, respectively, for controls, non-lesional, and lesional AE SC (P<0.03).* ***b-d****) Chain length distribution of saturated FFAs, MUFAs, and hydroxy-FFAs, respectively, in control SC, non-lesional, and lesional AE SC. There is a significant trend of increasing abundance of saturated FFAs with chain lengths of 16 and 18 carbon atoms between controls, non-lesional, and lesional AE SC (p<0.001). There is a significant trend of decreasing abundance of saturated FFAs with chain lengths of 22 and 30 carbon atoms (except C23) between controls, non-lesional, and lesional AE SC (p<0.04). There is a significant trend of increasing abundance of MUFAs with chain lengths of 16 and 18 carbon atoms between controls, non-lesional, and lesional AE SC (p<0.04). There is a significant trend of decreasing abundance of MUFAs with chain lengths of 20 and 30 carbon atoms (except C22, C23, C24) between controls, non-lesional, and lesional AE SC (p<0.05). There is a significant trend of decreasing abundance of all hydroxy-FFAs except C16 between controls, non-lesional, and lesional AE SC (p<0.04). The total relative amount of these three FFA classes is 100%. Controls, non-lesional AE skin, and lesional AE skin are shown in green, orange and red, respectively. Error bars represent the SD. The scatter dot plot in* ***e****) shows the average FFA chain length. Horizontal lines indicate the mean value ±SD. 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 ■/□,*

Results

Altered FFA composition in SC of AE patients

AE patients showed – in both lesional and non-lesional skin – an increase in MUFAs at the expense of hydroxy-FFAs (Figure 3a), whereas the total level of saturated (non-hydroxy) FFAs was unchanged. In lesional skin, changes in levels of the various FFA subclasses are more pronounced than in non-lesional skin. For the saturated FFAs, the chain length distribution is notably changed: very long chain FFAs (≥24 carbon atoms) were strongly reduced whereas shorter FFAs – in particular C16:0 and C18:0 – were increased (Figure 3b). These changes in FFA chain length distribution were more pronounced in lesional SC, but already of significance in non-lesional skin. The MUFAs showed higher levels of short FFAs C16:1 and C18:1 in non-lesional and lesional skin compared to the control (Figure 3c). Hydroxy-FFAs ≥18 carbon atoms were decreased, predominantly in lesional skin (Figure 3d). All these changes resulted in a significantly decrease in average FFA chain length in non-lesional as well as lesional AE skin compared to controls (20.3±0.9, 18.2±0.6 and 21.4±0.4 carbon atoms, respectively, $P<0.001$, Figure 3e). FFA profiles of AE patients from both lesional and non-lesional skin were compared per subject (pairing), and the

Parameters	Control		
	Noncarriers (n=13)	*FLG* mutation (n=2)	P-value
TEWL	6.5±1.7	6.5±1.6	0.933
FTIR Scissoring Bandwidth	11.6±0.9	11.4±0.2	0.410
FTIR Stretching peak position	2848.8±0.2	2848.6±0.1	0.305
NMF levels	1.064±0.217	0.98±0.042	0.800
Average CER chain length	47.0±0.7	47.4±1.0	0.549
Average FFA chain length	22.6±0.9	22.6±0.3	0.933
Abundance of MUFAs	0.129±0.081	0.123±0.055	0.933
Acyl-CERs total	9.3±2.6	11.6±2.6	0.361
C34 CERs total	0.87±0.16	0.90±0.76	>0.99

Table I: *Influence of loss-of-function FLG mutations on several parameters related to lipid parameters or the skin barrier function.*

Parameters	AE - Non-lesional			AE - Lesional		
	Noncarriers (n=14)	*FLG* mutation (n=14)	P-value	Noncarriers (n=6)	*FLG* mutation (n=5)	P-value
TEWL	11.4±6.8	13.1±6.2	0.246	25.7±12.1	29.8±12.0	0.662
FTIR Scissoring Bandwidth	11.1±1.1	10.1±1.4	0.089	10.5±0.8	7.8±1.8	**0.016**
FTIR Stretching peak position	2849.0±0.5	2849.3±0.4	0.052	2849.2±0.3	2849.8±0.6	0.151
NMF levels	0.875±0.431	0.451±0.195	**0.008**	0.454±0.18	0.37±0.168	0.662
Average CER chain length	46.5±0.8	46.3±0.6	0.603	45.0±2.1	43.4±2.4	0.413
Average FFA chain length	21.2±1.1	21.2±1.7	0.701	19.2±1.7	19.3±2.2	0.841
Abundance of MUFAs	0.184±0.091	0.203±0.081	0.329	0.205±0.087	0.225±0.144	0.841
Acyl-CERs total	8.3±2.9	8.0±2.0	0.713	4.0±0.9	5.8±3.4	0.556
C34 CERs total	1.62±0.95	2.04±0.68	0.066	6.38±2.55	7.11±1.82	>0.99

average FFA chain length was always lower in lesional skin compared to non-lesional skin (Supplementary Figure 2). No effects were observed of *FLG* mutation status on the FFA composition, neither in the FFA chain length, nor in the abundance of hydroxy-FFAs or MUFAs ($P>>0.1$, Table I).

Altered CER chain length in SC of AE patients

We compared changes in the FFA composition of AE patients in both lesional and non-lesional sites to changes in CER composition. Of all CER subclasses, the overall CER chain length distribution was determined in non-lesional and lesional skin (Figure 4a). Chain lengths of all CER subclasses showed a broad distribution mainly between 32 and 54 carbon atoms. The only exceptions were the acyl-CERs, which showed a range between 64 and 74 carbon atoms. This is mainly due to the additional fatty acid moiety attached to the CER. In AE patients, the level of long chain CERs (>42 carbon atoms) was reduced and the level of short chain CERs (<42 carbon atoms) was increased. Differences are most pronounced for lesional skin, but were also significantly altered in non-lesional skin. Among the short CERs, in particular the CERs with 34 carbon atoms (C34 CERs) are increased in AE (Figure 4b). To obtain more information on which CER subclasses were involved in this strong increase in C34 CERs, we also analyzed the level of all CER subclasses. The relative abundance of the 12 individual CER subclasses (see Figure 1) in lesional skin were combined with those in non-lesional skin previously reported[37]. The most prominent changes in lesional skin are an increase in CER [NS] and CER [AS], and a decrease in CER [NP] (Supplementary Figure 3). The CER chain length distribution shows a progressive increase in C34 CERs, predominantly in the CER [NS] and CER [AS] subclasses, and a reduction of almost 50% was observed for the long chain acyl-CERs in lesional AE SC (see Figures 4b and 4c). Together, this results in a significant reduction of the average CER chain length in both in non-lesional and lesional AE SC, compared to controls ($P<0.012$, Figure 4d).

A direct intra-subject comparison (paired statistics) between lesional and non-lesional skin from the same patient showed a lower average CER chain length in lesional skin as compared to the non-lesional skin sites (Supplementary Figure 2). Since we also observed this trend for the FFAs, we calculated the total lipid chain length and compared lesional skin with non-lesional skin sites (Figure 5). From these paired observations we noticed for each individual a reduction in total lipid chain length at lesional skin sites compared to non-lesional skin sites.

No indications were found that *FLG* mutations have an effect on the lipid composition in AE patients (Table I). No significant changes were observed between subjects with and

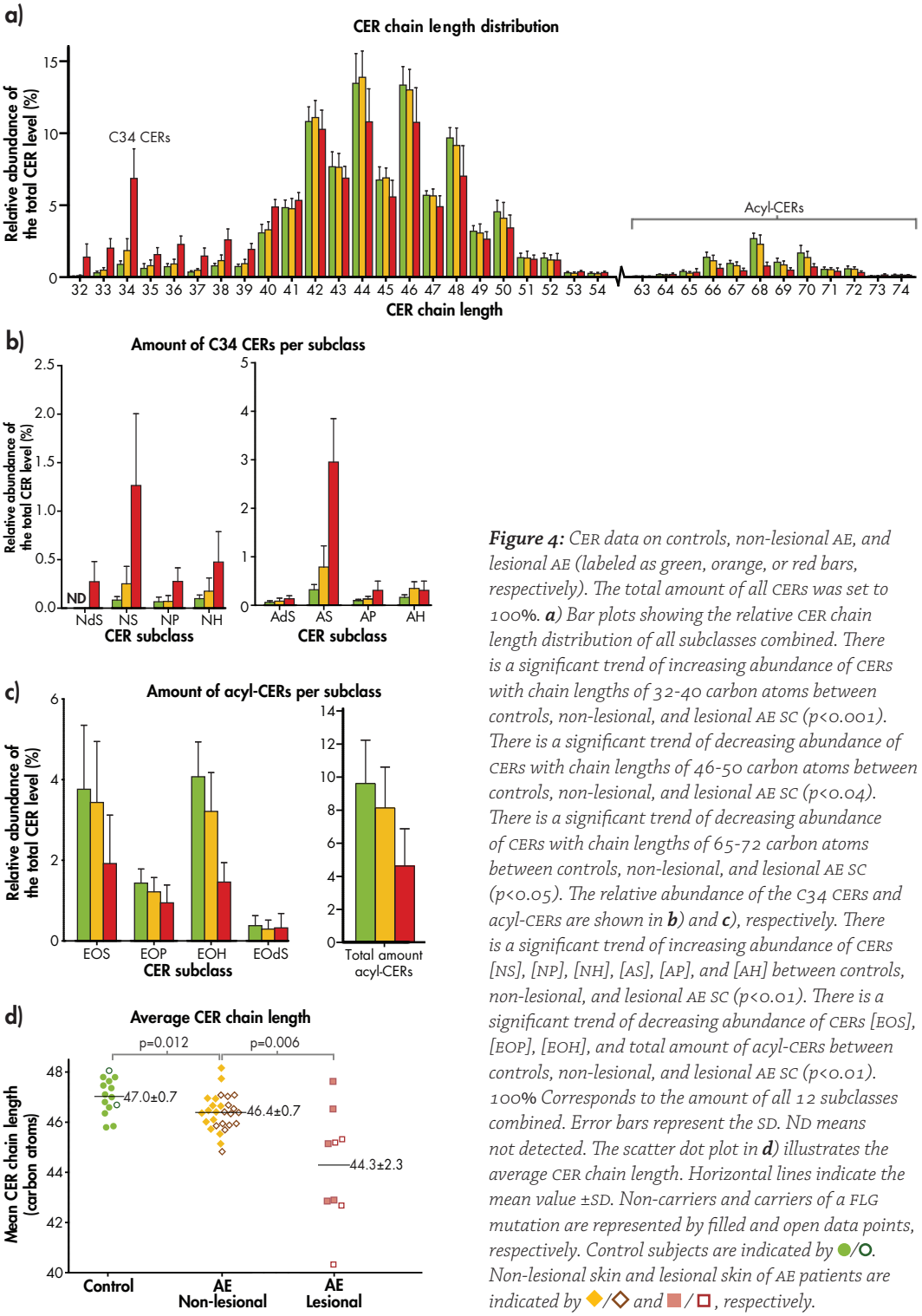

Figure 4: *CER data on controls, non-lesional AE, and lesional AE (labeled as green, orange, or red bars, respectively). The total amount of all CERs was set to 100%.* ***a****) Bar plots showing the relative CER chain length distribution of all subclasses combined. There is a significant trend of increasing abundance of CERs with chain lengths of 32-40 carbon atoms between controls, non-lesional, and lesional AE SC ($p<0.001$). There is a significant trend of decreasing abundance of CERs with chain lengths of 46-50 carbon atoms between controls, non-lesional, and lesional AE SC ($p<0.04$). There is a significant trend of decreasing abundance of CERs with chain lengths of 65-72 carbon atoms between controls, non-lesional, and lesional AE SC ($p<0.05$). The relative abundance of the C34 CERs and acyl-CERs are shown in* ***b****) and* ***c****), respectively. There is a significant trend of increasing abundance of CERs [NS], [NP], [NH], [AS], [AP], and [AH] between controls, non-lesional, and lesional AE SC ($p<0.01$). There is a significant trend of decreasing abundance of CERs [EOS], [EOP], [EOH], and total amount of acyl-CERs between controls, non-lesional, and lesional AE SC ($p<0.01$). 100% Corresponds to the amount of all 12 subclasses combined. Error bars represent the SD. ND means not detected. The scatter dot plot in* ***d****) illustrates the average CER chain length. Horizontal lines indicate the mean value ±SD. 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.*

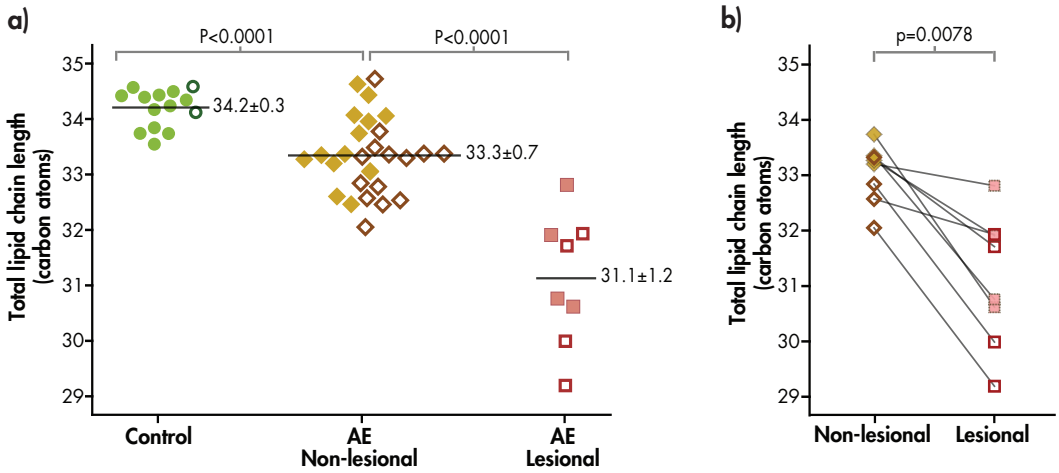

Figure 5: ***a****) Dot plots of the calculated total lipid chain length (FFAs + CERs) for all three groups: control, AE – non-lesional, and AE – lesional. Non-carriers and carriers of a FLG mutation are represented by filled and open data points, respectively. Control subjects are indicated by ●/○. Non-lesional skin and lesional skin of AE patients are indicated by ◆/◇ and ■/□, respectively.* ***b****) Paired plot of the calculated total lipid chain length of a selected subset of patients from which both non-lesional and lesional skin was analyzed.*

without a *FLG* mutation for either lesional and non-lesional skin: neither in the level of acyl-CERs ($P>0.1$), nor in the average CER chain length ($P>0.1$), nor in the total level of C34 CERs ($P>0.1$).

Relationship between FFA and CER composition

As presented in Table II, the average CER chain length correlated to the average FFA chain length ($r=0.60$, $P<0.001$). More precisely, a high abundance of short chain FFAs (<23 carbon atoms) is accompanied by a high abundance of short chain CERs (<42 carbon atoms), whereas an increased presence of long chain FFAs (>23 carbon atoms) correlates to an increase in long chain CERs (>42 carbon atoms), as illustrated by the correlation map in Supplementary Figure 4 and in Supplementary Table II.

Correlated Parameters	Correlation coefficient
Average CER chain length versus average FFA chain length	0.596*
Average CER chain length versus stretching peak position	-0.567*
Average CER chain length versus scissoring bandwidth	0.517*
Average FFA chain length versus stretching peak position	-0.683*
Average FFA chain length versus scissoring bandwidth	0.727*

Table II: *Spearman correlation coefficients of lipid composition and lipid organization parameters.*

Altered lipid composition in AE patients corresponds to a less ordered lipid organization

We investigated whether the observed changes in SC lipid composition in AE affect the lipid organization in these patients. The lateral lipid organization was assessed by attenuated total reflectance (ATR) FTIR spectroscopy, primarily focusing on lesional skin of AE patients. Principles of ATR-FTIR measurements are explained in the materials and methods section. The stretching peak position in AE patients was located at a higher wavenumber compared to control subjects in both non-lesional as well as lesional AE SC ($P<0.001$; Figure 6a). This indicates that the organization of SC lipids in AE patients is less ordered compared to control subjects. The difference in stretching peak position between lesional and non-lesional skin was not significant ($P=0.083$).

To obtain information about the hexagonal or orthorhombic organization of SC lipids, the scissoring bandwidth was determined. Lesional SC of AE patients shows a scissoring bandwidth of 9.1±1.9 cm^{-1}, which is significantly lower than the bandwidth in non-lesional skin of AE patients (10.6±1.3 cm^{-1}) and in control skin (11.6±0.8 cm^{-1}; $P<0.001$; Figure 6b). This indicates that the subpopulations of lipids in a highly ordered orthorhombic phase are less dominantly present in lesional skin in AE compared to non-lesional skin of AE patients and control subjects.

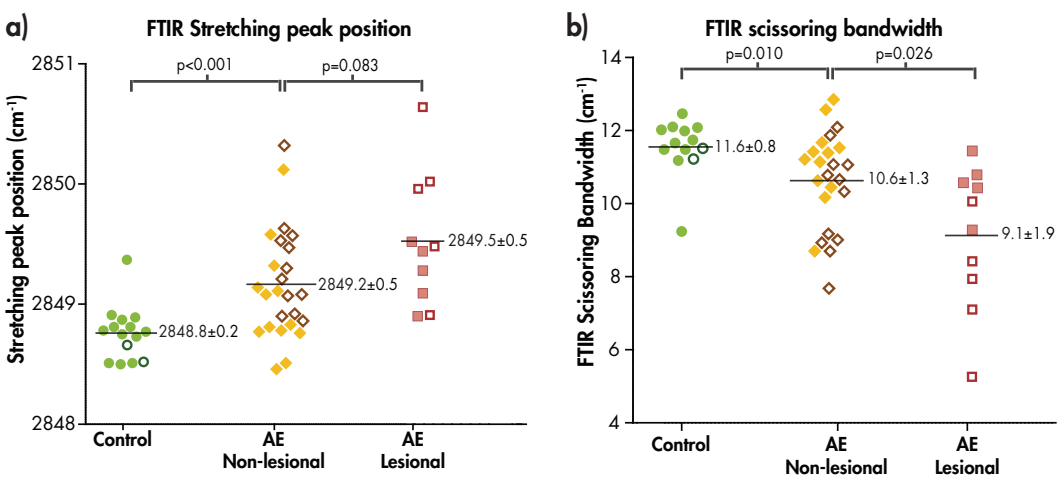

Figure 6: *Scatter dot plots of* ***a****) the FTIR CH_2 stretching peak position and* ***b****) the FTIR CH_2 scissoring bandwidth for all three groups: control, AE – non-lesional, and AE – lesional. Non-carriers and carriers of a FLG mutation are represented by filled and open data points, respectively. Control subjects are indicated by ●/○. Non-lesional skin and lesional skin of AE patients are indicated by ◆/◇ and ■/□, respectively.*

Regarding the influence of *FLG* mutations, a significant difference was observed for the scissoring bandwidth in lesional skin between AE patients with and without a *FLG* mutation: P=0.016. This was not found for the stretching peak position (P>0.05).

Table II shows that the average CER chain lengths correlate to a high extent with the change in lateral lipid ordering ($|r|$>0.51; P<0.001), and the correlation of FFA chain length with the change in lateral lipid ordering is even stronger ($|r|$>0.68; P<0.001). Supplemental Figures 5a and b show this correlation between FFA chain length and FTIR bandwidth in more detail (r=0.73; P<0.001): It becomes clear that FFAs with chain lengths ≤20 relate to a lower FTIR bandwidth (more hexagonal lipid organization) whereas FFAs with chain lengths ≥24 associates with a higher FTIR bandwidth and therefore more orthorhombic lipid organization.

Skin barrier function shows a high affinity with lipid chain length and lipid ordering

Supplemental Figure 6 shows that the TEWL is significantly increased in non-lesional as well as lesional AE SC compared to controls (12.2±6.5 g/m²/h, 27.6±11.6 g/m²/h, 6.5±1.7 g/m²/h, respectively, P<0.0005). Figure 7a shows that the increase in TEWL associates very well with the average lipid chain length: a reduction in average chain length corresponds to an increase in TEWL (r=-0.80, P<<0.001). Short lipid chain FFAs and CERs (<20 and <41 carbon atoms, respectively) correlate positively with TEWL, whereas long lipid chains (>23 and >42 carbon atoms for FFAs and CERs, respectively) are negatively correlated (Figures 7b and c). Also for the lipid organization a high correlation with TEWL was observed for both the scissoring bandwidth and the stretching peak position (Figure 7d and e): a decrease in lipid ordering corresponds to an increase in TEWL.

Discussion

As there is increasing evidence that SC lipids play a role in the impaired barrier function of AE[7,27], we investigated the lipid chain length distribution in SC of AE patients, focusing on FFAs. In addition, we examined the CER composition in lesional skin in these patients. A clear shift to shorter SC lipid chain lengths was observed in AE patients for both CERs and FFAs. Short chain FFAs (≤20 carbon atoms) showed a negative correlation with FTIR bandwidth and long chain FFAs (≥24 carbon atoms) showed a positive correlation with FTIR bandwidth. Previous *in vitro* studies with lipid model systems have shown that the formation of the hexagonal organization is strongly enhanced by a decreased FFA chain length[51]. This leads to the conclusion that the FFA chain length is an important determinant for the lipid organization in AE: short chain FFAs enhance the formation of a hexagonal lipid organization whereas long chain FFAs enhance the formation of an

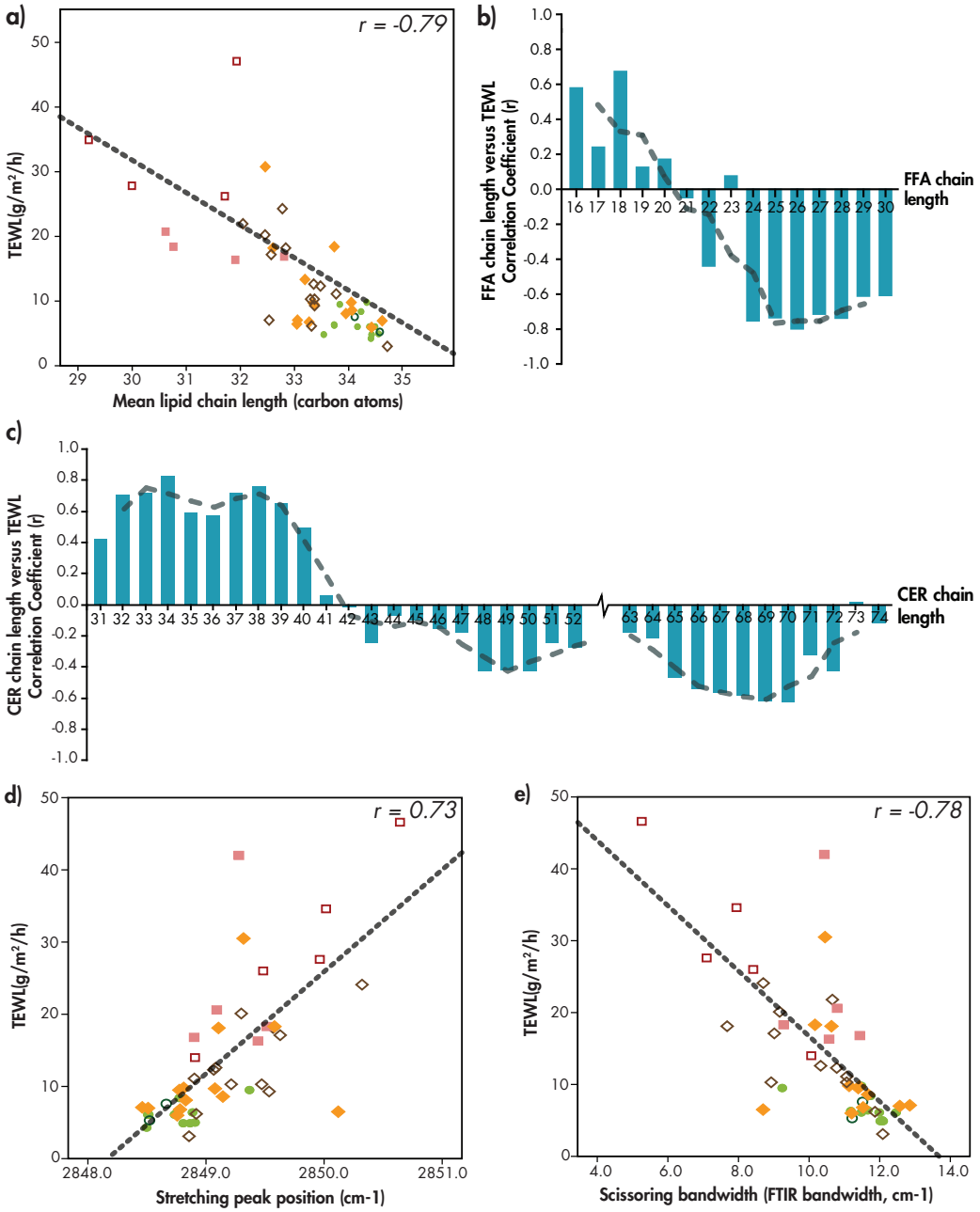

Figure 7: ***a****) Correlation plot in which the lipid chain length of all individual subjects is plotted versus the* TEWL*. The dashed line indicates the optimal fit through the data.* ***b****) and* ***c****) present bar plots in which each individual chain length of respectively* FFAs *and* CERs *is plotted versus their respective spearman correlation coefficients with* TEWL*. The dashed line indicates the moving average (period = 3).* ***d****) and* ***e****) are correlation plots of the* TEWL *vs either the stretching peak position or the scissoring bandwidth, respectively. The dashed lines indicate the optimal fit through the data. 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. Concerning the correlation plots, spearman correlation coefficients are plotted in the upper right corner.*

orthorhombic lipid organization. Furthermore, we observed an increased level of MUFAs in SC of AE patients, which also correlated to the presence of a hexagonal lipid organization in *in vitro* studies (unpublished results).

The reduction in FFA chain length matches the presence of shorter CERs: AE patients showed a shift from long chain CERs (≥43 carbon atoms) towards short chain CERs (≤42 carbon atoms). It has been reported that the sphingosine base in CERs is on average about 18-20 carbon atoms long[52]. Using these numbers in estimating the acyl chain length in the CERs, our results suggest that CERs having an approximate acyl chain length of ≥23 carbon atoms (42 minus 18-20 carbon atoms) are decreased in SC of AE patients. This matches our observations in the decreased levels of FFAs >23 carbon atoms. In addition, the high levels of C34 CERs in AE patients correspond to an acyl chain length similar to the 16 carbon atoms of the FFAs. The level of this particular FFA is also highly elevated in these patients. These correlations between CERs and FFAs demonstrate for the first time that the acyl chain length in CERs correlates with the FFA chain length, which is a strong argument for a common synthetic pathway[42,43].

The reduction in long chain FFAs corresponds to a reduction in long-chain acyl-CER levels in AE. These acyl-CERs are also crucial for a proper organization of the lipids: *in vitro* studies have shown that a reduced level of acyl-CERs results in a less thermostable orthorhombic organization[53]. The decreased amount of acyl-CERs in AE patients could also play an important role in the reduced skin barrier function, as permeability studies have shown that lower levels of acyl-CERs results in increased permeation[54], or could even lead to neonatal death by epidermal dehydration in mice completely lacking acyl-CERs[40]. Therefore, not only the changes in FFA composition, but also reduced levels of acyl-CERs may contribute to the increased hexagonal organization and impaired skin barrier function in AE.

TEWL is a commonly accepted measure of skin barrier integrity. The results demonstrate that the increase in TEWL is strongly associated with the decreased lipid chain length. In other words, an increased abundance of short lipid chains contributes to an increased TEWL in AE, whereas an increased presence of long chain lipids correspond to lower TEWL values. In addition, the increased TEWL values observed in AE patients show a strong association with the altered lipid organization in AE. This raises the question whether the changes in lipid chain length are causative for the changes in lipid organization and increased TEWL. Previous *in vitro* studies by Groen *et al*. with model lipid membranes demonstrate that a shorter FFA chain length leads to an increased permeability of benzoic acid[51].

These findings provide new fundamental insights for possible barrier repair: the

presence of long-chain lipids may be crucial for the lipid organization and therefore a proper skin barrier function. A possible treatment to compensate for the reduced long chain lipids may therefore focus on topical application of long-chain lipids. Our observed relation between the FFAs and CERs also imply that normalization of the FFA chain length distribution in AE skin will also contribute to normalization of the CER chain length distribution in these patients. As discussed below, most probably the elongase enzyme family will play an important role here.

One of the challenges is to unravel the changes in the biochemical synthesis of the lipids that are responsible for the altered SC lipid composition in AE, as this may result in new targets for normalizing the skin barrier function. The shift to a shorter FFA chain length distribution suggests that FFA elongation is hampered to some extent. It is known that elongation of FFAs ≥C16 occurs by a series of elongases, ELOVL 1-7[55], all having a certain selection towards a specific FFA chain length[43]. Currently, little is known about these elongases in the epidermis. It has been reported that ELOVL 1, 3, 4 and 6 are expressed in mammalian skin[56]. Only ELOVL4 is identified in the epidermis on a protein level and plays a crucial role in the elongation of FFAs ≥C24[40,57]. Our data on AE shows a decrease in FFAs ≥C24, from which we hypothesize that the ELOVL4 protein level and/or activity may be decreased in AE patients. Although no information has been reported on humans with respect to AE, two studies in ELOVL4 knockout mice demonstrate a strong reduction in very long chain FFAs, supporting our hypothesis[40,58]. In addition, the loss of functional ELOVL4 in mice has been related to a reduced level of hydroxy-FFAs, in particular long chain hydroxy-FFAs[40]. Interestingly, in our study a reduced level of hydroxy-FFAs is also observed. As we also observed increased levels in FFAs with 16 and 18 carbons, ELOVL6 and ELOVL1 – which are the major enzymes involved in elongation of respectively FFAs>C16 and FFAs>C18 – may also be reduced in activity in the epidermis of patients with AE. Furthermore, if the elongation of these short chain FFAs is hampered, an alternative metabolic pathway may also become more important. This pathway involves the synthesis of MUFAs by stearoyl-CoA desaturases (SCDs)[59]. Indeed an increased level of MUFAs with chain lengths of 16 and 18 carbon atoms is observed in SC of AE patients that may be caused by an increased activity of SCD. Another aspect that should be considered is the importance of the sebaceous lipids in relationship to the SC barrier. Previous studies have reported an altered level of sebaceous lipids in AE, which may affect the SC barrier function in these patients[60-62].

Eleven AE patients had both lesional and non-lesional sites at their ventral forearms. We could therefore perform an intra-subject comparison of non-lesional and lesional skin. The shift to shorter FFA and CER chain lengths was more pronounced in lesional

skin compared to non-lesional skin. These changes in lipid composition can mainly be attributed to inflammation, although increased levels of cutaneous microbes (e.g. *Staphylococcus aureus*), genetic mosaicism[18] or environmental factors could also play a role. Cytokines, which play a role in inflammation, are capable of interfering to a large extent with the lipid synthesis in multiple ways: they have shown to be involved in i) reduced levels of enzymes involved in CER synthesis (e.g. β-glucocerebrosidase and acid sphingomyelinase), thereby affecting the CER composition[63]; ii) peroxisome proliferator-activated receptors (PPARs), which are closely related to the skin lipid metabolic pathway and show a relation to AE[64]: PPAR-α and PPAR-β/δ are respectively down- and upregulated in AE patients[65,66]. These nuclear receptors promote SC barrier formation and are suggested to have anti-inflammatory properties in skin[67-70]; iii) TNF-α and several other cytokines, which downregulate filaggrin expression[71-73]. This may result in lower NMF levels thereby affecting the local pH. It has been suggested that such a pH change may alter the lipid synthesis in the epidermis[74]. This is emphasized by the importance of environmental factors that can also raise the pH of the skin, of which washing the skin with soap and other detergents may be the most important one[75,76].

Downregulation of filaggrin expression may be a factor interfering with *FLG* mutations and lipid parameters. This can explain the absence of a correlation between *FLG* mutations and lipid parameters. This suggests that mutations in the *FLG* genotype have no direct effect on the metabolic skin lipid pathways, but can indirectly affect these pathways via the filaggrin breakdown products (NMF)[3737]. Most enzymes involved in lipid synthesis are pH dependent, and changes in NMF levels caused by *FLG* mutations may therefore indirectly lead to a dysfunction in lipid synthesis and skin barrier function.

Summarizing, we analyzed the SC lipid chain length distribution in non-lesional and lesional skin of AE patients. The results demonstrate an increased presence of short chain FFAs and a decreased presence of long-chain FFAs in SC of AE patients. These changes are in line with the changes observed for the CERs, and lead to an altered lipid organization and decreased skin barrier function in AE. The outcomes of this study do not only give more insights in fundamental SC lipid knowledge, but also suggest possible new targets for future drug therapy for both lesional and non-lesional AE skin.

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References

1 Alanne S, Nermes M, Soderlund R *et al.* Quality of life in infants with atopic dermatitis and healthy infants: a follow-up from birth to 24 months. *Acta Paediatr* 2011; 100: e65-70.

2 Mozaffari H, Pourpak Z, Pourseyed S *et al.* Quality of life in atopic dermatitis patients. *J Microbiol Immunol Infect* 2007; 40: 260-4.

3 Slattery MJ, Essex MJ, Paletz EM *et al.* Depression, anxiety, and dermatologic quality of life in adolescents with atopic dermatitis. *J Allergy Clin Immunol* 2011; 128: 668-71 e3.

4 van Valburg RW, Willemsen MG, Dirven-Meijer PC *et al.* Quality of life measurement and its relationship to disease severity in children with atopic dermatitis in general practice. *Acta Derm Venereol* 2011; 91: 147-51.

5 Williams H, Flohr C. How epidemiology has challenged 3 prevailing concepts about atopic dermatitis. *Journal of Allergy and Clinical Immunology* 2006; 118: 209-13.

6 Di Nardo A, Wertz P, Giannetti A *et al.* Ceramide and cholesterol composition of the skin of patients with atopic dermatitis. *Acta Derm Venereol* 1998; 78: 27-30.

7 Elias PM, Schmuth M. Abnormal skin barrier in the etiopathogenesis of atopic dermatitis. *Current Opinion in Allergy and Clinical Immunology* 2009; 9: 437-46.

8 Seidenari S, Giusti G. Objective assessment of the skin of children affected by atopic dermatitis: A study of pH, capacitance and TEWL in eczematous and clinically uninvolved skin. *Acta Dermato-Venereol* 1995; 75: 429-33.

9 Werner Y, Lindberg M. Transepidermal water loss in dry and clinically normal skin in patients with atopic dermatitis. *Acta Derm Venereol* 1985; 65: 102-5.

10 Yoshiike T, Aikawa Y, Sindhvananda J *et al.* Skin barrier defect in atopic dermatitis: increased permeability of the stratum corneum using dimethyl sulfoxide and theophylline. *J Dermatol Sci* 1993; 5: 92-6.

11 Feingold KR, Jiang YJ. The mechanisms by which lipids coordinately regulate the formation of the protein and lipid domains of the stratum corneum: Role of fatty acids, oxysterols, cholesterol sulfate and ceramides as signaling molecules. *Dermato-endocrinology* 2011; 3: 113-8.

12 Addor FA, Aoki V. Skin barrier in atopic dermatitis. *An Bras Dermatol* 2010; 85: 184-94.

13 Elias PM, Schmuth M. Abnormal skin barrier in the etiopathogenesis of atopic dermatitis. *Current Allergy and Asthma Reports* 2009; 9: 265-72.

14 Palmer CN, Irvine AD, Terron-Kwiatkowski A *et al.* Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat Genet* 2006; 38: 441-6.

15 Sandilands A, Terron-Kwiatkowski A, Hull PR *et al.* Comprehensive analysis of the gene encoding filaggrin uncovers prevalent and rare mutations in ichthyosis vulgaris and atopic eczema. *Nat Genet* 2007; 39: 650-4.

16 Brown SJ, McLean WH. One remarkable molecule: filaggrin. *The Journal of investigative dermatology* 2012; 132: 751-62.

17 Sandilands A, Sutherland C, Irvine AD *et al.* Filaggrin in the frontline: role in skin barrier function and disease. *Journal of cell science* 2009; 122: 1285-94.

18 Brown SJ, Irvine AD. Atopic eczema and the filaggrin story. *Seminars in Cutaneous Medicine and Surgery* 2008; 27: 128-37.

19 Mao-Qiang M, Fowler AJ, Schmuth M *et al.* Peroxisome-proliferator-activated receptor (PPAR)-gamma activation stimulates keratinocyte differentiation. *The Journal of investigative dermatology* 2004; 123: 305-12.

20 Jakasa I, Koster ES, Calkoen F *et al.* Skin barrier function in healthy subjects and patients with atopic dermatitis in relation to filaggrin loss-of-function mutations. *J Invest Dermatol* 2011; 131: 540-2.

21 Angelova-Fischer I, Mannheimer AC, Hinder A *et al.* Distinct barrier integrity phenotypes in filaggrin-related atopic eczema following sequential tape stripping and lipid profiling. *Exp Dermatol* 2011; 20: 351-6.

22 Jungersted JM, Scheer H, Mempel M *et al.* Stratum corneum lipids, skin barrier function and filaggrin mutations in patients with atopic eczema. *Allergy* 2010; 65: 911-8.

23 Flohr C, England K, Radulovic S *et al.* Filaggrin loss-of-function mutations are associated with early-onset eczema, eczema severity and transepidermal water loss at 3 months of age. *Br J Dermatol* 2010; 163: 1333-6.

24 Proksch E, Folster-Holst R, Jensen JM. Skin barrier function, epidermal proliferation and differentiation in eczema. *J Dermatol Sci* 2006; 43: 159-69.

25 Elias PM, Menon GK. Structural and lipid biochemical correlates of the epidermal permeability barrier. *Adv Lipid Res* 1991; 24: 1-26.

26 Jiang YJ, Lu B, Kim P *et al.* PPAR and LXR activators regulate ABCA12 expression in human keratinocytes. *The Journal of investigative dermatology* 2008; 128: 104-9.

27 Proksch E, Jensen JM, Elias PM. Skin lipids and epidermal differentiation in atopic dermatitis. *Clin Dermatol* 2003; 21: 134-44.

28 Wertz PW. Lipids and barrier function of the skin. *Acta Derm Venereol Suppl* (Stockh) 2000; 208: 7-11.

29 van Smeden J, Hoppel L, van der Heijden R *et al.* LC/MS analysis of stratum corneum lipids: ceramide profiling and discovery. *J Lipid Res* 2011; 52: 1211-21.

30 Coderch L, Lopez O, de la Maza A *et al.* Ceramides and skin function. *Am J Clin Dermatol* 2003; 4: 107-29.

31 Downing DT, Stewart ME, Wertz PW *et al.* Skin lipids: an update. *J Invest Dermatol* 1987; 88: 2s-6s.

32 Bouwstra JA, Gooris GS, van der Spek JA *et al.* Structural investigations of human stratum corneum by small-angle X-ray scattering. *J Invest Dermatol* 1991; 97: 1005-12.

33 Madison KC, Swartzendruber DC, Wertz PW *et al.* Presence of intact intercellular lipid lamellae in the upper layers of the stratum corneum. *J Invest Dermatol* 1987; 88: 714-8.

34 Bommannan D, Potts RO, Guy RH. Examination of stratum corneum barrier function in vivo by infrared spectroscopy. *J Invest Dermatol* 1990; 95: 403-8.

35 Ongpipattanakul B, Francoeur ML, Potts RO. Polymorphism in stratum corneum lipids. *Biochim Biophys Acta* 1994; 1190: 115-22.

36 Pilgram GS, Vissers DC, van der Meulen H *et al.* Aberrant lipid organization in stratum corneum of patients with atopic

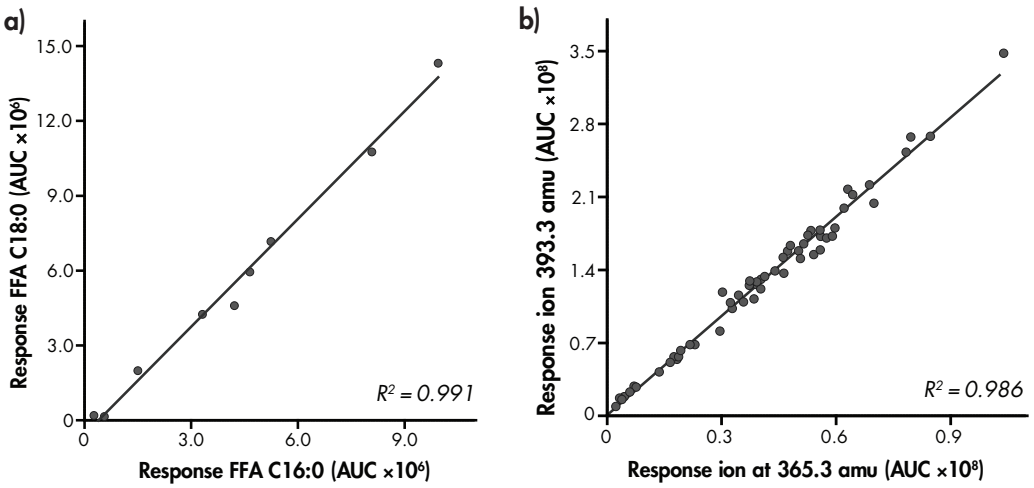
dermatitis and lamellar ichthyosis. *J Invest Dermatol* 2001; 117: 710-7.
37 Janssens M, van Smeden J, Gooris GS *et al.* Increase in short-chain ceramides correlates with an altered lipid organization and decreased barrier function in atopic eczema patients. *J Lipid Res* 2012; 53: 2755-66.
38 Ansari MN, Nicolaides N, Fu HC. Fatty acid composition of the living layer and stratum corneum lipids of human sole skin epidermis. *Lipids* 1970; 5: 838-45.
39 Norlen L, Nicander I, Lundsjo A *et al.* A new HPLC-based method for the quantitative analysis of inner stratum corneum lipids with special reference to the free fatty acid fraction. *Arch Dermatol Res* 1998; 290: 508-16.
40 Vasireddy V, Uchida Y, Salem N, Jr. *et al.* Loss of functional ELOVL4 depletes very long-chain fatty acids (> or =C28) and the unique omega-O-acylceramides in skin leading to neonatal death. *Hum Mol Genet* 2007; 16: 471-82.
41 Macheleidt O, Kaiser HW, Sandhoff K. Deficiency of epidermal protein-bound omega-hydroxyceramides in atopic dermatitis. *J Invest Dermatol* 2002; 119: 166-73.
42 Ohno Y, Suto S, Yamanaka M *et al.* ELOVL1 production of C24 acyl-CoAs is linked to C24 sphingolipid synthesis. *Proc Natl Acad Sci U S A* 2010; 107: 18439-44.
43 Uchida Y. The role of fatty acid elongation in epidermal structure and function. *Dermatoendocrinol* 2011; 3: 65-9.
44 Thakoersing VS, Ponec M, Bouwstra JA. Generation of human skin equivalents under submerged conditions-mimicking the in utero environment. *Tissue Eng Part A* 2010; 16: 1433-41.
45 Chang SS, Jiang WW, Smith I *et al.* Chronic cigarette smoke extract treatment selects for apoptotic dysfunction and mitochondrial mutations in minimally transformed oral keratinocytes. International journal of cancer. *Journal international du cancer* 2010; 126: 19-27.
46 Rivier M, Castiel I, Safonova I *et al.* Peroxisome proliferator-activated receptor-alpha enhances lipid metabolism in a skin equivalent model. *The Journal of investigative dermatology* 2000; 114: 681-7.
47 Motta S, Monti M, Sesana S *et al.* Ceramide composition of the psoriatic scale. *Biochim Biophys Acta* 1993; 1182: 147-51.
48 Boncheva M, Damien F, Normand V. Molecular organization of the lipid matrix in intact Stratum corneum using ATR-FTIR spectroscopy. *Biochim Biophys Acta* 2008; 1778: 1344-55.
49 Damien F, Boncheva M. The extent of orthorhombic lipid phases in the stratum corneum determines the barrier efficiency of human skin in vivo. *J Invest Dermatol* 2010; 130: 611-4.
50 Mendelsohn R, Liang GL, Strauss HL *et al.* IR spectroscopic determination of gel state miscibility in long-chain phosphatidylcholine mixtures. *Biophys J* 1995; 69: 1987-98.
51 Groen D, Poole DS, Gooris GS *et al.* Is an orthorhombic lateral packing and a proper lamellar organization important for the skin barrier function? *Biochim Biophys Acta* 2011; 1808: 1529-37.
52 Masukawa Y, Narita H, Sato H *et al.* Comprehensive quantification of ceramide species in human stratum corneum. *J Lipid Res* 2009; 50: 1708-19.
53 de Sousa Neto D, Gooris G, Bouwstra J. Effect of the omega-acylceramides on the lipid organization of stratum corneum model membranes evaluated by X-ray diffraction and FTIR studies (Part I). *Chem Phys Lipids* 2011; 164: 184-95.
54 de Jager M, Groenink W, Bielsa i Guivernau R *et al.* A novel in vitro percutaneous penetration model: evaluation of barrier properties with p-aminobenzoic acid and two of its derivatives. *Pharm Res* 2006; 23: 951-60.
55 Matsuzaka T, Shimano H, Yahagi N *et al.* Cloning and characterization of a mammalian fatty acyl-CoA elongase as a lipogenic enzyme regulated by SREBPs. *J Lipid Res* 2002; 43: 911-20.
56 Guillou H, Zadravec D, Martin PG *et al.* The key roles of elongases and desaturases in mammalian fatty acid metabolism: Insights from transgenic mice. *Prog Lipid Res* 2010; 49: 186-99.
57 Li W, Sandhoff R, Kono M *et al.* Depletion of ceramides with very long chain fatty acids causes defective skin permeability barrier function, and neonatal lethality in ELOVL4 deficient mice. *Int J Biol Sci* 2007; 3: 120-8.
58 Cameron DJ, Tong Z, Yang Z *et al.* Essential role of Elovl4 in very long chain fatty acid synthesis, skin permeability barrier function, and neonatal survival. *Int J Biol Sci* 2007; 3: 111-9.
59 Miyazaki M, Ntambi JM. Chapter 7 - Fatty acid desaturation and chain elongation in mammals. *In: Biochemistry of Lipids, Lipoproteins and Membranes (Fifth Edition) (Dennis EV, Jean EV, eds). San Diego: Elsevier.* 2008; 191-V.
60 Yamamoto A, Serizawa S, Ito M *et al.* Stratum corneum lipid abnormalities in atopic dermatitis. *Arch Dermatol Res* 1991; 283: 219-23.
61 Abe T, Ohkido M, Yamamoto K. Studies on skin surface barrier functions:--skin surface lipids and transepidermal water loss in atopic skin during childhood. *The Journal of dermatology* 1978; 5: 223-9.
62 Rajka G. Transepidermal water loss on the hands in atopic dermatitis. Arch Dermatol Forsch 1974; 251: 111-5.
63 Briot A, Deraison C, Lacroix M *et al.* Kallikrein 5 induces atopic dermatitis-like lesions through PAR2-mediated thymic stromal lymphopoietin expression in Netherton syndrome. J Exp Med 2009; 206: 1135-47.
64 Sertznig P, Seifert M, Tilgen W *et al.* Peroxisome proliferator-activated receptors (PPARs) and the human skin: importance of PPARs in skin physiology and dermatologic diseases. Am J Clin Dermatol 2008; 9: 15-31.
65 Staumont-Salle D, Abboud G, Brenuchon C *et al.* Peroxisome proliferator-activated receptor alpha regulates skin inflammation and humoral response in atopic dermatitis. *J Allergy Clin Immunol* 2008; 121: 962-8 e6.
66 Plager DA, Leontovich AA, Henke SA *et al.* Early cutaneous gene transcription changes in adult atopic dermatitis and potential clinical implications. *Exp Dermatol* 2007; 16: 28-36.
67 Hanley K, Jiang Y, Crumrine D *et al.* Activators of the nuclear hormone receptors PPARalpha and FXR accelerate the development of the fetal epidermal permeability barrier. *J Clin Invest* 1997; 100: 705-12.
68 Jiang YJ, Barish G, Lu B *et al.* PPARdelta activation promotes stratum corneum formation and epidermal permeability barrier development during late gestation. *J Invest Dermatol* 2010; 130: 511-9.

69 Man MQ, Barish GD, Schmuth M *et al.* Deficiency of PPARbeta/delta in the epidermis results in defective cutaneous permeability barrier homeostasis and increased inflammation. *J Invest Dermatol* 2008; 128: 370-7.
70 Schmuth M, Jiang YJ, Dubrac S *et al.* Thematic review series: skin lipids. Peroxisome proliferator-activated receptors and liver X receptors in epidermal biology. *J Lipid Res* 2008; 49: 499-509.
71 Kim BE, Howell MD, Guttman-Yassky E *et al.* TNF-alpha downregulates filaggrin and loricrin through c-Jun N-terminal kinase: role for TNF-alpha antagonists to improve skin barrier. *J Invest Dermatol* 2011; 131: 1272-9.
72 Hvid M, Vestergaard C, Kemp K *et al.* IL-25 in atopic dermatitis: a possible link between inflammation and skin barrier dysfunction? *J Invest Dermatol* 2011; 131: 150-7.
73 Gutowska-Owsiak D, Schaupp AL, Salimi M *et al.* Interleukin-22 downregulates filaggrin expression and affects expression of profilaggrin processing enzymes. *Br J Dermatol* 2011; 165: 492-8.
74 Elias PM, Schmuth M. Abnormal skin barrier in the etiopathogenesis of atopic dermatitis. *Curr Opin Allergy Clin Immunol* 2009; 9: 437-46.
75 Cork MJ, Robinson D, Vasilopoulos Y *et al.* Predisposition to sensitive skin and atopic eczema. *Community practitioner : the journal of the Community Practitioners' & Health Visitors' Association* 2005; 78: 440-2.
76 Mucke H, Mohr KT, Rummler A *et al.* [Skin pH value on hands after application of soap, cleaners and hand disinfectants]. *Die Pharmazie* 1993; 48: 468-9.

Supplementary data

Quantitative analysis of FFA C16:0 and C18:0, correcting for tape strip contamination
Recently, we developed a method that enables analysis of FFAs by LC/MS, and applied that method on tape-stripped SC. However, endogenous C16:0, C17:0, C18:0, and C18:1 FFAs are abundantly present on control tapes (blanks), thereby interfering with the results. To correct for the amount of these four endogenous lipids on tape, it was not possible to simply subtract a certain amount of these lipids as the tape appeared to be too inhomogeneous for a reliable correction: We observed that the amount could vary ~100 fold. Therefore, a more comprehensive correction method was needed that corrects for these 2 lipids for each individual tape strip.

Although the variation in absolute amounts of endogenous lipids is very high, we observed that the ratio of these lipids is always the same. In example, C16:0 is always linearly correlated to the amount of C18:0, which can be appreciated from Supplementary Figure 1a. We also analyzed two peaks which appear in blank tape strips but not in human SC, possibly exogenous lipids. Again, a very high correlation was observed between the amounts of the two unidentified peaks (Supplementary Figure 1b). The amount of these two lipid peaks also correlates very well (but not perfectly) to the amount of the four endogenous FFAs (Supplementary Table I). Hence, by quantifying the two exogenous lipids on tapes that do not interfere with SC from *in vivo* subjects, we could calculate the predicted amount of endogenous lipids C16:0, C17:0, C18:0, C18:1. Indeed, we were able to successfully correct for the amount of contamination of these lipids. However, we mention that the methodological error does increase since we did not observe perfect correlations: Since the R^2-values of the correlations are around 0.85-0.95, the expected increment in error is around 10%. Nevertheless, either taking into account or completely leaving out the C16:0 and C18:0 data did not change any of the conclusions that could be drawn from the data.

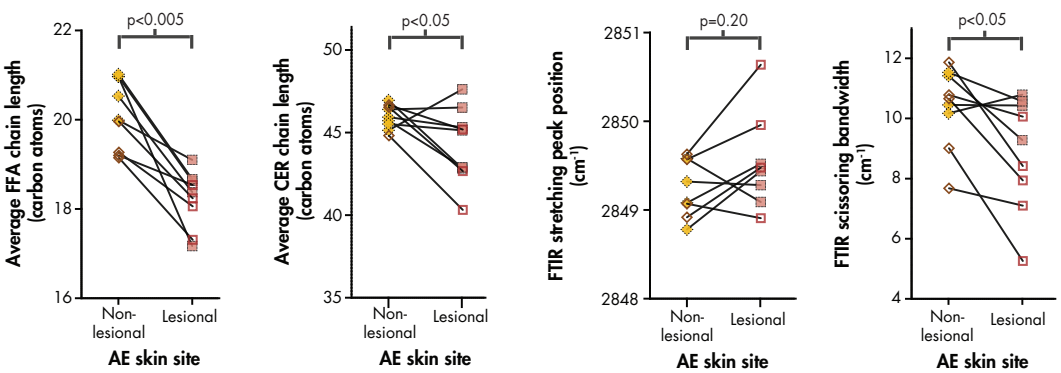


Supplementary Figure 1: *Tape strips were analyzed to obtain information on the amount of endogenous lipids that interfere with data from SC lipids, and how to correct for this contamination. Data are presented as scatter dot plots and their correlations of* ***a****) endogenous FFAs C16:0 versus C18:0 (R^2=0.991) analyzed from control tape strips.* ***b****) Exogenous ions m/z 365.3 amu versus 393.3 amu. Data points include control tape strips as well as tapes from all subjects.*

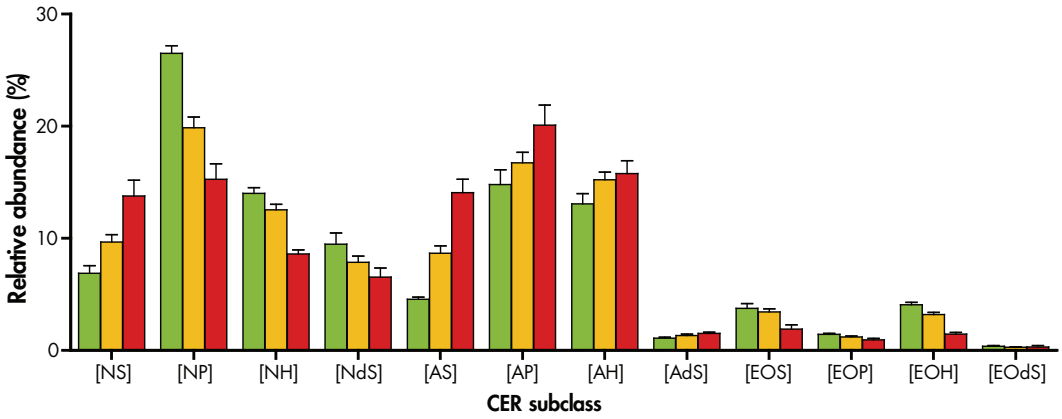
Supplementary Table I: *Correlation coefficients (R^2) between the endogenous lipids and the two exogenous lipids.*

Endogenous lipid	Correlation Coefficient	
	Exogenous ion 1 (m/z = 365.3 amu)	Exogenous ion 2 (m/z = 393.3 amu)
C16:0	0.917	0.852
C17:0	0.862	0.855
C18:0	0.942	0.903
C18:1	0.944	0.890

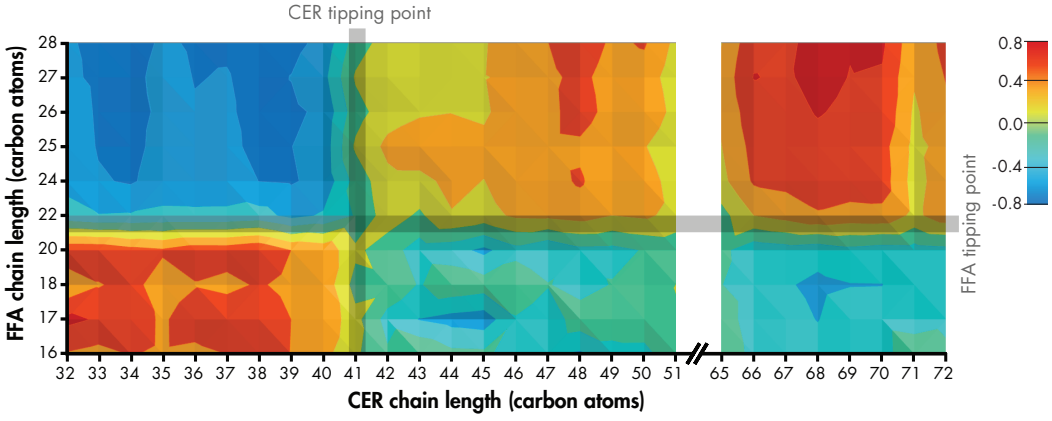
The correlation coefficients state the accuracy in which the amounts of the two exogenous ions (viz. m/z 365.3 and 393.3 amu) are correctly predicting the amount of endogenous lipids related to the tape. In general, exogenous ion 1 was chosen to predict the amount of endogenous lipids, as the correlation coefficient was higher compared to exogenous ion 2.



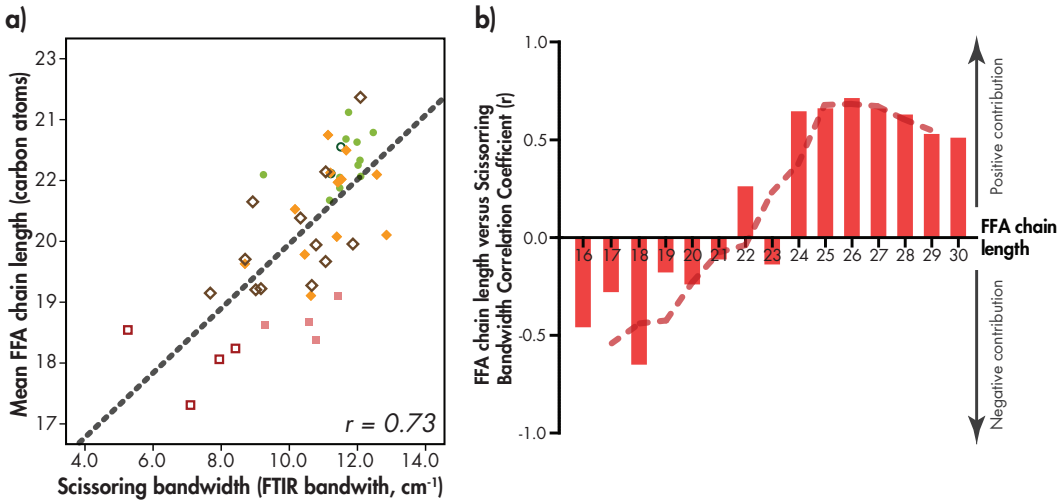
Supplementary Figure 2: *Paired data of non-lesional and lesional AE skin for the FFA chain length, CER chain length, FTIR stretching peak position and FTIR bandwidth. P-values are calculated from the Wilcoxon signed rank test. Non-lesional skin and lesional skin of AE patients are indicated by ◆/◇ and ■/□, respectively. Open and filled data points indicate carriers and non-carriers of FLG mutations, respectively.*



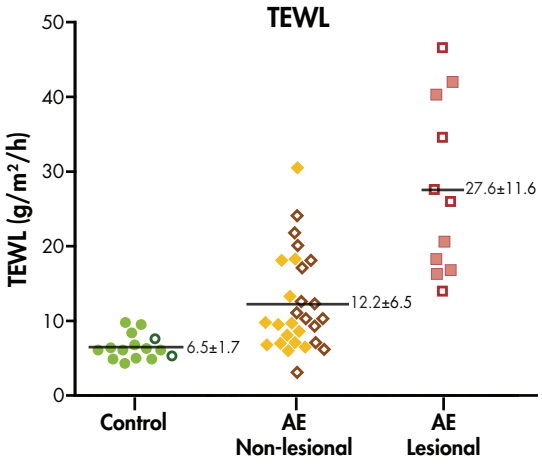
Supplementary Figure 3: *Relative CER composition of each subclass for control (green), AE – non-lesional (orange), and AE – lesional (red).*



Supplementary Figure 4: *Correlation map. On the horizontal axis are the individual CER chain lengths, whereas on the vertical axis are the individual FFA chain lengths. Strong negative or positive correlations are depicted in blue or red, respectively. Gray strokes indicate the tipping point of the chain length correlations; in other words, where positive correlations turn into negative correlations and vice versa.*



Supplementary Figure 5: ***a****) Correlation plot in which the scissoring bandwidth of all individuals is plotted versus the mean FFA chain length. The gray dashed line indicates the optimal linear fit. Control subjects are indicated by ●/○. Non-lesional skin and lesional skin of AE patients are indicated by ◆/◇ and ■/□, respectively. Open and filled data points indicate carriers and non-carriers of FLG mutations, respectively.* ***b****) Bar plots demonstrating the correlation between each individual FFA chain length (x-axis) versus the FTIR scissoring bandwidth. Positive correlation coefficients indicate that these FFAs contribute to a more ordered lateral lipid packing, whereas FFAs with negative correlation coefficients contribute to a less ordered packing of the lipids. The dashed line indicates the moving average (period = 3).*



Supplementary Figure 6: *TEWL levels in control subjects, non-lesional SC and lesional SC of AE patients. Control subjects are indicated by ●/○. Non-lesional skin and lesional skin of AE patients are indicated by ◆/◇ and ■/□, respectively. Open and filled data points indicate carriers and non-carriers of FLG mutations, respectively. Means are indicated by gray horizontal lines and their corresponding values (±SD). Significant differences were observed between control subjects, non-lesional, and lesional SC of AE patients (P<0.0005).*

Supplementary Table II: *Correlations between short/long CERs versus short/long FFAs.*

	FFAs <C23	FFAs >C23
CERs <C42	0.496*	-0.541*
CERs >C42	-0.429*	-0.481*

*Spearman correlations showing that short CERs correlate positively with short FFAs and negatively with long FFAs, and vice versa for long CERs. *:P<0.01.*