Increase in short-chain ceramides correlates with an altered lipid organization and decreased barrier function in atopic eczema patients

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**Abbreviations:** AE, atopic eczema; CER, ceramide; FLG, filagrin; FTIR, Fourier transform infrared spectroscopy; LC-MS, liquid-chromatography-mass-spectrometry; LPP, long periodicity phase; NMF, natural moisturizing factor; SAXD, small angle X-ray diffraction; SC, stratum corneum; SCORAD, SCORing Atopic Dermatitis; SPP, short periodicity phase; TEWL, transepidermal water loss.
Abstract

A hallmark of atopic eczema (AE) is skin barrier dysfunction. Lipids in the stratum corneum (SC), primarily ceramides, fatty acids and cholesterol, are crucial for the barrier function, but their role in relation to AE is indistinct. Filaggrin is an epithelial barrier protein with a central role in the pathogenesis of AE. Nevertheless, the precise causes of AE-associated barrier dysfunction are largely unknown. In this study a comprehensive analysis of ceramide composition and lipid organization in non-lesional SC of AE patients and control subjects was performed by means of mass spectrometry, infrared spectroscopy and X-ray diffraction. In addition, the skin barrier and clinical state of the disease were examined. The level of ceramides with an extreme short chain length is drastically increased in SC of AE patients, which leads to an aberrant lipid organization and a decreased skin barrier function. Changes in SC lipid properties correlate with disease severity but are independent of filaggrin mutations. We demonstrate for the first time that changes in ceramide chain length and lipid organization are directly correlated with the skin barrier defects of non-lesional skin of AE patients. We envisage that these insights provide a new therapeutic entry in therapy and prevention of AE.

Supplementary key words: ceramide composition, lipid organization, lamellar phase, lateral packing
Introduction

The skin offers a protective barrier against allergens, irritants, microorganisms and prevents excessive transepidermal water loss (TEWL). The barrier function strongly relies on the outermost layer of the skin, the stratum corneum (SC), which consists of corneocytes embedded in a highly organized lipid matrix (1, 2). This lipid matrix is considered to be important for a proper skin barrier function.

Ceramides (CERs), cholesterol and free fatty acids are the main lipid classes in SC. To date, 12 CER subclasses in human SC have been identified with a wide chain length distribution (3, 4). An explanation of the CER nomenclature is given in Figure 1. The aim of the present study was to determine the chain lengths of each CER subclass in non-lesional skin of atopic eczema (AE) patients and to correlate these with the lipid organization, skin barrier function and disease severity, see Figure 2.

AE is a chronic relapsing inflammatory skin disease characterized by a broad spectrum of clinical manifestations such as erythema, dryness and intense pruritus (5, 6). AE affects over 15% of Caucasian children and 2-10% of adults, and its prevalence is increasing rapidly especially in developed countries (7-11). Patients have a decreased skin barrier function in lesional and non-lesional skin (12-16).

In previous studies it has been shown that AE is strongly associated with mutations in the filaggrin gene (FLG) (17-19) but the role of FLG mutations for the barrier dysfunction is yet inconclusive (20-25). Other factors, such as aberrations in the SC lipids, may play a role in the decreased skin barrier in AE (12, 26, 27). In healthy SC, lipids form two lamellar phases with repeat distances of approximately 6 and 13 nm. These are referred to as the short periodicity phase (SPP) and long periodicity phase (LPP), respectively (28, 29). A schematic presentation of the lipid organization is provided in Figure 3. Within the lipid lamellae, the lipids have a dense (orthorhombic) lateral organization, although a subpopulation of the lipids can be less densely packed in a hexagonal organization (30-32).
CERs play a crucial role in the lipid organization (33) and have a characteristic molecular architecture. Several studies have reported significant changes in CER subclasses in non-lesional SC of AE patients: reduced CER [NP], increased CER [AS] and reduced long chain CERS [EOH] and [EOS] (12, 23, 34-36). Some of these changes could be correlated with changes in skin barrier function. However, no information was reported on the effect of chain length distribution of CERs on the skin barrier, until recently. Ishikawa et al. showed that lesional skin has a significantly increased level of short-chain CERs (with a total chain length of 34 carbon atoms) in one specific CER subclass, which correlates with the impaired skin barrier function (37). These results suggest that CER chain length may be an important factor in skin barrier dysfunction of AE patients. These findings were the starting point of the present study in which we performed a detailed analysis on CER composition, focusing in particular on the CER chain length distribution in non-lesional SC of AE patients in relation to lipid organization and skin barrier dysfunction.

We studied SC of non-lesional skin as we aimed to monitor the changes in lipid properties in the absence of inflammation. We have identified several CER subclasses that exhibit an increased level of extremely short C34 chains in AE and we demonstrate that the overall level of C34 is increased in AE. The changes in CER chain length distribution correlated with changes in lipid organization, skin barrier function, disease severity and levels of natural moisturizing factor (NMF, composed of filaggrin-derived amino acids, their metabolites, specific sugars and salts). Changes in CER chain length distribution did not correlate with FLG genotype. These results demonstrate for the first time that CER chain length is an important factor in skin barrier dysfunction in non-lesional skin of AE.

**Material and Methods**

**Study population and general study setup**

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethical Committee of the Leiden University Medical Center. All subjects gave written informed consent. 15 Caucasian subjects without (history of) dermatological disorders (25.0±5.2
years; 5 males) and 28 Caucasian AE patients (25.6±5.6 years; 11 males) were included. The group of AE patients consists of 14 patients with and 14 patients without the presence of common FLG genotype mutations (see FLG mutation analysis below). Subjects did not apply any dermatological products to their forearms for at least one week prior to the study. The study itself was performed in a temperature and humidity controlled room and subjects were acclimatized for 45 minutes prior to the measurements. Per subject, all measurements were performed on a single day on one of the ventral forearms, which was observed by a dermatologist at the start of the study to carefully depict an area of non-lesional skin, which was marked accordingly. At this area, NMF levels were determined with Raman spectroscopy followed by subsequent tape stripping, TEWL and Fourier transform infrared spectroscopy (FTIR) measurements, as described below. At the end of the study day, buccal mucosa cells were collected with a cotton swab and SCORing Atopic Dermatitis (SCORAD) was performed by a dermatologist to determine the severity of the disease. Finally, a 4 mm biopsy was harvested close to the area where all measurements were performed.

**FLG mutation analysis**

The influence of FLG mutations on the lipid properties was studied. We screened all subjects on the four most prevalent mutations found in European Caucasians (2282del4, R501X, S3247X and R2447X), covering around 93% of all FLG mutations known to date (38). 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 (39).

**Skin barrier function assessment by TEWL**

A Tewameter TM 210 (Courage+Khazaka, Köln, Germany) was used to measure TEWL on the marked area on the ventral forearm of the subject. The forearm was placed in an open chamber and TEWL 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 to have an indication of the amount of removed SC.

**SCORAD**

SCORAD was performed by the dermatologist to determine the severity of the disease (40).

**Determination of NMF levels in SC**

Confocal Raman microspectroscopy (3510 Skin Composition Analyzer, River Diagnostics, Rotterdam, The Netherlands) was used to measure NMF in the SC of the ventral forearm. The principles and experimental details of this method and the procedure have been described elsewhere (41, 42). Depth profiles of Raman spectra were measured at 2 µm intervals from the skin surface to 20 µm below the skin surface. In each subject 15 profiles were measured at different spots within the marked area on the ventral forearm. Raman spectra were recorded between 400 to 1800 cm\(^{-1}\) with a 785 nm laser. Laser power on the skin was 25 mW. NMF levels relative to keratin were determined from the Raman spectra measured between 4-8 µm by means of classical least-squares fitting. Relative NMF to keratin levels were calculated from the recorded Raman spectra by using SkinTools 2.0 (River Diagnostics, Rotterdam, The Netherlands).

**Tape stripping procedure**

To harvest SC lipids, the following tape stripping procedure was performed on both control subjects and non-lesional regions of AE patients: multiple poly(phenylene sulfide) tape strips (Nichiban, Tokyo, Japan) were successively applied at the same area (4.5 cm\(^2\)) on the ventral forearm. All tapes were pressed to the targeted skin with a pressure of 450 g/cm\(^2\) using a D-Squame pressure instrument (Cuderm Corp., Dallas, USA). Tweezers were used to remove the tape in a fluent stroke, using alternating directions for each successive tape strip. The
Squamescan 850A (Heiland electronic, Wetzlar, Germany) was used to determine the amount of SC removed, in order to obtain a good indication of the depth of each tape strip taken (43, 44). Calibration was performed by a bicinchoninic acid (BCA) assay using bovine serum albumin (BSA). The predicted total amount of protein in the SC was calculated by plotting 1/TEWL against the cumulative amount of protein removed. The intercept with the x-axis is indicative for the total amount of protein in the SC according to Kalia (43). Tapes 6 to 9 were selected for lipid composition analysis, since these tapes do not show surface contamination (observed when analyzing tape strips from the surface of the SC). All tapes were punched to a circular area of 2 cm², put individually into glass vials containing 1 mL chloroform/methanol/water (1:2:½) and stored at -20°C under argon atmosphere prior to lipid extraction.

**Lipid extraction and ceramide analysis by LC/MS**

Lipid extraction was performed on 4 tape strips (numbers 6 to 9) of each subject. Before lipids were extracted from tape strips by liquid-liquid extraction, 2 deuterated internal ceramide standards (CER [NS] C24 and, CER [EOS] C30 linoleate) were added to compare CER levels between control group and non-lesional skin of AE patients. Then, a slightly enhanced extraction procedure of the commonly used Bligh and Dyer method was performed on all 4 selected tape strips individually: 3 different ratios of solvent mixtures Chloroform/Methanol/Water (1:2:½; 1:1:0; 2:1:0) were used sequentially to extract all lipids. A detailed procedure is described elsewhere (45, 46). Afterwards, lipid extracts from all 4 individual tapes were pooled, dried under N₂ gas and resolved in 100 µl C:M:H (2½:2½:95) to obtain a total lipid concentration around 1.0 mg/mL. Samples were stored at -20°C until use. The analysis was performed by LC-MS using a recently developed LC-MS method described in detail elsewhere (4). Briefly, 10 µl of each lipid sample was automatically injected and separated onto an analytical normal phase column (PVA-bonded column; 100 x 2.1 mm i.d., 5 µm particle size, YMC (Kyoto, Japan)) by a gradient solvent system from heptane to heptane/IPA/EtOH at a flow rate of 0.8 mL/min using an Alliance 2695 HPLC system (Waters Milford, USA). The HPLC was coupled to a mass
spectrometer (TSQ Quantum, Thermo Finnigan, San Jose, CA, USA) in APCI-positive mode with a scan range set from 360-1200 amu. The temperature of the source heater and heated capillary were set to 450°C and 250°C respectively, while the discharge current was set to 5 µA. The ceramide analysis was performed using Xcalibur software version 2.0, and its nomenclature used throughout this article is according to Motta (47) in which ceramide subclasses are classified by letter abbreviations according to their two individual chains: the sphingoid base (either dihydrosphingosine (dS), sphingosine (S), 6-hydroxy sphingosine (H) or phytosphingosine (P)), chemically linked to the fatty acid chain (either an α-hydroxy fatty acid (A), an esterified ω-hydroxy fatty acid (EO) or a non-hydroxy fatty acid (N)). This results in 12 different CER subclasses, namely [AdS], [AS], [AH], [AP], [EOdS], [EOS], [EOH], [EOP], [NdS], [NS], [NH] and [NP]. The CER nomenclature and its molecular structure are explained in Figure 1.

**Lateral organization and conformational ordering of the lipids**

To obtain information on the lateral organization and conformational ordering of the lipids, FTIR spectra were recorded in the same skin region also used for lipid analysis. FTIR spectra of the SC were collected after each 2 tape strips using a Varian 670-IR spectrometer (Varian Inc., Santa Clara, USA) equipped with a broad band Mercury-Cadmium-Telluride (MCT) detector and an external sample compartment containing a GladiATR (Pike, Madison, USA) attenuated total reflection (ATR) accessory with a single reflection diamond. The spectral resolution was 2 cm⁻¹. The instrument was continuously purged with dry N₂. Each spectrum was an average of 150 scans. For data treatment the instrument software Resolutions Pro 4.1 (Varian Inc., Santa Clara, USA) was used. We calculated positions of the CH₂ symmetric stretching vibration and second derivatives of the scissoring bandwidth 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 CH₂ symmetric stretching vibration positions of spectra recorded between the removal of 2 to 10 tape strips.
Biopsy and small angle X-ray diffraction measurements

After tape stripping, 4 mm biopsies were taken from the ventral forearm close to the region of the tape stripping. SC was isolated by trypsin digestion as described earlier (50). This procedure does not affect the lipid organization in SC (51). The SC sheets were measured by small angle X-ray diffraction (SAXD) performed at the European Synchrotron Radiation Facility (ESRF, Grenoble, France) using station BM26B. Prior to the measurements, SC was hydrated over a 27% NaBr solution during 24h. To obtain high quality diffraction patterns SC was carefully oriented parallel to the primary X-ray beam. SAXD patterns were detected with a Frelon 2000 CCD detector at room temperature for a period of 10 minutes using a microfocus beam, similarly as described elsewhere (52). Samples were checked for evidence of radiation damage and the exposure time to the X-rays was kept to a minimum. From the scattering angle, the scattering vector \( q \) was calculated by \( q = 4\pi \sin \theta / \lambda \), in which \( \lambda \) is the wavelength of the X-rays at the sample position and \( \theta \) the scattering angle.

Statistical analysis

Statistical analysis was performed using SPSS Statistics. Non-parametric Mann-Whitney tests were performed when comparing 2 groups and stated significant when \( P<0.05 \). When the effect of FLG was taken into account and trends were observed, Kruskall-Wallis tests and eventually an additional Mann-Whitney test was performed. Bivariate analysis was performed to analyze which parameters showed a significant correlation, and their respective Spearman's \( \rho \) correlation coefficients were calculated. Univariate general linear model analysis was performed to correlate the biologically and clinically relevant parameters to 2 independent lipid parameters, as well as the predicted and the observed average chain length.
Results

Description of study population

Fourteen out of 28 AE patients were carriers of at least one of the four most common FLG mutations (details in Supplementary Table I). Patient and control subject characteristics are provided in Supplementary Table II. In addition, two control subjects were heterozygous for FLG mutations. Severity of the disease was scored by an experienced dermatologist using SCORAD (Figure 4a). AE patients showed an elevated TEWL in non-lesional skin compared to control subjects (12.2±6.5 g/m²/h and 6.5±1.7 g/m²/h, respectively, P<0.0005, Figure 4b) and lower NMF levels compared to control subjects (0.66±0.39 and 1.05±0.20, respectively, P<0.01, Figure 4c).

Reduced CER chain length correlates with a decreased barrier function.

From the LC/MS data, lipid profiles were constructed and examples are shown in Supplementary Figure I. From manual integration of these lipid profiles, the relative abundance of all CER subclasses could be calculated, which is shown in Supplementary Figure II. Statistical differences between control subjects and AE patients were observed in 7 subclasses: CERs [EOP], [EOH], [NS], [NP], [NH], [AS] and [AH] (P<0.05). No difference was observed between carriers and non-carriers of FLG mutations. The total CER level in the control group and in non-lesional skin of AE patients was not significantly different 37.0±3.6 and 38.5±2.4 ng/μg protein, respectively (P>0.1).

The average CER chain length was significantly decreased by 0.64±0.23 total carbon atoms in AE patients (mean±SEM; P=0.012, Figure 5a). No difference was observed between carriers and non-carriers of FLG mutations (P>0.1). Figure 5a shows that in non-lesional skin of AE patients, extremely short C34 CERs were increased within several CER subclasses. This was primarily observed in CER subclasses [AS], [AH], [NS] and [NH] (Figure 5b, P<0.05). The increase in total C34 CERs in AE (P<0.0001) contributes to a reduction in overall chain length. In addition,
the very long chain CERs belonging to the [EO] subclass are significantly reduced, which is primarily caused by significantly decreased levels of CER [EOH] and [EOP] ($P=0.019$ and $P=0.040$, respectively, Figure 5a and Supplementary Figure IIb). Univariate analysis shows that an increased level of C34 CERs and decreased level of CERs [EO] largely contribute to a reduction in average CER chain length, as can be observed from Figure 5c. The influence of FLG mutations on any of the CER chain length parameters was not significant ($P>0.1$; detailed overview in Supplementary Tables III-V).

The observed changes in CER chain length were compared to changes in barrier function as assessed by TEWL. The results in Figure 5d show a strong correlation between TEWL and the levels of C34 CERs and CER [EO]: univariate analysis of TEWL versus the total C34 CERs and total CER [EO] levels shows a correlation coefficient of 0.77 ($P<0.0001$).

Correlations between the relative abundances of the various CER subclasses with TEWL are shown in Table I. CER [EOH] and CER [AS] are the two subclasses which are most significantly associated with TEWL. This again indicates the importance of the chain length for the skin barrier in AE: the exceptional long CER [EOH] is decreased while CER [AS] (the CER subclass with the highest abundance of exceptionally short C34 CERs) is increased. The changes in CER composition are irrespective of FLG mutation status ($P=0.58$).

**Altered lamellar and lateral lipid organization correlates with a decreased barrier function.**

SAXD gives information about the lamellar organization of intercellular lipids in SC (28) (Explanation in Supplementary Figure III). The lipids form two lamellar phases, the SPP and LPP. Figure 6a shows three examples of SAXD curves of SC. The upper curve is a typical example from a control subject. The central curve is typical for AE. The bottom curve is representative for a subgroup of AE patients with an aberrant SAXD profile. The upper two curves show lipid-based features as two weak diffraction peaks (labeled I and III) and a strong peak (labeled II). The LPP contributes to all three peaks, while the SPP contributes only to peak II (Supplementary Figure
In the bottom curve both peaks I and III are absent and peak II is shifted to higher q-values. Peaks I and III were not present in 5 out of 28 patients. As peak I and peak III only attribute to the LPP, this indicates that there is a drastic reduction in the presence of the LPP in those patients.

Figure 6b shows the position of the strong peak (II) in the SAXD curves from control subjects (left) and AE patients (right). The peak position shows a larger variance within the group of AE patients compared to the group of control subjects. The average position of the strong peak is located at significantly higher q-values for AE patients as compared to control subjects (1.03 nm⁻¹ and 1.00 nm⁻¹, respectively, P=0.046). The position of peak II showed no difference between AE patients with and patients without FLG mutations (P=0.76).

FTIR was used to obtain information on the lateral lipid organization. Two types of vibrations were monitored, the CH₂ symmetric stretching vibrations and the CH₂ scissoring vibrations (Supplementary Figure IV). A low (~2848 cm⁻¹) wavenumber of the CH₂ symmetric stretching vibrations indicates the presence of a highly ordered lipid organization (either hexagonal or orthorhombic), while a high (2853 cm⁻¹) wavenumber indicates a liquid disordered phase (53). The mean value of the position of the CH₂ symmetric stretching vibrations of AE patients shows a small but significant shift to higher values compared to control subjects (2849.2 cm⁻¹ versus 2848.8 cm⁻¹, respectively, P=0.0013, Figure 6c). In addition, the variance in the group of AE patients is larger than in control subjects. In order to distinguish between an orthorhombic (dense) or hexagonal (less dense) lateral organization, the bandwidth of the CH₂ scissoring vibrations was monitored. A narrow bandwidth (typically 8 cm⁻¹) indicates the presence of only a hexagonal lipid organization, while a large bandwidth of typically 11 cm⁻¹ is indicative for the presence of mainly an orthorhombic organization (49). The average bandwidth of the scissoring vibrations was significantly lower in AE patients compared to control subjects (10.6 cm⁻¹ versus 11.6 cm⁻¹, respectively, P=0.010, Figure 6d), demonstrating a reduction of lipids in an orthorhombic organization and thus a less dense lipid organization. No significant
influence of FLG mutations on the lipid organization in AE patients was found ($P>0.05$, Figures 6b-d).

Univariate analysis was performed between TEWL and two independent lipid organization parameters: a lamellar organization component (SAXD peak II position) and a lateral organization component (FTIR scissoring bandwidth). The correlation coefficient was $r=0.76$ ($P<0.0001$, Figure 6e), which demonstrates that skin barrier function as measured by TEWL is significantly influenced by lipid organization.

**Altered CER composition correlates with aberrant lipid organization.**

As CER composition and lipid organization both show a relationship with a reduced skin barrier (TEWL), the relation between the lipid parameters are summarized in Table II. The levels of C34 CERs and CER [EO] associate with both the lamellar organization (SAXD) and lateral lipid organization (FTIR scissoring bandwidth and stretching vibrations position). A powerful correlation of 0.71 was observed when both the lamellar and lateral organization components (SAXD and CH$_2$ scissoring) were plotted versus the two components of the CER composition ([EO] CERs and C34 CERs). Supplementary Figure 5 illustrates this correlation.

**Altered CER composition and aberrant lipid organization correlate with NMF levels and SCORAD.**

A detailed overview of correlation coefficients between different parameters is presented in Table III. NMF levels correlate ($r>0.4$, $P<0.01$) with both lamellar and lateral lipid organization as well as with chain length of the CERs. SCORAD (disease severity) was associated with CER composition (i.e. total C34 CERs and total CER [EO] content) and lipid organization (i.e. SAXD peak II position and FTIR scissoring bandwidth), with correlation coefficients of 0.56 ($P<0.01$) and 0.58 ($P<0.01$), respectively.
*FLG* mutations correlate with NMF levels, but not with SCORAD and TEWL levels.

NMF levels were significantly lower in *FLG* carriers than in non-carriers (0.45±0.19 and 0.87±0.43, respectively, *P*<0.005). Both SCORAD and TEWL values were independent of *FLG* genotype (*P*=0.34 and *P*=0.23).
Discussion

We performed an integral analysis of CER composition focusing on the chain length distribution of each of the CER subclasses in relation to lipid organization and their correlation with skin barrier function (TEWL), disease severity (SCORAD), FLG mutations and NMF levels. This provides detailed information about the role of lipids in the impaired skin barrier function of non-lesional AE skin.

The results show a reduced average CER chain length in non-lesional skin of AE patients. This reduction in chain length can be attributed to an increase in extremely short C34 CERs as well as a reduction in very long CER [EO] subclasses. The increment in C34 CERs has recently been reported by Ishikawa et al. for a single subclass (CER [NS]) in lesional skin of AE patients (37). Here we show in non-lesional skin a largely increased level of C34 CERs in four CER subclasses: CER [NS], [NH], [AS] and [AH]. In addition, the results show changed levels in some of the CER subclasses consistent with previous reports: a decrease in CER [NP] level and an increase in CER [AS] level (12, 34-37, 54, 55). We did not observe a change in CER/protein levels between AE patients and controls. Interestingly, the reduction in CER chain length found in the present study had a much stronger impact on the skin barrier function than the changes in CER subclass levels: the TEWL increases proportionally with decreasing chain length. These findings are in excellent agreement with earlier in vitro studies showing that a reduction in chain length of CERs has a stronger impact on the lipid organization and permeability than a change in the ratio between CER subclasses keeping the chain length approximately equal (56-58), unpublished results M. Oguri, G.S. Gooris, J.A. Bouwstra).

Di Nardo et al. observed a reduction in CER/CHOL ratio in non-lesional skin of AE patients, while other studies do not report a decrease in CER content in non-lesional AE skin (20, 35-37, 54). Groen et al. (58) observed that when increasing the CER or FFA level while keeping the level of the other two main lipid classes equal, did not affect the permeability in vitro. Therefore these studies suggest that the CER composition and chain length rather than the ratio between lipid classes does play a major role in the increased TEWL in non-lesional skin in patients with AE.
The increment in C34 CERs and decrement in CER [EO] suggest that elongation of the acyl chains is reduced. As the elongase family plays an important role in the elongation of fatty acids in the viable epidermis, we hypothesize that the higher abundance of C34 CERs may be due to a misbalance in the activity of some of the members of the elongase family (59, 60).

Several publications report on the lipid organization in AE patients: Pilgram et al. reported changes in the lateral packing when comparing three AE patients with three controls (32). In a very recent study, we reported the first results on a very limited number of subjects focusing on the lamellar phases and ceramide subclasses without examining the CER chain lengths (61). In addition, several studies focused on the (delayed or incomplete) lamellar body extrusion process in AE (62-64) possibly caused by a reduced peroxisome proliferator-activated receptor activation (65). In the present study we were particularly interested in the influence of CER chain length on the lipid organization in AE patients.

With respect to the lamellar lipid organization, we observed a shift in peak II position of the SAXD curves of SC of AE patients. This indicates a reduced value of the repeat distances of the lamellar phases and/or a reduced formation of the LPP (28). The correlation between SAXD peak II position with CER [EO] and C34 CER levels indicates that changes in these CER levels affect the lamellar organization. When focusing on the lateral organization, AE patients show a less dense lipid packing compared to controls that correlates strongly with a higher level of C34 CERs. This shows that CER chain length is also an important determinant of the lateral lipid organization in SC. The observed changes in lamellar and lateral organization correlate with the increased TEWL levels and thus with an abnormal skin barrier function in patients with AE. The findings in this study strongly support the hypothesis that in AE patients a reduction in CER chain length leads to a change in lipid organization, which in turn leads to an impaired barrier function. In addition, the present study shows that this impaired barrier function is also correlated to the disease severity as determined by SCORAD, which is supported by literature (66, 67). This may indicate that as a result of inflammation, the lipid synthesis is influenced (even at non-lesional sites), and subsequently the barrier function is decreased.
Since FLG mutations are known to be predisposing factors for AE (17), an interesting question is whether lipid changes are associated with the presence of FLG mutations. We screened our subjects for four of the most prevalent FLG mutations, accounting for 93% of the European FLG mutation spectrum (38). In our study cohort there is no evidence that FLG mutations have an effect on CER composition and lipid organization. In contrast, in a recent study in ichthyosis vulgaris patients (68), changes in the lamellar organization were observed between the patients and controls. In that investigation, however, the majority of the patients was homozygote or compound heterozygote with respect to FLG mutations and no inflammation was observed in these patients.

In previous studies, as well as in the current study, AE patients with FLG mutations showed significantly reduced NMF levels (22, 69). Remarkably, in this study changes in lipids correlated with NMF levels but not with the presence or absence of FLG mutations. This suggests that between FLG gene (genotype) and NMF (phenotype), other (translational and environmental) factors may also influence NMF levels. These factors may include FLG copy numbers (repeat alleles on the FLG gene) (70) and interleukin levels, which can downregulate filaggrin expression (71). Changes in NMF levels are suggested to lead to a change in pH, and together with altered interleukin levels and protease activity this may affect enzymes involved in CER biosynthesis (5, 68, 72-74) and therefore change the CER composition and lipid organization. Thus, despite the fact that we did not find a correlation between the lipids and FLG mutation status, filaggrin might play an indirect role in the decreased barrier function of AE patients, although the underlying mechanism remains unclear. Besides, other barrier proteins may be involved. This will be subject of future studies in our group.

In conclusion, in this study we have shown that the CER chain length is altered in AE patients by elevated C34 CERs levels and reduced CER [EO] levels. These changes correlate with an altered lipid organization and a decreased barrier function in AE patients. In addition, a significant correlation was observed between disease severity and change in lipid composition and organization. Our results suggest a novel therapeutic entry to repair skin barrier defects in
AE patients, aiming at normalizing CER chain length distribution. Such a treatment could improve the SC lipid organization and restore the skin barrier function of AE patients.

Conflict of interest

At the time of the study P.J. Caspers was employee of River Diagnostics B.V.

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References


Figure legends

Figure 1: Structure and nomenclature of CERs. All CERs bear a polar head group and two long carbon chains. The polar head group may vary in molecular architecture (at the carbon positions marked in red), resulting in 12 different subclasses in human SC. Both chains in every CER subclass show varying carbon chain lengths (marked by red arrows). Each CER subclass is denoted by its sphingoid base (blue) and fatty acid chain (gray) resulting in the 12 CER subclasses. The abbreviations are as follows: For the sphingoid base: Dihydrosphingosine (dS), Sphingosine (S), Phytosphingosine (P), 6-hydroxy sphingosine (H). The various acyl chains are denoted by: Non-hydroxy fatty acid (N), α-hydroxy fatty acid (A) and esterified ω-hydroxy fatty acid (EO). This results in the 12 CER subclasses notations: [NdS], [AdS], [EOdS], [NS], [AS], [EOS], [NP], [AP], [EOP], [NH], [AH], [EOH]. The number of total carbon atoms in the CERs (e.g. C34 CERs) is the number of carbon atoms in the fatty acid chain plus the number of carbon atoms in the sphingoid base.

Figure 2: Schematic overview of the SC lipid parameters, clinical parameters and the determinants of the filaggrin content discussed in this article. These different parameters may all affect the skin barrier and are therefore investigated in this study. Arrows indicate possible correlations that are studied throughout the manuscript.
Figure 3: Lamellar and lateral organization in human stratum corneum. (1) The outermost layer of the epidermis, the stratum corneum (SC), consists of dead cells (corneocytes) embedded in a lipid matrix, also referred to as the brick (corneocytes) and mortar (lipids) structure (2). The intercellular lipids are arranged in layers (lamellae) (3), with two coexisting lamellar phases. These lamellar phases have a repeat distance of 6 nm (referred to as the short periodicity phase (SPP)) or 13 nm (referred to as the long periodicity phase (LPP)). The lateral organization 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.

Figure 4: SCORAD, TEWL and NMF levels in control subjects and AE patients. Dot plots showing individual control subjects (○ and ●) and AE patients (◇ and ▲) of the measured parameters (a) SCORAD, (b) TEWL and (c) NMF levels. 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 and AE patients for both TEWL and NMF. FLG mutations were associated with reduced NMF levels in AE patients (P<0.005) but not with SCORAD and TEWL.
Figure 5: CER composition in control subjects and AE patients. (a) Dot plot showing the average chain length of all CERs in total; the relative abundance of total C34 CERs; and the relative abundance of total [EO] CERs. (b) Dot plots indicating the relative abundance of C34 CER species for each subclass. (c) Scatter plot of univariate analysis of the predicted average chain length (by the abundance of C34 CERs and [EO] CERS) versus the observed average chain length. Gray dotted line represents the optimal fit (r=0.94):
\[
\text{Average chain length} = (0.33 \cdot \text{C34 CERs}) + (0.24 \cdot \text{CER}[EO]).
\]
(d) Scatter plot of univariate analysis of C34 CER and CER [EO] versus the TEWL. Insets show the residuals of the respective plots. Gray dotted line represents the optimal fit (r=0.77):
\[
\text{TEWL} = 8.2 + (4.6 \cdot \text{C34 CERs}) - (0.6 \cdot \text{CER}[EO]).
\]
Control subjects are indicated by ○ and ●. AE patients with are indicated by ◇ and ◆. Open and filled data points indicate carriers and non-carriers of FLG mutations, respectively.

Figure 6: Lipid organization in control subjects and AE patients. (a) The upper SAXD curve of a control subject shows the 1st (I), 2nd (II) and 3rd (III) order peak positions of the LPP. # indicates phase separated cholesterol. The middle diffraction curve is from an AE patient and resembles the pattern of SC of the control subject. The bottom curve of an AE patient shows only the presence of peak II. (b) Position of peak II in SAXD curves. (c) Position of the stretching vibrations in the FTIR spectrum. (d) Scissoring bandwidth in the FTIR spectrum. (e) Correlation between lipid organization and TEWL. Scatter plot of univariate analysis of SAXD peak II position + bandwidth of scissoring vibrations versus the TEWL. The inset shows the residuals of this plot. The gray dotted line represents the optimal fit (r=0.76):
\[
\text{TEWL} = -30.1 + (64 \cdot \text{SAXD peakII position}) - (2.3 \cdot \text{Bandwidth}).
\]
The correlation coefficient is 0.76. Control subjects are indicated by ○ and ●. AE patients with are indicated by ◇ and ◆. Open and filled data points indicate carriers and non-carriers of FLG mutations, respectively.
Tables

Table I: Correlation coefficients of the various CER subclasses versus TEWL. The table contains all 12 CER subclasses as well as the CERs that strongly influence the chain length (i.e. total CER [EO] and total C34 CERs).

<table>
<thead>
<tr>
<th>CER subclass</th>
<th>TEWL</th>
</tr>
</thead>
<tbody>
<tr>
<td>CER [EOdS]</td>
<td>-0.245</td>
</tr>
<tr>
<td>CER [EOS]</td>
<td>-0.177</td>
</tr>
<tr>
<td>CER [EOP]</td>
<td>-0.434**</td>
</tr>
<tr>
<td>CER [EOH]</td>
<td>-0.617***</td>
</tr>
<tr>
<td>CER [NdS]</td>
<td>-0.226</td>
</tr>
<tr>
<td>CER [NS]</td>
<td>0.407**</td>
</tr>
<tr>
<td>CER [NP]</td>
<td>-0.483**</td>
</tr>
<tr>
<td>CER [NH]</td>
<td>-0.420**</td>
</tr>
<tr>
<td>CER [AdS]</td>
<td>0.214</td>
</tr>
<tr>
<td>CER [AS]</td>
<td>0.650***</td>
</tr>
<tr>
<td>CER [AP]</td>
<td>0.255</td>
</tr>
<tr>
<td>CER [AH]</td>
<td>0.195</td>
</tr>
<tr>
<td>Total CER [EO]</td>
<td>-0.441**</td>
</tr>
<tr>
<td>Total C34 CERs</td>
<td>0.738***</td>
</tr>
<tr>
<td>Average CER chain length</td>
<td>-0.528***</td>
</tr>
</tbody>
</table>

Statistical differences on the $P<0.01$ level are labeled **.

Statistical differences on the $P<0.001$ level are labeled ***.
Table II: Correlation coefficients of lipid composition and lipid organization parameters.

<table>
<thead>
<tr>
<th>Lipid organization / CER composition parameters</th>
<th>Correlation coefficients (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAXD peak II position vs total C34 CER content</td>
<td>0.432**</td>
</tr>
<tr>
<td>SAXD peak II position vs total CER [EO] content</td>
<td>-0.393**</td>
</tr>
<tr>
<td>SAXD peak II position vs average CER chain length</td>
<td>-0.370*</td>
</tr>
<tr>
<td>FTIR scissoring bandwidth vs total C34 CER content</td>
<td>-0.669***</td>
</tr>
<tr>
<td>FTIR scissoring bandwidth vs total CER [EO] content</td>
<td>0.267</td>
</tr>
<tr>
<td>FTIR scissoring bandwidth vs average CER chain length</td>
<td>0.386*</td>
</tr>
<tr>
<td>FTIR stretching vs total C34 CER content</td>
<td>0.607***</td>
</tr>
<tr>
<td>FTIR stretching vs total CER [EO] content</td>
<td>-0.399*</td>
</tr>
<tr>
<td>FTIR stretching vs average CER chain length</td>
<td>-0.471**</td>
</tr>
</tbody>
</table>

Statistical differences on the $P<0.05$ level are labeled *.
Statistical differences on the $P<0.01$ level are labeled **.
Statistical differences on the $P<0.001$ level are labeled ***.
Table III: Correlations between the various parameters.

<table>
<thead>
<tr>
<th>Correlated parameters</th>
<th>Correlation coefficients (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEWL vs SCORAD</td>
<td>0.560**</td>
</tr>
<tr>
<td>TEWL vs SAXD peak II position</td>
<td>0.450**</td>
</tr>
<tr>
<td>TEWL vs FTIR scissoring bandwidth</td>
<td>-0.735**</td>
</tr>
<tr>
<td>TEWL vs FTIR stretching</td>
<td>0.645**</td>
</tr>
<tr>
<td>TEWL vs total C34 CER content</td>
<td>0.738**</td>
</tr>
<tr>
<td>TEWL vs total CER [EO] content</td>
<td>-0.441**</td>
</tr>
<tr>
<td>TEWL vs average CER chain length</td>
<td>-0.528**</td>
</tr>
<tr>
<td>TEWL vs NMF</td>
<td>-0.643**</td>
</tr>
<tr>
<td>SCORAD vs SAXD peak II position</td>
<td>0.164</td>
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<tr>
<td>SCORAD vs FTIR scissoring bandwidth</td>
<td>-0.474*</td>
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<tr>
<td>SCORAD vs FTIR stretching</td>
<td>0.534**</td>
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<tr>
<td>SCORAD vs total C34 CER content</td>
<td>-0.470*</td>
</tr>
<tr>
<td>SCORAD vs total CER [EO] content</td>
<td>-0.238</td>
</tr>
<tr>
<td>SCORAD vs average CER chain length</td>
<td>-0.265</td>
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<tr>
<td>SCORAD vs NMF</td>
<td>-0.362*</td>
</tr>
<tr>
<td>NMF vs SAXD peak II position</td>
<td>-0.501**</td>
</tr>
<tr>
<td>NMF vs FTIR scissoring bandwidth</td>
<td>0.697**</td>
</tr>
<tr>
<td>NMF vs FTIR stretching</td>
<td>-0.778**</td>
</tr>
<tr>
<td>NMF vs total C34 CER content</td>
<td>-0.611**</td>
</tr>
<tr>
<td>NMF vs total CER [EO] content</td>
<td>0.416**</td>
</tr>
<tr>
<td>NMF vs average CER chain length</td>
<td>0.456**</td>
</tr>
</tbody>
</table>

Note: the SCORAD indicates the severity of AE, and gives only valid values when AE is diagnosed. Therefore all control subjects were excluded in these specific correlations.

* Correlation is significant at the 0.05 level.

** Correlation is significant at the 0.01 level.
### Figure 1

<table>
<thead>
<tr>
<th></th>
<th>Non-hydroxy fatty acid</th>
<th>α-hydroxy fatty acid CERs</th>
<th>Esterified α-hydroxy fatty acid CERs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dihydrosphingosine [dS]</td>
<td>[dS]</td>
<td>[dA]</td>
<td>[dEO]</td>
</tr>
<tr>
<td>Sphingosine [S]</td>
<td>[S]</td>
<td>[S]</td>
<td>[EO]</td>
</tr>
<tr>
<td>Phytosphingosine [P]</td>
<td>[P]</td>
<td>[P]</td>
<td>[EOP]</td>
</tr>
<tr>
<td>6-hydroxy sphingosine [H]</td>
<td>[H]</td>
<td>[H]</td>
<td>[EOH]</td>
</tr>
</tbody>
</table>
Figure 2

Clinical parameters
- Skin barrier function: TEWL
- AE severity: SCORAD

FLG content
- FLG genotype
- NMF levels

Ceramide composition
- CER subclasses
- CER chain length

Lipid organization
- Lamellar organization
- Lateral organization
- Conformational ordering
Figure 3
Figure 4

(a) SCORAD

(b) TEWL

(c) NMF
Figure 6

(a) FTIR stretching peak position (cm⁻¹) and FTIR scissoring bandwidth (cm⁻¹) for Control and AE.

(b) SAXD peak II position (nm⁻¹) for Control and AE.

(c) FTIR stretching peak position (cm⁻¹) for Control and AE.

(d) FTIR scissoring bandwidth (cm⁻¹) for Control and AE.

(e) TEWL (g/m²) vs. Lipid organization (SAXD Peak II position [lamellar organization] and FTIR scissoring bandwidth [lateral organization]).