



Electron diffraction study of lipids in non-lesional stratum corneum of atopic eczema patients

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ABSTRACT

Skin barrier impairment is thought to be an important factor in the pathogenesis of atopic eczema (AE). The skin barrier is located in the stratum corneum (SC), consisting of corneocytes embedded in lipids. Ceramides, cholesterol and free fatty acids are the major lipid classes and are crucial for the skin barrier function, but their role in relation to AE is indistinct. Filaggrin is an epidermal barrier protein and common mutations in the filaggrin gene strongly predispose for AE. However, there is no strong evidence that filaggrin mutations are related to the reduced skin barrier in AE. In this study, electron diffraction is used in order to study the lipid organization of control SC and non-lesional SC of AE patients *in vivo*. An increased presence of the hexagonal lipid organization was observed in non-lesional SC of AE patients, indicating a less dense lipid organization. These changes correlate with a reduced skin barrier function as measured with transepidermal water loss but do not correlate with the presence of filaggrin mutations. These results are indicative for the importance of the lipid organization for a proper skin barrier function.

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1. Introduction

The barrier function of the skin is located in the stratum corneum (SC) which consists of corneocytes embedded in lipids. Several papers report on an impaired skin barrier function not only in lesional, but also in non-lesional skin in patients with atopic eczema (AE) [1–8]. AE is an inflammatory skin disease affecting almost 20% of Caucasian children and 2–10% of adults, with increasing prevalence [9,10]. Patients with active AE have lesional skin regions that appear red, itchy and flaky. The causes of AE are still unknown, but its prevalence has been associated with loss-of-function mutations of the filaggrin gene (*FLG*) [11–14].

In human SC, lipids are organized in layers in between the corneocytes, forming two lamellar phases with repeat distances of approximately 6 and 13 nm, referred to as the short periodicity phase (SPP) and long periodicity phase (LPP), respectively [15,16].

Abbreviations: AE, Atopic eczema; BSA, Bovine serum albumin; BCA, Bicinchoninic acid; CER, Ceramide; ED, Electron diffraction; FEG, Field emission gun; FLG, Filaggrin; FTIR, Fourier transform infrared spectroscopy; HEX, Hexagonal; LPP, Long periodicity phase; ORTH, Orthorhombic; SC, Stratum corneum; SPP, Short periodicity phase; TEM, Transmission electron microscope; TEWL, Transepidermal water loss

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Within these stacked lipid layers, the lipids form mainly a dense, orthorhombic lateral organization, although a subpopulation of the lipids also forms a less dense hexagonal lateral organization, as determined with Fourier transform infrared spectroscopy (FTIR) and electron diffraction (ED) [17–19]. Ceramides (CERs), cholesterol and free fatty acids are the three most abundant lipid classes in SC. Several studies report that the lipid composition is altered in AE patients compared to controls [4,20–23]. In addition, it was found that the CER chain length is important for a proper lipid organization and skin barrier function [24,25].

In the present study we used ED as a tool to examine the lateral lipid organization of healthy SC and non-lesional SC of AE patients *in vivo*. This method is used in combination with non-invasive tape-stripping. The advantage of ED over FTIR is that it provides more detailed information as it samples the lateral organization very locally.

In a previous study Pilgram et al. [19] used ED to study the lipid organization in 3 controls and 3 AE patients. However, we recently showed that there is a large variation in the lipid organization in patients with AE [25]. Therefore, a larger number of patients is warranted. In addition, in the study of Pilgram et al. the changes in lipid organization were not related with the skin barrier function and presence of filaggrin mutations [19]. Therefore, besides the larger patient population, the lateral organization was correlated with the skin barrier function as assessed by transepidermal water loss (TEWL) and the presence of *FLG* mutations.

In our studies we observed a higher abundance of diffraction patterns that indicate the presence of a hexagonal lipid organization in non-lesional AE skin compared to control SC. These changes in lipid organization correlated with an increased TEWL observed in AE patients. The presence of *FLG* mutations did not affect the lipid organization.

2. Materials and methods

2.1. 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 control 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 carriers of *FLG* 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 took place in a humidity and temperature controlled room.

2.2. *FLG* mutation analysis

We screened all subjects on the four most prevalent mutations found in European Caucasians, covering around 93% of all *FLG* mutations known to date [26]: 2282del4, R501X, S3247X and R2447X. 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. After DNA extraction mutations were determined by genotyping [27]. Here, subjects with one (or more) of the above mentioned mutations are called 'carriers' of *FLG* mutations and subjects without the studied mutations are called 'non-carriers' of *FLG* mutations.

2.3. Tape stripping procedure

Multiple poly(phenylene sulfide) tape strips (Nichiban, Tokyo, Japan) were successively applied at the same area (4.5 cm^2) on the 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 proteins removed, in order to obtain a good indication of the depth the SC for ED was harvested [28,29]. After 10 tape strips, grid strips for ED analysis were taken. Calibration was performed by a bicinchoninic acid (BCA) assay using bovine serum albumin (BSA). The total amount of protein in SC was calculated by plotting $1/\text{TEWL}$ against the cumulative amount of protein removed. The intercept with the x-axis after extrapolation is indicative for the total amount of proteins in the SC according to Kalia [28].

2.4. Skin barrier function assessment by TEWL

A Tewameter TM 210 (Courage + Khazaka, Köln, Germany) was used to measure TEWL. 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 s of the measurement was calculated. This procedure was performed before tape stripping and after each two tape strips.

2.5. Electron diffraction

Grids (thin bar mesh-700 hexagonal, Agar scientific, Stansted, UK) were dipped into a 3% w/v glue/chloroform solution (glue: T406-30, Avery Dennison, Leiden, The Netherlands in chloroform: Biosolve, Valkenswaard, The Netherlands). After blotting the excess of chloroform

with a filter paper, the grids were air dried, leaving a sticky layer of glue on the grid bars.

A total of 12 grids were pressed to the arm during 5 s and taken off and directly cryo-fixed in liquid nitrogen and stored until use. Upon use, grids were put into a pre-cooled Gatan 626 cryo holder (Gatan, München, Germany) and examined in a FEI Tecnai 200 kV cryo transmission electron microscope (TEM) (FEI Company, Eindhoven, The Netherlands) with a Field Emission Gun (FEG) source at 200 kV with spotsize 11. No selected area aperture was used. Diffraction patterns were recorded at a camera length of 1000 mm.

A fixed area of approximately $0.78 \mu\text{m}^2$ was selected for diffraction. This small area was chosen in order to decrease the number of exposed lipid crystals and therefore increase the possibility of obtaining diffraction spots instead of rings. This allows discrimination between the orthorhombic and hexagonal lipid organization.

The electron dose rate was $0.75 \text{ e}^- \text{ \AA}^{-2} \text{ s}^{-1}$. In order to prevent electron beam damage and disappearance of diffraction spots an exposure time of 2 s was used, resulting in a dose of $1.5 \text{ e}^- \text{ \AA}^{-2}$. A longer exposure time between 4 and 6 s resulted in weaker diffraction patterns and subsequent disappearance of spots. Diffraction patterns were digitally recorded on a Gatan $4\text{K} \times 4\text{K}$ CCD camera (Gatan, München, Germany), with binning 2. Pictures were stored as a DM3 file and examined with Image J. Spacing of the reflections in the ED patterns was calculated using the equation $Rd = \lambda L$, which is deduced from Bragg's Law. The known ED pattern of gold was used to calibrate the constant factor λL , in which λ is the wavelength of electrons and L the camera length. The distance of a recorded reflection to the central beam spot (R) was determined with Image J. In this way the repeat distance (d) could be calculated. ED patterns were scored independently by three persons into five categories as described in the results section.

2.6. Statistical analysis

Statistical analysis was performed using SPSS Statistics. A repeated measurement generalized linear model analysis was performed to investigate the difference in the ED patterns of control subjects and AE patients as well as the influence of *FLG* mutations. Significance level was set at $p < 0.05$.

3. Results

ED patterns were obtained from grids taken after removal of 10 tape strips. Fig. 1 shows an example of a TEM picture from a grid



Fig. 1. Grid containing SC visualized with TEM. The scale bar is $10 \mu\text{m}$.

containing SC. First, we compared the depth at which the grids were taken. For controls, the grids were taken after removing $42.5 \pm 2.6\%$ of the total mass of SC proteins and in AE patients this was $46.9 \pm 2.5\%$. This indicates that the grids for ED analysis were taken at a similar SC depth in the two groups ($p > 0.05$).

3.1. Interpretation of ED patterns

When the lipids are oriented perpendicular to the incident electron beam (Fig. 2a) this results in reflections that provide information about the lateral lipid organization.

Fig. 2b shows schematic pictures of the arrangement of the lipid acyl chains (blue circles) in a hexagonal lattice (left) and an orthorhombic lattice (right) when the lipid chains are oriented perpendicular to the incident electron beam. In a hexagonal organization the distances

between the lattice planes in various directions are equal, being 0.41 nm. In the orthorhombic lattice the lipids are denser packed, in which in one direction the lattice planes have a distance of 0.37 nm, while in the other two directions this distance is 0.41 nm. The corresponding diffraction patterns when only one orientation of the crystals is in the electron beam are provided in Fig. 2c. In a hexagonal organization (middle) the three pairs of reflections have interplanar angles of 60° . In the orthorhombic lattice the diffraction pattern consists of two reflections with interplanar angles of 68° and 56° (right). Here, two out of six reflections are located at a larger distance from the primary electron beam.

When a large number of differently oriented crystals are exposed the large number of spots forms rings (Fig. 2d); in a hexagonal arrangement one strong ring at a spacing of 0.41 nm is visible (middle). However the presence of an orthorhombic lipid organization cannot be excluded as

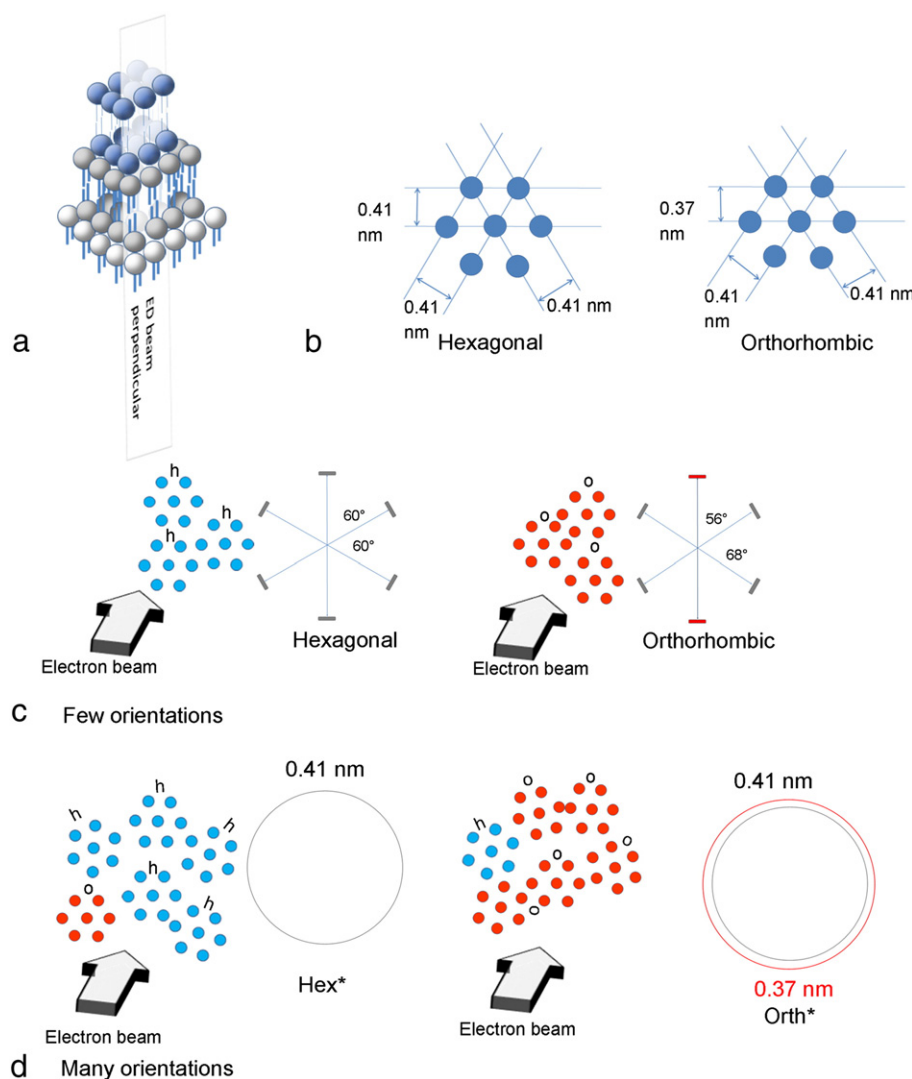


Fig. 2. Explanation of hexagonal and orthorhombic lipid organization and corresponding ED patterns. (a) Perpendicular orientation of the lipids with respect to the incoming electron beam. In this orientation there are various lattice planes that give rise to reflections in the ED pattern simultaneously. The positions of these reflections give information about the lateral lipid organization. (b) The ordering of the hydrocarbon chains of the lipids (blue and red circles). In a hexagonal organization, the spacings between the lattice planes are 0.41 nm (left). In the orthorhombic lattice the lipids are tighter organized, resulting in a spacing of 0.37 nm in one direction while in the other two directions this is 0.41 nm (right). (c) The corresponding diffraction patterns when only a few orientations of the crystals are in the electron beam. In a hexagonal lattice, the three pairs of reflections have interplanar angles of 60° (left). In the orthorhombic lattice the diffraction pattern consists of reflections with interplanar angles of 68° and 56° (right). (d) The corresponding diffraction patterns consist of rings when a large number of differently oriented crystals are exposed. In a hexagonal arrangement one strong ring at a spacing of 0.41 nm is visible (left). However the presence of an orthorhombic lipid organization cannot be excluded as the outer ring in the orthorhombic pattern is less intense than the inner ring. This pattern is therefore referred to as *hex**. When dealing with an orthorhombic lattice the diffraction pattern consists of two strong rings at spacings of respectively 0.41 nm and 0.37 nm (right). As the inner reflection is at the same spacing as the hexagonal pattern it is not possible to determine whether also a hexagonal lateral packing is present. This category is therefore referred to as *orth**. h = hexagonal, o = orthorhombic.

the outer ring in the orthorhombic pattern is less intense than the inner ring. This pattern is therefore referred to as *hex**. When dealing with an orthorhombic lattice the diffraction pattern consists of two strong rings at spacings of respectively 0.41 nm and 0.37 nm (right). As the inner reflection is at the same spacing as the hexagonal pattern it is not possible to determine whether also a hexagonal lateral packing is present. This pattern is therefore referred to as *orth**.

Occasionally, higher order reflections with spacings of 0.22 and 0.24 nm are also observed.

We used an exposure time of 2 s (electron dose $1.5 \text{ e}^- \text{ \AA}^{-2}$) in order to obtain the ED patterns. We confirmed that this did not lead to radiation damage by making series of diffraction patterns with exposure times varying between 1 to 10 s. A too high electron beam intensity damages the lipids leading to disappearance of the crystal structure and broadening and fading away of the ED patterns. This occurs at exposure times higher than 4 s.

The obtained ED patterns were classified into five groups: *hex* = hexagonal; *hex** = hexagonal, however the presence of an orthorhombic organization cannot be excluded; *hex + orth* = combination of hexagonal and orthorhombic organization; *orth** = orthorhombic, however the presence of hexagonal organization cannot be excluded; *orth* = orthorhombic.

Fig. 3 shows representative examples of ED patterns in each of the above mentioned categories. Fig. 3a and b show three pairs of reflections at 0.41 nm which have an interplanar angle of 60° indicative for the presence of mainly one hexagonal lattice in the beam area. Fig. 3c shows an orthorhombic ED pattern with two pairs of reflections at 0.41 nm (red arrows) and one pair of reflections at 0.37 nm (green arrows), with

interplanar angles of 68° and 56° , respectively. This indicates the presence of one orthorhombic orientation in the beam. Fig. 3d shows an ED pattern of three different orthorhombic lattices that are rotated over an angle of 60° relative to each other (indicated by red, green and blue arrows), also demonstrating only the presence of an orthorhombic lateral packing, but now in at least three different orientations.

In addition to the above mentioned categories, combinations of orthorhombic and hexagonal patterns exist. Fig. 3e and f show ED patterns in which both the hexagonal and orthorhombic lattice are observed; both reflections with an interplanar angle of 60° (indicative for the hexagonal lipid organization, see white arrows) as well as reflections with interplanar angles of 68° and 56° (indicative for the orthorhombic lipid organization, see red arrows) are present. In Fig. 3g and h two rings are observed located at 0.41 and 0.37 nm. This indicates that the orthorhombic lipid organization is present. However the hexagonal lattice cannot be excluded, as the hexagonal pattern consists of a single ring at a spacing of 0.41 nm that might be obscured by the inner diffraction ring of the orthorhombic packing. Fig. 3i and j show examples of hexagonal ED patterns in which the presence of an orthorhombic lipid organization cannot be excluded: only one ring at a spacing of 0.41 nm is observed. In this case it might be possible that only a few lipids are in the beam and that the orthorhombic reflections at 0.37 nm are therefore too weak to be visible.

3.2. ED patterns in control SC and SC of AE patients

Out of 28 patients and 15 control subjects, we could not obtain ED patterns from 4 AE patients and 3 controls as their grids did not

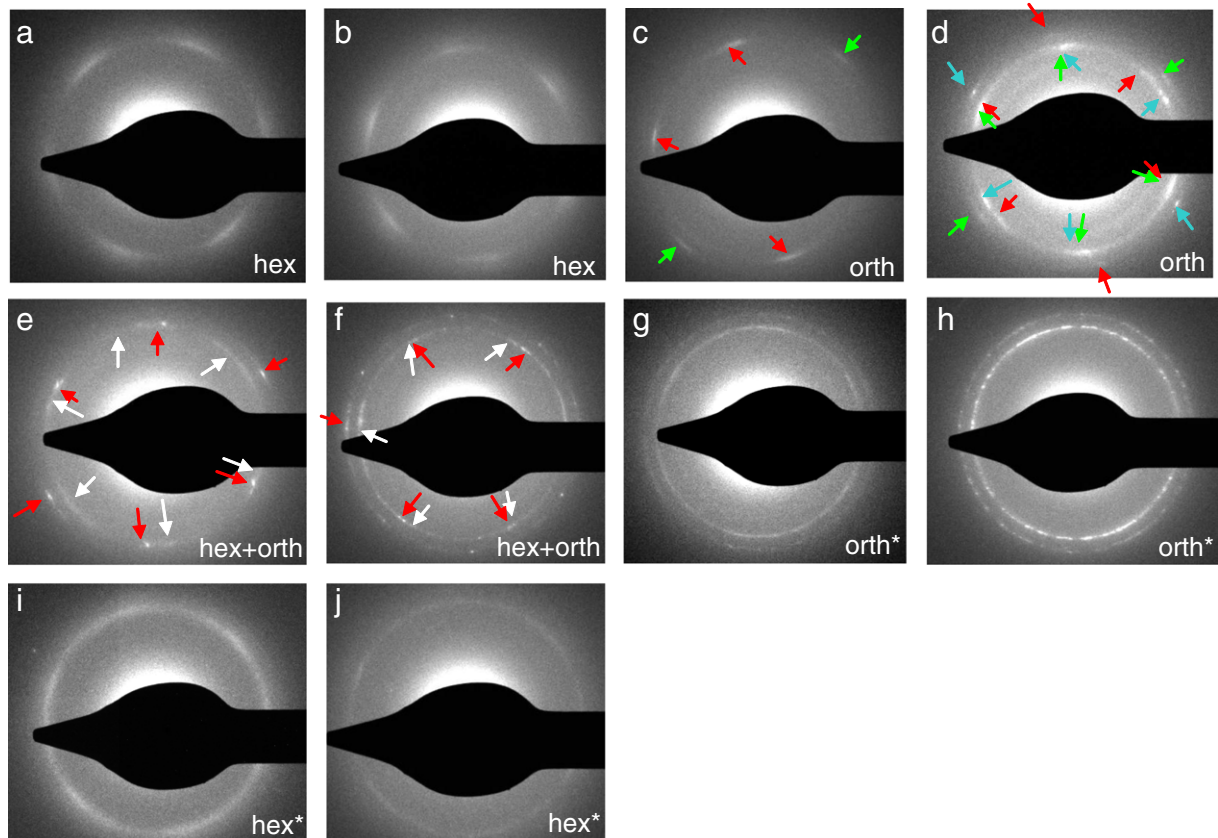


Fig. 3. Representative ED patterns recorded in human SC. (a) and (b) Three pairs of reflections at 0.41 nm which have an interplanar angle of 60° indicative for the presence of a hexagonal lattice. (c) Orthorhombic ED pattern with two pairs of reflections at 0.41 nm (red) and one pair of reflections at 0.37 nm (green) with interplanar angles of 66° and 57° . (d) ED pattern in which three differently oriented orthorhombic lattices are present in the beam area (indicated by red, green and blue arrows). (e) and (f) Combination of hexagonal and orthorhombic organization. The orthorhombic reflections appear as spots (red arrows) and the hexagonal reflections appear as arcs (white arrows). In f at least three different orthorhombic orientations are observed. (g) and (h) Two rings located at 0.41 and 0.37 nm, indicative for an orthorhombic lipid organization, however the presence of a hexagonal organization cannot be excluded. (i) and (j) Ring located at 0.41 nm indicative for a hexagonal lipid organization, however the presence of orthorhombic organization cannot be excluded.

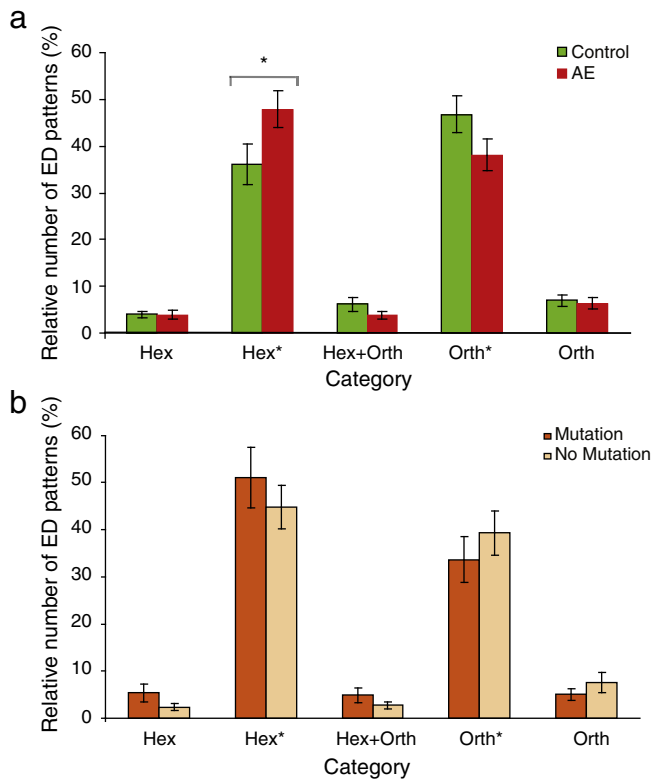


Fig. 4. Occurrence of ED patterns in the different categories for controls and AE patients. (a) Relative number of ED patterns ($\pm$ S.E.M.) in control SC ($n = 12$) and AE SC ($n = 24$). (b) Relative number of ED patterns ($\pm$ S.E.M., $n = 12$ per group) in carriers and non-carriers of *FLG* mutations. *Hex* = hexagonal; *hex** = hexagonal, however the presence of orthorhombic organization cannot be excluded; *hex + orth* = combination of hexagonal and orthorhombic organization; *orth** = orthorhombic, however the presence of hexagonal organization cannot be excluded; *orth* = orthorhombic. * indicates a statistical difference on the $p < 0.05$ level.

contain sufficient SC. At least 50 ED patterns were recorded and scored of all the other subjects. A total of 956 ED patterns of control subjects were scored, as well as 1662 ED patterns of AE patients. Per group, the percentage of ED patterns was calculated for each of the

5 described categories above. Fig. 4a shows the distribution profile of the ED patterns that have been obtained from controls (green) and non-lesional SC of AE patients (red). As can be observed from Fig. 4a, the highest percentage of ED patterns was either *hex** or *orth**. There is a significant increase in the relative occurrence of *hex** ED patterns in AE patients compared to controls ($47.9 \pm 3.9\%$ and $36.1 \pm 4.3\%$, respectively, $p < 0.05$). A trend was observed for the category *orth**: this pattern was found less frequently in AE SC compared to controls, although not significant ($38.1 \pm 3.4\%$ and $46.8 \pm 3.9\%$, respectively, $p = 0.08$). For the other categories there were no significant differences found: $3.9 \pm 1.0\%$ *hex* in AE compared to $4.0 \pm 1.0\%$ *hex* in controls; $3.8 \pm 0.9\%$ *hex + orth* in AE compared to $6.1 \pm 1.5\%$ *hex + orth* in controls and $6.3 \pm 1.3\%$ *orth* in AE compared to $6.9 \pm 1.2\%$ *orth* in controls (all $p > 0.1$). Overall, the orthorhombic organization predominates in control SC (categories *orth** and *orth*), whereas in non-lesional AE SC the hexagonal ED patterns predominate (categories *hex** and *hex*).

Furthermore, we determined the spacings of the hexagonal and orthorhombic reflections in 5 controls and 5 AE patients. In controls these spacings were 0.409 ± 0.01 nm and 0.363 ± 0.009 nm, respectively. In AE patients these were 0.412 ± 0.01 nm and 0.368 ± 0.009 nm. No significant difference in spacing between controls and AE was observed ($p > 0.14$).

When investigating the effect of *FLG* mutations on the ED patterns, no relation was observed between the frequency of occurrence of the various diffraction patterns and *FLG* mutations ($p > 0.13$ Fig. 4b).

Besides scoring the ED patterns into the above mentioned categories, the relative number of ED patterns in which cholesterol reflections were observed was also determined.

Fig. 5a shows an example of an ED pattern in which cholesterol reflections are visible reflecting the presence of phase separated cholesterol. The cholesterol reflections are located at a spacing of 0.51 nm (see arrow). The presence of the ring demonstrates that the cholesterol crystals are randomly oriented. Fig. 5b shows the relative number of ED patterns in which cholesterol reflections were observed in control SC (green) and SC of AE patients (orange). The percentage of ED patterns containing cholesterol reflections was not different between controls and AE SC: $15.8 \pm 4.2\%$ and $15.6 \pm 2.5\%$, respectively ($p > 0.85$). In addition, there was no difference between carriers and non-carriers of *FLG* mutations in the percentage of ED patterns containing cholesterol ($14.0 \pm 3.1\%$ and $17.2 \pm 4.1\%$, respectively, $p > 0.60$).

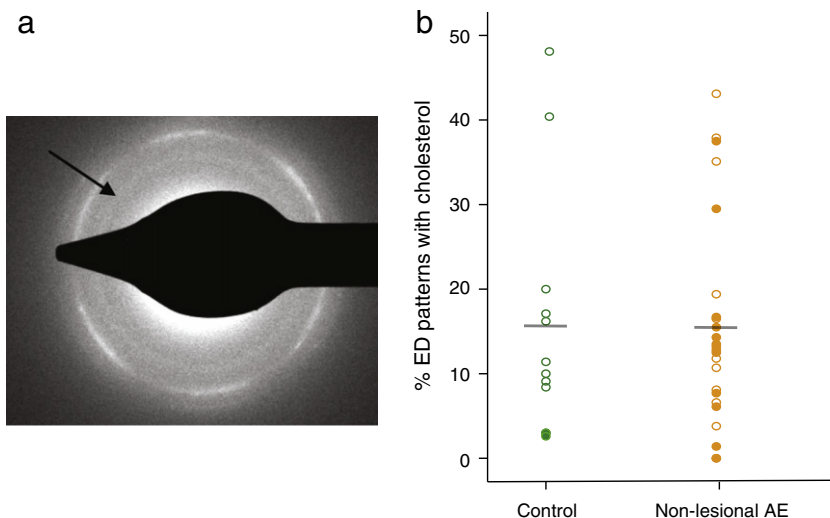


Fig. 5. Presence of cholesterol in ED patterns. (a) Diffraction pattern in which cholesterol reflections with spacings of 0.51 nm are observed (see arrow). (b) Percentage of ED patterns in which cholesterol spacings were observed in control SC (green) and AE SC (orange). Open and filled dots indicate non-carriers and carriers of *FLG* mutations, respectively.

3.3. Correlation between lipid organization and skin barrier function

Next, we studied whether these changes in lipid organization result in a reduced skin barrier function. As the grid strips for ED analysis were taken after removal of 10 tape strips, we correlated TEWL values at this depth with ED results.

Fig. 6a shows the TEWL after removal of 10 tape strips. After removal of 10 strips the TEWL is significantly higher in AE compared to controls: $16.5 \pm 1.6 \text{ g}^{-2} \text{ h}^{-1}$ and $9.0 \pm 0.7 \text{ g}^{-2} \text{ h}^{-1}$ in AE patients and control subjects, respectively, $p < 0.001$.

As hexagonal patterns predominate in the categories *hex* and *hex**, we combined the percentages of these categories in order to study the correlation between TEWL and hexagonal lipid organization. In order to study the correlation between TEWL and the presence of an orthorhombic lipid organization, we also combined the categories *orth* and *orth**, as these categories mainly consist of orthorhombic organized lipids.

The correlation between the relative number of hexagonal ED patterns and TEWL is plotted in Fig. 6b. The correlation coefficient is $r = 0.61$ ($p < 0.001$). From the correlation it becomes clear that TEWL is higher when the amount of lipids that is predominantly hexagonally organized, is increased. The correlation coefficient between TEWL and orthorhombic ED patterns is $r = -0.52$ ($p < 0.005$, Fig. 6c), indicating a lower TEWL when the amount of lipids with an orthorhombic organization is increased.

4. Discussion

We investigated the lipid organization in SC of controls and non-lesional SC of AE patients with ED. This technique gives information on the local variation of the SC lipid organization. We studied the effect of *FLG* mutations on the ED patterns and correlated the obtained results to the skin barrier function.

With ED it is possible to study non-invasively the lateral lipid organization of SC *in vivo*. It is used in combination with tape-stripping and can therefore give information about the lipid organization at various depths in SC. Here, we studied the lipid organization after removal of 10 tape-strips. In this way we made sure not to measure surface contamination or sebum, which may result in a relatively higher amount of obtained hexagonal ED patterns [30].

As the lipid organization is dependent on the SC depth [19], we first checked whether the grids for ED analysis were taken in the same range for both groups. The depth range at which the grids were taken for ED analysis was comparable between controls and AE patients. This demonstrates that we can directly compare the results obtained from the

controls with those from the AE patients and that observed changes in lipid organization between controls and AE patients are not a result of different SC depths at which grids were taken. In addition we checked whether the procedure of cryo-fixation and exposing to the electron beam would change the lipid organization in our samples. In a previous publication of Pilgram et al. we already showed that when using lipid mixtures consisting of CERs, cholesterol and fatty acids (mimicking the lipid composition in SC) equilibrated either at room temperature or at 32°C , the lipid organization after cryo-fixation was orthorhombic and hexagonal, respectively, similar as prior to cryo-fixation [30]. A similar control experiment was performed in this study, but now varying the lipid composition: mixtures prepared from CERs and cholesterol resulted in an ED pattern of an hexagonal packing, while mixtures prepared with CERs, cholesterol and fatty acids resulted in an ED pattern of an orthorhombic packing. This is the same phase behavior as detected by X-ray diffraction, again confirming that the lateral packing is preserved during the procedure we use.

We classified the obtained ED patterns into 5 categories. As the amount of SC on the grids varied between subjects we did not obtain the same number of ED patterns for every subject (between 50 and 120 patterns). In order to ensure equal contribution of every subject within each group the total amount of ED patterns per subject was included in the statistical model that was used.

Our results show that the most frequently encountered lateral packing is orthorhombic in controls. *Orth** was scored most often in controls. In this category the presence of a hexagonal lipid organization cannot be excluded, however it is very likely that most of the patterns in this category indicate mainly orthorhombic organized lipids. We did not observe the orthorhombic packing as frequently as previously reported by Pilgram et al. [19,30]. This difference might be caused by the smaller area ($0.78 \mu\text{m}^2$) that was selected for diffraction in our studies compared to theirs (1 and $20 \mu\text{m}^2$). Due to the smaller area selected for diffraction there is i) a higher probability to have only hexagonal organized lipids in the beam and ii) a lower number of exposed lipids. Therefore, in order to detect the outer less intense diffraction ring in the diffraction pattern, the fraction of lipids forming the orthorhombic lateral packing that is exposed to the electron beam should be higher compared to that in the study of Pilgram et al. If this ring is not detectable, the pattern is scored as being in the *hex** category.

We observed an increase in the relative number of hexagonal ED patterns in non-lesional SC of AE patients, indicating a less dense lipid organization in SC of AE patients. These changes in lipid organization can be explained by an altered lipid composition in non-lesional SC of AE patients. Several studies have reported significant changes in CER subclass composition between the lipid classes in non-lesional SC of

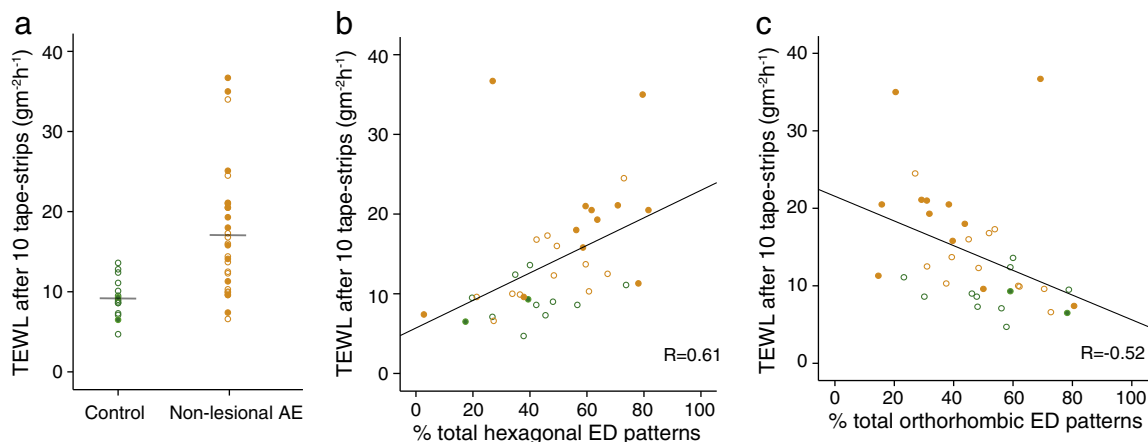


Fig. 6. Correlation between skin barrier function and lipid organization. (a) TEWL in control SC and AE SC after removal of 10 tape strips. (b) Correlation between TEWL and presence of hexagonal ED patterns ($r = 0.61$). (c) Correlation between TEWL and presence of orthorhombic ED patterns ($r = -0.52$). Open and filled dots indicate non-carriers and carriers of *FLG* mutations, respectively and green and orange dots represent controls and AE patients, respectively.

AE patients [4,20–23]. Furthermore, a decreased chain length of CERs and free fatty acids as well as a higher degree of unsaturated fatty acids are observed in AE [24,25] (and unpublished results). Previous *in vitro* studies have shown that i) shorter lipid chains, ii) a lower percentage of free fatty acids and iii) an increase in unsaturated lipid chains induce a hexagonal lipid organization [31,32] (and unpublished results).

The occurrence of the ED patterns (rings, arcs) depends on the number of lipid crystals that is exposed in the area selected for diffraction; when only a few crystals are exposed the diffraction pattern consists of arcs. As we did not observe significant differences in the categories *orth* and *hex* (both containing ED patterns with arcs) between both groups this indicates that there are no large differences in the size of the lipid crystals in SC of controls and AE patients.

The next step was to determine whether changes in lipid organization as observed with ED correlate with the impaired skin barrier function in AE. The observed correlations show clearly that an increased percentage of ED patterns indicating a predominant hexagonal lipid organization corresponds to a higher TEWL. In the same line, the increased ED patterns reflecting mainly a dominant orthorhombic packing correlated with a lower TEWL. This corresponds excellently with *in vivo* FTIR measurements of Damien et al. [33]. They observed a correlation between the skin barrier function and extent of SC lipids organized in orthorhombic lattices. This indicates that the less dense lipid organization in AE patients plays a role in the decreased barrier function in these patients. The study of Groen et al. [34] showed that a change from an orthorhombic to hexagonal lipid organization did not affect the permeability of the model compound benzoic acid. However, these experiments were carried out with *in vitro* lipid mixtures. This difference may be explained by physical properties (water vs benzoic acid) and inside out (TEWL) vs outside-in permeability (benzoic acid). Also, the lipid mixture that Groen et al. used has a high uniformity in lipid chain length and degree of saturation, which may lead to a more crystalline structure, whereas *in vivo* there is a large range of chain lengths of the CERs.

ED is a complementary method to FTIR to study the lateral lipid organization in SC. In case of ED, the method is sampling the local lateral packing, while FTIR is an averaging technique. As a result ED provides very local information on the SC lipid organization. Our previous FTIR results showed a significant decrease in the bandwidth of the CH₂ scissoring peak in AE SC. This bandwidth, the maximum of which is around 12 cm⁻¹, is affected by the fraction of lipids forming an orthorhombic packing as well as the size of orthorhombic domains [35]. The maximum bandwidth due to the domain sizes is already achieved when the orthorhombic domains are only around 100 lipid chains, thus being still rather small. The bandwidth is around 5 cm⁻¹ when all lipids are organized in a hexagonal packing [35]. With ED, more detailed information is obtained about the local variability of the lipid organization in SC; it provides information on the presence of orthorhombic as well as hexagonal lipid domains, but also lipid domains containing a combination of both types of lipid organization. Our results demonstrate that in around 6.5% of the patterns only an orthorhombic lateral packing is detected in the electron beam, suggesting that areas of at least 0.78 μm² with only an orthorhombic lateral packing exist in both AE and control skin. In addition such regions with only a hexagonal packing are also observed, but the frequency is only ~4% of the total number of patterns in AE and controls. As in our studies the majority of the ED patterns consists of rings, this at least suggests that the size of the crystal domains in these regions is rather small.

To date, *FLG* mutations are a well known predisposing factor for AE [11–14]. However, until now no clear correlation has been observed between *FLG* mutations and an impaired skin barrier function in AE [3,5,36]. Subjects were screened for four of the most prevalent *FLG* mutations, accounting for 93% of the European *FLG* mutation spectrum [26]. Our results show that the distribution of ED patterns and thus the lateral lipid organization does not differ between

carriers and non-carriers of *FLG* mutations within the group of AE patients. This is in agreement with a previous study in which no correlations were found between *FLG* mutation status and lipid organization measured with FTIR [25].

In conclusion, the results described in this paper show that there is an increased amount of hexagonally organized lipids in non-lesional SC of AE patients. This change in lipid organization leads to a decreased skin barrier function as measured by TEWL. The observed changes are not correlated with the presence of *FLG* mutations. Taken together, these results suggest the important role of the lipids in the skin barrier and future treatment may aim at normalizing lipid composition, and therefore lipid organization and skin barrier function in AE patients.

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