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Lipid to protein ratio plays an important role in the skin barrier function of atopic eczema patients

Running head: Lipid/protein ratio in atopic eczema skin

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Conflict of interest

The authors P.J. Caspers and G.J. Puppels are employees of RiverD International BV.

Bulleted statements

What's already known about this topic?

Filaggrin mutations are a predisposing factor for atopic eczema (AE).

In non-lesional and lesional stratum corneum (SC) of AE patients the intercellular lipid composition and organisation are altered.

Changes in the SC lipid properties correlate with a reduced skin barrier function, but are not associated with filaggrin mutations.

What does this study add?

The dry SC mass, being a measure for SC thickness, is only lower in lesional AE SC compared with SC from controls.

The lipid/protein ratio in SC is reduced in both non-lesional and lesional SC of AE patients and strongly correlates with the impaired skin barrier function and disease severity of AE patients.

The lipid/protein ratio plays a more important role in the impaired skin barrier of AE than the SC thickness.

Abbreviations: AE, atopic eczema; CER, ceramide; *FLG*, filaggrin; NMF, natural moisturising factor; SC, stratum corneum; SC_{mass/area}, dry SC mass per surface area; SCORAD, SCORing Atopic Dermatitis; TEWL, transepidermal water loss.

Summary

Background: The barrier function of the skin is primarily provided by the stratum corneum (SC), the outermost layer of the skin. Skin barrier impairment is thought to be a primary factor in the pathogenesis of atopic eczema (AE). Filaggrin is an epidermal barrier protein and common mutations in the filaggrin gene strongly predispose for AE. However, the role of filaggrin mutations in the decreased skin barrier in AE is not fully understood. It was recently shown that changes in SC lipid composition and organisation play a role in the reduced skin barrier in AE.

Objectives: To determine whether the lipid/protein ratio and the total dry SC mass per surface area are related with the skin barrier function of controls and AE patients.

Methods: A case-control study was performed to compare non-lesional and lesional skin of AE with skin of controls. The dry SC mass was determined by tape-stripping and squamescan. The ratio between lipid and protein bands in the Raman spectrum was used to determine the lipid/protein ratio. Skin barrier function was assessed by transepidermal water loss.

Results: The results show that the dry SC mass per skin area is altered only in lesional SC of AE patients compared with control subjects. The observed reduction in the lipid/protein ratio in SC of AE patients was more pronounced, both in lesional and non-lesional SC and correlated strongly with the skin barrier function and disease severity.

Conclusions: The lipid/protein ratio plays a role in the reduced skin barrier function in AE.

Introduction

Atopic eczema (AE) is a chronic relapsing inflammatory skin disease characterized by several clinical manifestations such as erythema, pruritus and xerosis. AE is currently affecting over 15% of Caucasian children and 2-10% of adults, and its prevalence is increasing.¹⁻⁴ There is

increasing evidence that AE is related to an impaired skin barrier function.^{5,6} AE is strongly associated to mutations in the filaggrin gene (*FLG*) encoding the epidermal barrier protein filaggrin.⁷ Filaggrin is part of the cornified envelope and contributes to the alignment of keratin filaments, facilitating the flattening of keratinocytes during terminal cornification.⁸ In addition, filaggrin is metabolized into hygroscopic molecules being part of the natural moisturizing factor (NMF), which is important for SC hydration.⁹ Despite the fact that an impaired barrier function has been demonstrated in adult AE patients, the precise role of filaggrin for the skin barrier function is not completely understood and compelling evidence that *FLG* mutations are the main cause of the reduced barrier function in AE is missing.¹⁰⁻¹²

The skin functions as a permeability barrier provided by the stratum corneum (SC), the outermost layer of the skin. It contains nonviable corneocytes oriented parallel to the skin surface and surrounded by a cornified cell envelope. This envelope reduces the uptake of most substances into the corneocytes and therefore redirects the transport of most substances along the intercellular regions.¹³ The intercellular regions are filled with highly organized lipids with a characteristic composition and organisation, which are considered to be crucial for an adequate skin barrier function. The major lipid classes in the SC are ceramides (CERs), free fatty acids and cholesterol.

In previous studies, changes have been observed in the CER composition of non-lesional as well as lesional SC of AE patients.^{5,14-20} Recently, strong evidence was observed that besides changes in CER subclasses, the CER chain length plays a prominent role in the impaired barrier function of AE skin.^{20,21}

Besides the lipid composition and organisation, the lipid/protein ratio in the SC as well as the SC thickness may also affect the skin barrier function. The aim of the present study was to

determine the lipid/protein ratio in SC of control subjects and AE patients. In addition, the dry SC mass per surface area (referred to as $SC_{\text{mass/area}}$) was determined, being a measure for the SC thickness. Subsequently, these parameters were related to the barrier function of the skin, as measured by transepidermal water loss (TEWL).

The results show that the $SC_{\text{mass/area}}$ is altered in lesional AE SC but not in non-lesional AE SC, as compared with controls. The lipid/protein ratio is lower in both non-lesional and lesional SC in AE compared with that in SC of control subjects and correlates strongly with inverse TEWL values. Therefore, besides lipid composition and organisation, the lipid/protein ratio may be an important factor for the impaired skin barrier function in AE patients.

Materials and Methods

Study population and study setup

The study was a case-control study and was approved by the Medical Ethics Committee of the Leiden University Medical Center (protocol number P09.171) and conducted in accordance with the Declaration of Helsinki Principles. All subjects gave written informed consent. In total twenty-eight Caucasian AE patients (25.6 ± 5.6 years; 11 males) and fifteen Caucasian control subjects without (history of) dermatological disorders (25.0 ± 5.2 years; 5 males) were selected using the diagnostic criteria of Hanifin and Rajka.²² This cohort included fourteen patients that were carrier of at least one prevalent *FLG* null allele. A total of 11 AE patients suffered from eczematous lesions on at least one of the ventral forearms. The overall disease severity was evaluated by using SCORAD.²³ Subjects did not apply any dermatological products to their forearms for at least one week prior to the study. Measurements were performed in a temperature and humidity controlled room. Subjects were acclimatized for 45 minutes prior to the

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measurements. A dermatologist marked an area ($\sim 4.5 \text{ cm}^2$) of non-lesional skin and, if applicable, an area ($\sim 4.5 \text{ cm}^2$) of lesional skin on the forearm of each AE patient at the start of the study day. Raman measurements were performed on these marked areas as function of depth. Hereafter, the same skin areas were tape stripped while TEWL was monitored after each two tape strips (more details below and in the Supplementary material).

***FLG* mutation analysis**

All subjects were screened for the four most prevalent mutations found in the European Caucasian population (2282del4, R501X, R2447X, S3247X). Mutations were determined by genotyping after DNA extraction as described before.^{20,24}

Skin barrier function assessment

TEWL was monitored using a Tewameter TM 210 (Courage+Khazaka, Köln, Germany), the method of which was reported earlier and explained in detail in the Supplementary material.^{20,24}

Confocal Raman microspectroscopy

Confocal Raman microspectroscopy (Model 3510 Skin Composition Analyzer, RiverD International, Rotterdam, The Netherlands) was used to determine the lipid/protein ratio of the SC on the ventral forearm. The principles of Raman spectroscopy and the procedure have been described elsewhere and are included in the Supplementary material.^{25,26}

SC isolation and lipid extraction

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In order to obtain Raman spectra of human SC lipids and extracted human SC, human SC was isolated by trypsin digestion as described elsewhere.²⁷ A short description of SC isolation and lipid extraction has been provided in the Supplementary material.

Tape stripping procedure

Multiple poly(phenylene sulfide) tape strips (Nichiban, Tokyo, Japan) were successively applied at the same area of the ventral forearm. All tapes were applied on the skin and pressed for 5 seconds at 450 g/cm² 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 subsequent tape strip. Afterwards, the tapes were punched to a surface area of 2 cm². The Squamescan 850A (Heiland electronic, Wetzlar, Germany) was used to determine the dry SC mass removed by each tape strip. This is described in detail in the Supplementary material.

Statistical analysis

Statistical analysis was performed using SPSS Statistics version 17.0. Data are shown as mean $\pm$ one standard deviation (SD). As the data show a non-normal distribution, non-parametric Mann-Whitney tests were performed for comparison of different groups with a significance level of $P < 0.05$. For the paired comparison of non-lesional and lesional SC, a Wilcoxon signed rank test was used. Bivariate analysis and Spearman's ρ correlation coefficient were used to analyse correlations between parameters.

Results

From the TEWL values and the cumulative amount of stripped SC, the $SC_{\text{mass/area}}$ was calculated. Fig. 1 shows typical examples of three AE patients. The abscissa intercept by extrapolation is indicative for the $SC_{\text{mass/area}}$ (cm^2), as described earlier for the SC thickness.²⁸ In the examples in Fig. 1, the $SC_{\text{mass/area}}$ varies between $1195.2 \mu\text{g}/\text{cm}^2$ and $1802.0 \mu\text{g}/\text{cm}^2$. For calculating the SC thickness we assume that Fick's first law holds for water transport in the SC.

Fig. 2a shows the $SC_{\text{mass/area}}$ in control SC ($1619 \pm 400 \mu\text{g}/\text{cm}^2$), non-lesional SC of AE patients ($1407 \pm 340 \mu\text{g}/\text{cm}^2$), and lesional SC of AE patients ($1200 \pm 168 \mu\text{g}/\text{cm}^2$). The results show a significant difference in the dry skin mass per surface area only between controls and lesional AE skin ($P = 0.01$). However, there is a trend showing a reduced $SC_{\text{mass/area}}$ in non-lesional skin compared with controls ($P = 0.08$). Open and filled circles indicate carriers and non-carriers of *FLG* mutations, respectively. There was no difference in $SC_{\text{mass/area}}$ from AE patients with or without a *FLG* mutation ($P > 0.1$). In Fig. 2b, $1/\text{TEWL}$ values are plotted as function of the $SC_{\text{mass/area}}$ ($\mu\text{g}/\text{cm}^2$). The correlation coefficient between these parameters is $r = 0.67$ ($P < 0.001$), indicating that there is a correlation between the skin barrier function and total $SC_{\text{mass/area}}$.

Raman spectroscopy was used to determine the lipid/protein ratio in the SC. The method is explained in detail in the Supplementary material. Briefly, the ratio between the lipid peak (at 2880 cm^{-1}) and the protein peak (2930 cm^{-1}) was determined. These peaks were selected as Raman spectra of SC lipids show that a clear CH_2 stretching vibration peak is detected at 2880 cm^{-1} , not being present in the spectrum of the extracted SC. In these spectra a clear peak at 2930 cm^{-1} is detected due to the proteins, which is only weakly present in the spectrum of the extracted lipids.

Fig. 3a shows the lipid/protein ratio as a function of depth (between 0 and 40 μm), with a maximum between 0 and 4 μm and a gradual decrease deeper than 8 μm . Fig. 3b shows the average lipid/protein ratio between 4 and 10 μm in the SC in control SC, non-lesional AE SC, and lesional AE SC. Non-lesional skin shows a significantly lower lipid/protein ratio than the skin of control subjects (0.24 ± 0.05 and 0.28 ± 0.03 , respectively, $P<0.005$). In lesional skin, the lipid/protein ratio is even further reduced (0.19 ± 0.03 , $P<0.001$). Fig. 3c shows the intra-subject comparison between lesional and non-lesional skin, paired per patient. In 9 out of 11 AE patients, the lipid/protein ratio is lower in lesional skin compared with non-lesional skin ($P<0.01$).

The lipid/protein ratio in AE patients showed no difference between carriers and non-carriers of *FLG* mutations (0.24 ± 0.04 in non-carriers and 0.23 ± 0.06 in carriers $P>0.1$ for non-lesional AE SC; and 0.19 ± 0.02 in non-carriers and 0.18 ± 0.04 in carriers, $P>0.1$ for lesional AE SC). Fig. 3d shows a representative example of a high wavenumber Raman spectra from a patient with a large difference in lipid/protein ratio between non-lesional and lesional SC.

The relationship between skin barrier properties and lipid/protein ratio is presented in Fig. 4a, showing the correlation between $1/\text{TEWL}$ and the lipid/protein ratio in control subjects and in non-lesional and lesional SC of AE patients (correlation coefficient 0.86, $P<0.001$). This shows that the lipid/protein ratio is an important determinant for the skin barrier function. In addition, the disease severity (SCORAD) and lipid/protein ratio show a weaker negative correlation of -0.52 ($P = 0.005$, Fig. 4b), which indicates a lower lipid/protein ratio in SC of patients with a higher disease severity.

Discussion

We determined the lipid/protein ratio in SC of control subjects and in non-lesional as well as lesional SC of AE patients. In addition, the $SC_{\text{mass/area}}$ was determined in control subjects and in non-lesional skin of AE patients. Finally, we studied the correlations of these parameters with the skin barrier function as assessed by TEWL.

The Raman spectra of extracted SC lipids and extracted SC demonstrate that the ratio between the peaks located at 2880 cm^{-1} and 2930 cm^{-1} is an excellent measure for the lipid/protein ratio in SC: the contribution of the lipids to the 2930 cm^{-1} peak as well as the contribution of the proteins to the 2880 cm^{-1} peak is negligible. Based on a spectral comparison we were able to confirm that the difference between spectra with a high lipid/protein ratio and spectra with a low lipid/protein ratio consistently show a residual spectrum with strong lipid contributions. An increase in lipid to protein ratio was noticed towards the skin surface. This may be caused by i) the presence of surface lipids (sebum). Although the skin has been wiped with water before measurements, this will not completely remove sebum. ii), The SC contains many proteases that degrade SC proteins and leads to less (intact) protein in the outermost layers of the SC.²⁹ These hydrophilic compounds are easily washed out, leading to a higher lipid/protein ratio at the SC surface.

It was decided to use lipid/protein peak ratios as these are less sensitive to artifacts than the peak intensities themselves. Little is known about the total protein content as function of depth in the SC of AE patients or control subjects, but it is known that the water level in SC changes as a function of depth in SC.^{25,30,31} A change in water level is not expected to change the lipid/protein ratio, as both peaks in the spectrum are insensitive to the water level in the SC. When comparing SC protein structure in healthy and diseased skin as function of depth, little is

known in diseased skin on spectroscopic levels. On a spectroscopic level, protein structures have been reported by Osada et al., analyzing SC of psoriasis patients and healthy subjects.³² They found a small shift in the CH₃ protein peak (between 2910 to 2966 cm⁻¹) in the Raman spectrum of psoriatic scales, not observed in our study.

We observed that the lipid/protein ratio in both lesional and non-lesional SC of AE patients is significantly lower than that in SC of control subjects. This may be partly explained by changes in SC lipid composition in AE patients.^{5,14-18,20,21,33} Some studies revealed that the level of long-chain CERs is reduced in non-lesional AE skin and the level of very short chain CERs (with a total chain length of 34 carbon atoms, referred to as C34 CERs) is increased.^{16,20,21} However, the total level of C34 CERs and long-chain CERs in non-lesional AE skin are accounting for about 10 mol% of the total CER content in non-lesional skin.²⁰ Assuming an equimolar CER:Cholesterol:Free fatty acids ratio this is only 3 mol% of the total lipids, whereas the lipid/protein level has decreased by ~14% in non-lesional AE and ~32% in lesional AE. Hence the changes in CER composition do not account for the complete reduction in lipid/protein ratio, which indicates another, yet unknown factor. A possible candidate is a delayed or incomplete lamellar body extrusion process and/or a reduced transport of lipids from the Golgi complex into the lamellar bodies.³⁴⁻³⁷

We have performed an intra-subject comparison of non-lesional and lesional skin for eleven subjects who had also lesional regions on their ventral forearms. Within these subjects in most cases the lipid/protein ratio was lower in lesional skin than in non-lesional skin. These differences may primarily be attributed to inflammation, the presence of microorganisms and/or an altered pH in lesional skin. These factors may affect the lipid synthesis^{38,39} and result in a lower lipid/protein ratio.

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A measure of SC thickness is the total $SC_{\text{mass/area}}$, obtained by extrapolation of $1/TEWL$ against the cumulative amount of tape-stripped SC. These data do not all fall in a perfect straight line, especially the data of lesional SC, which may explain the large SDs. No significant difference in the $SC_{\text{mass/area}}$ was observed between non-lesional and control SC. In lesional AE, the total $SC_{\text{mass/area}}$ was lower compared with controls. In line with these observations, Voegeli et al. have reported a decreased SC thickness in lesional AE SC.⁴⁰ They suggested increased serine protease activity in lesional SC of AE patients as a possible explanation for the observed differences. We noticed that SC thickness has an effect on the TEWL values and thus the skin barrier function as observed by the correlation between the total $SC_{\text{mass/area}}$ and TEWL.

An alternative method to determine SC thickness is based on the water concentration profile in the SC measured by confocal Raman spectroscopy.^{30,41-44} The advantage of Raman spectroscopy is that it enables the direct monitoring *in vivo* without damaging the SC. The method determines the actual SC thickness, which includes the effects of swelling of SC as a result of water uptake. As the total amount of tape-stripped dry SC mass and subsequent extrapolation does not change upon swelling, we used this method to determine the $SC_{\text{mass/area}}$. However, when comparing this method with the SC thickness determined by confocal Raman microscopy, a weak correlation exists between the SC thickness and $SC_{\text{mass/area}}$ ($r = 0.54$, $P < 0.001$, not shown). When performing a multivariate analysis on the $SC_{\text{mass/area}}$, SC thickness and NMF levels (as an indicator of hydration level), a better correlation is observed ($r = 0.68$, not shown). This indicates that swelling or change in water content could at least partially explain the difference between the two methods. Also a change in water distribution due to the stripping procedure may affect the TEWL values and thus the extrapolated cumulative amount of SC. It has been reported that water distribution changes in a subpopulation of healthy subjects³⁰.

Both SC thickness and lipid/protein ratio are factors that affect the TEWL. However, the association between lipid/protein ratio and 1/TEWL was much stronger than the correlation between $SC_{\text{mass/area}}$ (as a measure of SC thickness) and 1/TEWL. This suggests that the level of lipids in SC plays a more prominent role in the skin barrier function than the $SC_{\text{mass/area}}$. Our previous study shows a strong correlation between CER chain length and skin barrier function in patients with AE²⁰, also indicating the importance of the SC lipids in the impaired skin barrier function of AE. In the present study we noticed that the lipid/protein ratio correlates to a weak but significant extent with SCORAD, whereas in the previous study the lipid chain length also correlated with SCORAD. Therefore both the lipid/protein and lipid chain length contribute not only to the impaired skin barrier function, but may also play a role in disease severity.

FLG mutations are the strongest risk factor known to date for the development of AE.^{7,45} However, the role of *FLG* mutations in the decreased skin barrier function in AE is yet inconclusive.^{10-12,46-48} *FLG* mutations were not associated with lipid/protein ratio or with $SC_{\text{mass/area}}$ in AE. In a previous study we found that lipid organisation and CER composition are altered in AE, but that these changes were not associated with *FLG* mutations.²⁰ Our results show the importance of the SC lipids in the decreased skin barrier function in AE patients. The finding that the lipid/protein ratio is reduced in lesional skin and to a lesser extent in non-lesional SC of AE patients gives a basis for the use of topical supplementation of lipids and to prevent further damage to the skin lipids by avoidance of soap and other irritants. This may improve the skin barrier function of AE patients.

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Figure legends

Fig. 1. Three examples of $1/\text{TEWL}$ plotted against the cumulative amount of tape-stripped SC. The intercept of the line with the x-axis is indicative for the dry SC mass per cm^2 . Based on the assumption that Fick's first law holds for water transport in the SC, an approximately straight line is observed between the reciprocal flux ($1/\text{TEWL}$) and the dry SC mass per cm^2 removed by tape stripping.²⁸ Three different symbols indicate data of different AE patients.

Fig. 2. Dry SC mass and correlation with skin barrier function. (a) SCmass/area ($\mu\text{g}/\text{cm}^2$) in control subjects, non-lesional SC of AE patients and lesional SC of AE patients. (b) Correlation between $1/\text{TEWL}$ and dry SC mass per cm^2 in control subjects and AE patients. Non-carriers and carriers of *FLG* mutations 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.

Fig. 3. Lipid/protein ratio in SC of control subjects and AE patients. (a) Lipid/protein ratio in the range of 0-40 μm . The green, orange and red lines indicate the average ($\pm\text{SD}$) of control SC, non-lesional AE SC and lesional AE SC, respectively. (b) Average lipid/protein ratio (averaged between 4-10 μm in the SC) of the individual patients and controls. (c) Paired lipid/protein data of non-lesional and lesional AE skin. (d) High wavenumber spectrum of both non-lesional (blue, lipid/protein ratio = 0.26), lesional AE skin (green, lipid/protein ratio = 0.18), and the residual (non-lesional minus lesional skin, red). The residual spectrum is very similar to the spectrum of

extracted SC lipids (Fig. 3b), which confirms that the high lipid/protein ratio corresponds to a higher lipid content in non-lesional SC as compared with lesional SC. This example is representative for all patients. Non-carriers and carriers of *FLG* mutations 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.

Fig. 4. Correlation between lipid/protein ratio and skin barrier function and that with SCORAD (a) Correlation between lipid/protein ratio and skin barrier function. (b) Correlation between lipid/protein ratio and SCORAD. Green, orange and red data points belong to control SC, non-lesional AE SC and lesional AE SC, respectively. Non-carriers and carriers of *FLG* mutations 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.





