

## Ceramide and Cholesterol Composition of the Skin of Patients with Atopic Dermatitis

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Atopic dermatitis skin tends to be easily irritated and appears dry. These clinical peculiarities correspond to impaired barrier function and to increased transepidermal water loss (TEWL) values. A few studies suggest that a reduced amount of total ceramides (especially of ceramide 1) is responsible for functional abnormalities of the skin of atopic dermatitis patients. The aim of this study was to analyze the relationship between epidermal lipids and barrier impairment in the skin of patients with atopic dermatitis. The quantity of ceramides, cholesterol sulphate and free cholesterol of 47 patients with atopic dermatitis and 20 age- and sex-matched healthy subjects was assessed by cyanoacrylate stripping and thin layer chromatography. Capacitance and TEWL were recorded at the same site of the lipid sample. In patients with atopic dermatitis, the levels of ceramide 1 and 3 were significantly lower and values of cholesterol significantly higher with respect to healthy subjects.

Moreover, the CER/CH ratio was significantly lower with respect to normal skin. Patients with active signs of eczema also had higher TEWL values and lower capacitance values. By contrast, patients with no active signs of atopic dermatitis had a normal barrier function and intermediate values of ceramides and cholesterol, when compared to patients with atopic dermatitis with active lesions and normal subjects. The quantity of ceramide 3 was significantly correlated with TEWL impairment. These findings suggest that a decrease in ceramides in the stratum corneum is involved in barrier impairment in atopic dermatitis skin.

Our data confirm those of other authors and support the view that impaired metabolism of ceramides may be the cause of dry skin and impaired barrier function in atopic dermatitis. **Key word: barrier function.**

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Atopic dermatitis (AD) skin tends to be easily irritated and appears dry. These clinical traits correspond to an impaired barrier function and to increased TEWL values, as recorded by non-invasive methods (1–6).

It has been proved that increased TEWL and reduced hydration values with respect to skin of healthy subjects are present in patients with AD both in eczematous and apparently normal skin, supporting the view that barrier impairment represents a basic defect in AD (4–7).

Recent biochemical findings indicate that disturbances of epidermal lipid compartment structures, and particularly of ceramides, account for the defects in barrier function of atopic dry skin (8–14). With respect to healthy subjects, a decrease in the ceramide lipid fraction and a diminished ratio of ceramides and free sterols have been demonstrated (9). Moreover, a reduction in ceramide 1 in diseased and healthy

skin (10) and a decrease in the proportion of ceramide 1 together with an increased level of esterified C18:1 fatty acids (oleate) of ceramide 1 are observable in patients with AD (11).

Studies performed so far concern various lipid classes, investigated by different analytical methods; therefore, the results are difficult to compare and need to be confirmed. Moreover, correlations between biochemical data and biophysical parameters have not been investigated so far.

The aim of our study was to assess the relationship between epidermal lipids and barrier impairment in AD skin, by analyzing six ceramide fractions, cholesterol sulphate and free cholesterol in 47 patients with AD and 20 healthy subjects.

### MATERIALS AND METHODS

#### Study population

Forty-seven patients (23 males and 24 females), aged 3 to 30 (mean age  $10.3 \pm 9.3$ , with 8 subjects aged more than 20), affected by AD, diagnosed according to the criteria of Hanifin & Rajka (15), and 20 healthy age- and sex-matched subjects entered the study, after informed consent. At the moment of investigation 19 patients presented skin lesions at sites different from forearm skin. The assessed skin appeared normal or dry. No patients showed clinical signs of concomitant ichthyosis vulgaris.

The subjects were instructed to refrain from using topical drugs or moisturizers for 3 days prior to the study.

#### Instruments and study procedure

The study was carried out from October 1994 to March 1995.

Evaluations and collection of skin lipid samples were performed on forearm skin, 10 cm below the antecubital crease.

TEWL was measured using the Evaporimeter EPI (Servo Med, Sweden), which is based on vapour pressure gradient estimation. All TEWL recordings were performed according to the guidelines of the standardization group of the European Society of Contact Dermatitis (16). TEWL was recorded in 40 patients with AD (19 with active lesions) and in 7 healthy subjects.

The skin surface hydration was determined by the Corneometer CM 820 (Courage-Khazaka, Germany). The instrument measures the electrical capacitance of the stratum corneum. Since water has the highest dielectric constant in the skin, an increase in the water content will raise capacitance values, which are displayed in arbitrary units by the instrument. Capacitance was recorded in 42 patients with AD (19 with active lesions) and in 7 healthy volunteers.

All evaluations were performed after a 30-min acclimatization period in a room with temperature set at 21–22°C and humidity at 45–50% in the following order: TEWL measurements and capacitance measurements.

#### Stratum corneum samples

Stratum corneum sheets were removed from the volar site of the forearm, 10 cm below the antecubital fossa (close to the area on which capacitance and TEWL were assessed) by a single stripping with cyanoacrylate resin, two drops of which were placed on a glass slide adhering to an area of approximately  $2 \times 2$  cm<sup>2</sup> of the skin, until dry. The method of extracting lipids from stratum corneum sheets has

been described in detail elsewhere (10, 17, 18). Briefly, the slides were incubated in hexane/ethanol 95/5 (v/v) under ultrasonication.

Extracted stratum corneum lipids were filtered through a solvent resistant filter (Millex-SR 0.5 µm, Millipore, Molsheim, France), partially dried under nitrogen, subjected to measurement of lipid and weighed after final drying under vacuum for 24 h (freeze dryer, Labconco Corp., Kansas City, Mi.).

The residue on the glass slide, composed of stratum corneum and cyanoacrylate resin, was treated with dimethyl formamide (DMF). This caused the solubilization of cyanoacrylate resin and the dispersion of stratum corneum. Dispersed stratum corneum was separated from the DMF solution by filtration. The residue on the filter was dried in vacuum and weighed. The ratio of extracted lipids to the dispersed stratum corneum was calculated in µg lipids per mg stratum corneum.

#### Thin layer chromatography: sample preparation

Samples were stored at  $-80^{\circ}\text{C}$  until prepared for thin layer chromatography (TLC).

They were redissolved with 5 ml chloroform/methanol (2:1 v/v) and 1 ml of 2 M KCl (19). Each sample was then centrifuged and the upper phase discarded, while the lower was dried under nitrogen and finally redissolved in 100 µl of chloroform/methanol (2:1), containing methyl oleate (0.25 mg/ml), as an internal standard.

#### TLC plates

Glass plates, 20 × 20 cm and coated with 0.25-mm-thick silica gel G (Adsorbosil-plus-1; Alltech Associates; Deerfield, IL, USA), were washed with chloroform:methanol, 2:1, and activated in a  $110^{\circ}$  oven, and the absorbent was scored into 6-mm-wide lanes (20), which limits radial diffusion and allows up to 30 samples and standards to be analyzed simultaneously. One standard or 20 µl of sample was applied per lane 2–3 cm from the bottom edge of the plate, using a Hamilton syringe. The chromatogram was developed first with a mobile chloroform:methanol:water phase, 40:10:1, to 10 cm, followed by chloroform:methanol:acetic acid, 190:9:1, to 16 cm, and hexane:ethyl ether:acetic acid, 70:30:1 to 20 cm (21). After development, chromatograms were air-dried, sprayed with 50% sulphuric acid, and slowly heated to  $220^{\circ}\text{C}$  on an aluminium slab placed on a hot plate (20). After 2 h, charring was complete, and the chromatogram was quantified with a BioRad model 620 scanning video densitometer in absolute reflectance mode (Bio Rad Videodensitometry Model 620, Richmond, Ca). A mixture of porcine epidermal ceramides (22) was also included on each plate and served as a guide to identification.

#### Statistics

ANOVA for unpaired comparisons was used to compare mean values referring to different groups of subjects for the same parameter; Student Neuman Keuls test was used as a post hoc test.

A regression test was used to correlate biophysical parameters (capacitance and TEWL) with lipid surface composition. The 95% level of confidence interval was employed to accept or reject the null hypothesis.

## RESULTS

Considering the absolute amount of the whole population of atopic subjects, compared to non-atopics, significant differences were found for cholesterol sulphate, ceramide 3, ceramide 2 and ceramide 1, which were lower in patients with AD.

The compositional data for the ceramide percentage amount showed that ceramide 3 and ceramide 1 were lower in AD than in control subjects, whereas free cholesterol was higher. Moreover, the ceramides/cholesterol free ratio ( $1.53 \pm 0.46$  in AD and  $2.24 \pm 0.61$  in healthy subjects) was significantly lower in the AD group with respect to control subjects.

Fig. 1b illustrates lipid values in the skin of AD patients, subdivided into subjects with (ADL) and without lesions

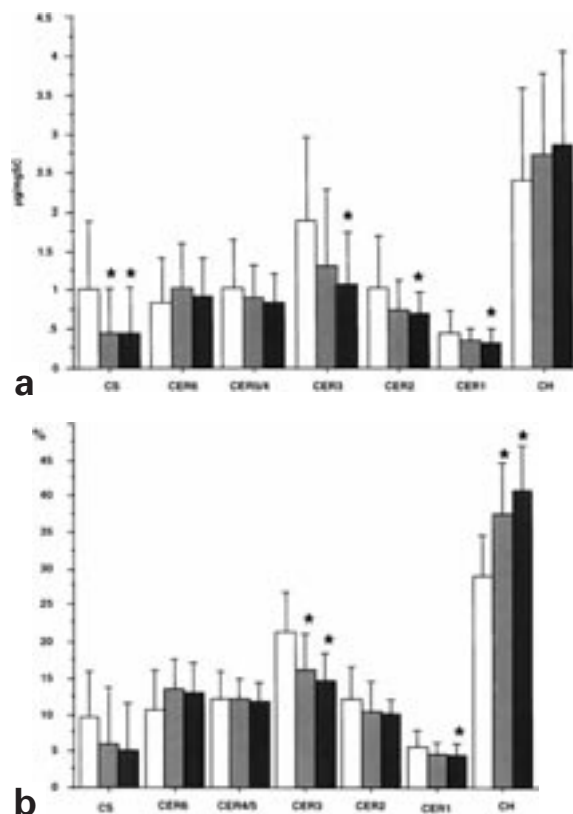


Fig. 1. Ceramide and cholesterol composition expressed in (a) µg/mg stratum corneum and (b) percentage composition in healthy subjects (□), patients with AD without active lesions (▨) and patients with AD with active lesions (■). The analyzed lipids are shown according to increasing polarity. \* $p < 0.05$  in comparison with healthy subjects.

(ADWL) at the moment of the investigation. Between the two AD groups no significant difference in lipid composition was observed; however, ADWL showed values which were intermediate between ADL and healthy subjects. In AD patients, besides a significant reduction in ceramide 1, ceramide 2 and ceramide 3 (Fig. 1a), significant differences with respect to healthy subjects were found for cholesterol sulphate both in ADL and ADWL, considering absolute values, whereas according to compositional data significances were observable for cholesterol-free values (Fig. 1b).

Capacitance values referring to AD skin ( $56 \pm 7.2$  and  $53 \pm 10$  for ADWL and ADL, respectively) did not show any significant difference in comparison with normal skin ( $56 \pm 9.7$ ), although a decrease was evident for ADL.

TEWL mean values are shown in Fig. 2.

No significant difference between values referring to healthy subjects and ADWL subjects was found, whereas TEWL values referring to ADL patients were significantly higher in comparison with ADWL patients.

#### Correlations

A positive correlation between TEWL and capacitance was present in healthy subjects ( $r = 0.9$ ,  $p < 0.05$ ). However, this

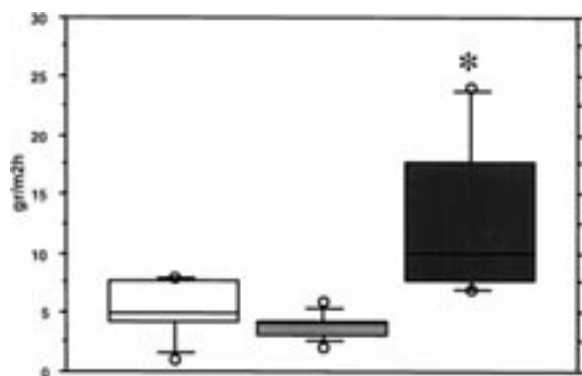


Fig. 2. Transepidermal water loss (TEWL) in healthy subjects (□), patients with AD without active lesions (▤) and patients with AD with active lesions (■). The values are expressed as cell plot of the populations and represent the distribution of the values, the median values and the standard deviations. \* $p < 0.05$  in comparison with healthy subjects.

correlation was not present in atopic patients (ADWL  $r = 0.05$ ,  $p > 0.05$  and ADL  $r = -0.008$ ,  $p > 0.05$ ).

Correlations between capacitance and lipid values were not significant for the three populations. Considering the whole AD population, TEWL and cholesterol sulphate values (percentage) and TEWL and ceramide 3 (percentage and weight) showed a significant negative correlation ( $r = -0.3$ ,  $p < 0.05$  and  $r = -0.3$ ,  $p < 0.05$ , respectively).

Considering only ADL patients, a stronger negative correlation between cholesterol sulphate percentage and TEWL ( $r = -0.48$ ,  $p < 0.05$ ) and a positive correlation with ceramide 6 percentage ( $r = 0.58$ ,  $p < 0.05$ ) were calculated.

## DISCUSSION

Owing to a specific alteration in the skin of patients with AD, which is easily irritated and appears dry (2), environmental factors are more effective in inducing damage in atopic than in normal skin, causing the phenotypic expression of eczema (1, 6). Functionally, this alteration corresponds to an impaired barrier function, as measured by TEWL and capacitance (3–8, 23). Previous studies performed on normal and diseased skin demonstrated an increase in TEWL and a decrease in capacitance both in subjects with active AD and in those without eczematous lesions (7), indicating that also the clinically normal skin of AD patients is functionally abnormal.

In this study, too, we found increased TEWL values at apparently healthy skin sites, more marked in patients with active lesions. Moreover, a relationship between the functional alterations, as recorded instrumentally, and the biochemical composition of the skin in AD patients was observable.

At an ultra-structural level, the water permeability barrier is represented by the intercellular multilamellar lipid sheets of the stratum corneum, deriving from exocytosis of lamellar bodies, and by ceramides that represent one of the major lipid constituents of epidermal lipids (24–27). Ceramides, consisting of different sub-fractions, are functionally important for the stability of the intercellular multilamellar lipid sheets. In particular, ceramide 1, owing to its very long chain, ensures the connection between adjacent bilayers (25), contributing to

the correct maintenance of water homeostasis and inhibiting water loss. When the barrier is experimentally damaged using solvents or detergents capable of removing ceramides, the skin appears xerotic and TEWL increases (28).

The relationship between skin surface lipids and TEWL in children with AD was investigated by Abe et al. (8), who demonstrated a modification of the correlation between lipid levels, especially cholesterol, and TEWL in AD patients in comparison with the normal population. Other authors investigated lipid classes (9–11), without, however, assessing skin barrier function in the same patient groups.

Melnik et al. (9) found a decrease in ceramide weight percent ratio in plantar and lumbar stratum corneum of AD patients in comparison with normal subjects. Subsequently, a decrease in the weight of total lipids, an increase in ceramide weight percent ratio and a slight decrease in the ratio of ceramide 1 to total lipids were described (10). Finally, a lower proportion of ceramide 1 and increased levels of esterified C18:1 fatty acids (oleate) of ceramide 1 were demonstrated in AD patients (11).

In our AD population we observed a decrease in the amount of ceramides 1 and 3 and a reduction in cholesterol sulphate, associated with an increase in cholesterol-free values and a decrease in the ceramide/cholesterol ratio. We also defined a clinically and functionally normal “in between” population, corresponding to subjects with AD in a silent phase of the disease showing intermediate levels of lipid classes when compared to ADL and healthy subjects.

Moreover, we found an inverse correlation between ceramides and TEWL. The correlation between TEWL and ceramide 3 and cholesterol sulphate was stronger when we considered the ADL group alone, stressing the connection between ceramides and barrier alteration in the AD population.

According to the suggestions of Mustakallio et al. (29) and Melnik et al. (9), the decrease in ceramides 1 and 3 may account for skin susceptibility to irritants and increased TEWL, whereas the increase in cholesterol-free values and the decrease in the cholesterol-ceramide ratio could represent a response to elevated TEWL. Our data also support the correlation between the decrease in ceramide 3 and the increase in cholesterol-free values.

Long et al. (30) found that cholesterol sulphate is lower in desquamated material and suggested that cholesterol sulphate serves as an intercellular cement in the stratum corneum and must be hydrolyzed to free cholesterol, to permit the shedding of individual corneocytes and, in fact, the presence of a decrease in cholesterol sulphate in AD patients accompanies desquamation.

It is not known whether atopic dry skin results from a sub-clinical dermatitis leading to secondary defective barrier function or whether it derives from primary barrier defects, characteristics of AD skin. In fact, recent observations on the uninvolved skin of AD patients have demonstrated that levels of enzymes implicated in ceramide metabolism, like sphingomyelinase (13, 14) and beta glucocerebrosidase (12), are lower or changed, suggesting that the metabolic chain is altered in apparently normal AD skin, too. Our data show that ceramides are reduced in the whole atopic population but particularly in those subjects in an active phase of the disease.

In vitro studies conducted on human fibroblast cultures using ceramide analogues have shown that ceramides modulate secretion of PGE<sub>2</sub> in response to the action of IL-1 (31).

Moreover, they enhance the secretion of IL-2 in lymphocytes, as demonstrated by *in vitro* studies (32); this, in addition to the role of ceramides in mediating TNF- $\alpha$  action (33), suggests that ceramides may be involved in modulating local immune functions (30). Thus, modifications in ceramide profiles are not only linked to disturbances in the differentiation of keratinocytes (34), which are responsible for the functional alterations of AD skin, but may also be responsible for the amplification of the inflammatory response to environmental stimuli.

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