

# Two Ceramide Subfractions Detectable in Cer(AS) Position by HPTLC in Skin Surface Lipids of Non-Lesional Skin of Atopic Eczema

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The non-involved skin of atopic eczema (NEAE) is characterized by severe dryness and an impaired barrier function of the stratum corneum as indicated by an increased transepidermal water loss. Previous studies have demonstrated that this barrier impairment coincides with marked alterations in the amount and composition of stratum corneum ceramides. The aim of this study was to identify specific alterations in NEAE that may be used in the diagnosis of the atopic eczema. Using a classical procedure for high performance thin layer chromatography we could confirm earlier results: apart from Cer(EOH), which contains  $\omega$ -hydroxy fatty acid (O) ester-linked to linoleic acid (E) and amide-linked to 6-hydroxy-4-sphingene (H), the quantities of all ceramide fractions were significantly decreased. Furthermore, Cer(EOH)/Cer<sub>total</sub> was significantly increased, whereas the percentage of Cer(EOS), which contains sphingosine (S), and Cer(NP), which contains non-hydroxy fatty acid (N) amide-linked to phyto-sphingosine (P), were significantly decreased. Using a modified procedure for high performance thin layer chromatography we could demonstrate the for-

mation of a double peak in the position of Cer(AS), which contains  $\alpha$ -hydroxy fatty acid (A), in lipids of NEAE. The subfractions of the double peak comprised 15% and 12% of Cer<sub>total</sub>. MALDITOF mass spectrometry suggested that the double peak was formed by a homologous series of mono-hydroxylated and mono-unsaturated ceramides of different chain length, e.g., Cer(AS) subfractions containing either (C<sub>16,18</sub>) or (C<sub>22,24,26</sub>)  $\alpha$ -hydroxy fatty acids. In contrast, in normal skin a single peak in Cer(AS) position, which comprised 22% of Cer<sub>total</sub>, was mainly formed by the long chain subfraction. In some cases this single peak displayed a small shoulder at its right flank, but never showed a clear peak separation when developed with NEAE samples. Furthermore, even in senile xerosis, or in either non-involved skin of psoriasis or seborrheic eczema, only a single peak occurred in Cer(AS) position. Accordingly, the double peak might be specific for NEAE and turn out to be a marker for atopic eczema. **Key words:** atopic eczema/ceramides/epidermal barrier. *J Invest Dermatol* 113:894-900, 1999

**T**he stratum corneum (SC) constitutes the main barrier for diffusion of substances into the skin (Elias and Friend, 1975; Landmann, 1986). It consists of corneocytes and intercellular lipids, mainly ceramides, sterols, and free fatty acids. The integrity of the SC requires the organization of the lipids into multilamellar intercellular membranes (ICM), which form from polar precursors (glycerophospholipids, glucocerebrosides, sphingomyelin) subsequent to extrusion at the stratum granulosum-SC interface

(Grayson and Elias, 1982; Elias and Menon, 1991). Once incorporated into ICM the precursors are converted to ceramides and free fatty acids by the action of co-extruded hydrolytic enzymes (Holleran *et al*, 1992; Mao-Qiang *et al*, 1996).

Atopic eczema (AE) is a common disease with increasing prevalence (Ring, 1991; Kay *et al*, 1994; Schäfer and Ring, 1995). Dry skin is a clinical feature of AE with increased transepidermal water loss (TEWL) levels even in non-eczematous skin sites (NEAE), which is a direct reflection of the impaired barrier function of the SC (Werner *et al*, 1982; Werner and Lindberg, 1985; Tupker *et al*, 1990; Gfesser *et al*, 1997). Previous studies have demonstrated changes in epidermal lipid metabolism (Schäfer and Kragballe, 1991) and a general reduction in the level of SC ceramides (Imokawa *et al*, 1991; Di Nardo *et al*, 1998). This deficiency of ceramides cannot be attributed to an altered function of the  $\beta$ -glucocerebrosidase, which liberates ceramides from glucocerebrosides (Jin *et al*, 1994). Additionally, there is a disturbed formation and extrusion of lamellar bodies in the stratum granulosum (Werner *et al*, 1987; Fartasch *et al*, 1991, 1992); however, perturbances of the epidermal barrier by tape stripping or

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Abbreviations: AE, atopic eczema; Cer<sub>total</sub>, total amount of all ceramides species ( $\mu$ g per mg SC protein); HPTLC, high performance thin layer chromatography; ICM, intercellular membranes; NEAE, non-eczematous skin of atopic eczema; NSK, normal skin; TEWL, transepidermal water loss; SC, stratum corneum; SSL, skin surface lipids.

acetone treatment results in an enhanced or normal rate of recovery of TEWL in NEAE, respectively, which could be caused by a permanently activated repair mechanism in NEAE (Tanaka *et al*, 1997; Gfesser *et al*, 1997). A complete restoration of the epidermal barrier function was not achieved, however, as the TEWL after barrier repair is on the same level as before perturbation. This in part can be explained by the reduced steady state quantity of SC ceramides shown by Imokawa and Di Nardo.

Besides the decrease in the levels of total ceramides in comparison with age-matched controls, a significant reduction of Cer(EOS), which contains  $\omega$ -hydroxy fatty acid (O) ester-linked to linoleic acid (E) and amide-linked to sphingosine (S), and Cer(NP), which contains non-hydroxy fatty acid (N) amide-linked to phytosphingosine (P), has been previously demonstrated (Imokawa *et al*, 1991; Yamamoto *et al*, 1991; Di Nardo *et al*, 1998). Cer(EOS), the least polar ceramide, is suggested to have an important function in stabilizing the multilayered lipid membranes and the ordering of lipids therein (Gray and White, 1978; Wertz and Downing, 1982; Bouwstra *et al*, 1998).

Deficiencies of Cer(EOS) and Cer(NP) have also been detected in psoriatic scale (Motta *et al*, 1993). Even xerotic types of normal skin (NSK) sometimes show deficiencies of Cer(NP) and more often of Cer(EOS) (unpublished results). Accordingly, the aim of this study was to detect more specific alterations of the composition of skin surface lipids (SSL) of NEAE, which might prove to be suitable as an additional feature for early diagnosis of the disease using modified procedures for high performance thin layer chromatography (HPTLC). As we could demonstrate, certain analytical conditions favour the resolution of a double peak in the position of Cer(AS), which contains  $\alpha$ -hydroxy fatty acid (A). As this chromatographic feature cannot be detected in SSL of NSK, senile xerosis, or even non-involved skin of other eczematous skin diseases, it might prove to be specific for NEAE.

## MATERIALS AND METHODS

**Chemicals and reagents** Chloroform (LiChrosolv, 102444), acetone (pa, 100014), methanol (LiChrosolv, 106018), ethanol (pa, 100983), acetic acid (pa, 100062), n-hexane (pa, 104374), propionic acid (pa, 800605), and diethylether (pa, 100921) were purchased from Merck (Darmstadt, Germany), dimethylformamide (reagent-grade, 7032) was obtained from J.T. Baker (Deventer, Netherlands), phosphoric acid (775899-8) was from Aldrich Chemie (Steinheim, Germany). Disperse I (210455) and trypsin/EDTA solution (210242) for SC separation were purchased from Boehringer (Mannheim, Germany). Dulbecco's modified Eagle's medium, phosphate-buffered saline, and fetal calf serum were purchased from Life Technologies (Eggenstein, Germany). Cupric sulfate pentahydrate/CuSO<sub>3</sub> (C-6283), ninhydrine (N4876), L-ascorbic acid (A5960), propionic acid, sodium salt (P1880), ethylene glycol monomethylether (E5378),  $\alpha$ -cyano-4-hydroxycinnamic acid (C2020), Cer(AS) (C-2512), Cer(NS) (C-2137), palmitoleic acid (P-9417), and cholesterol (C-8667) were from Sigma (Deisenhofen, Germany). Cer(NP) was obtained from Gist-Brocades (Delft, Netherlands).

**Lipid depletion from skin surface** Skin surface lipids (SSL) of NEAE (n=14) and of NSK (n=14) were extracted directly near the elbow region from the volar side of both forearms with 3 ml acetone:ethanol (1:1, by vol) using a Teflon funnel with a rotating paddle (300 rpm). Extraction time was 3 min on each site. Topical skin treatment with any ointment was stopped at least 24 h prior to investigation. Informed consent was obtained from all subjects. Diagnosis of AE was made according to the criteria of Hanifin and Rajka (1980). The mean age of the atopic individuals was 28 y (two male, 12 female) and 37 y in the control group (seven male, seven female), respectively. The SSL samples were dried under nitrogen and resolved in chloroform:methanol (2:1, by vol). Samples were stored at -20°C until further analysis.

**SC lipids of whole skin biopsies** Punch biopsies were taken from non-lesional atopic skin of the elbow region in five individuals and in six control subjects with NSK. Separation of SC from the epidermis was done as described previously (Wolber, 1996). First, the epidermis was separated from the dermis by dispase digestion at 37°C in phosphate-buffered saline for 2 h. Living keratinocytes were separated from the SC by a 10 min

incubation in a solution of trypsin (1% by weight) in phosphate-buffered saline at 37°C under gentle shaking. Trypsin digestion was stopped by adding Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (by vol). Isolated SC samples were dried on silk. Lipid extraction of samples was performed by chloroform:methanol (2:1, by vol) according to a modified procedure described by Bligh and Dyer (1959) using an ultrasonication bath for 20 min.

**Extraction of SC lipids from cyanacrylate strippings taken from long-term untreated skin sites** For the determination of lipid quantities ( $\mu$ g lipid per mg SC proteins) in the SC of different skin types, cyanacrylate strippings were taken from the long-term untreated shoulder region of 10 volunteers of either skin type. The mean age of both groups was around 20 y. Lipids of cyanacrylate strippings were extracted twice with hexane:ethanol (95:5, by vol; Imokawa *et al*, 1991).

**Quantification of SC proteins from cyanacrylate strippings** For quantification of SC proteins cyanacrylate glue was removed by extraction with 3 ml dimethylformamide (DMF) for 10 min under steady and gentle shaking until the stripped SC layers lost contact with the slide. The SC layers were then transferred to a Teflon-vial and washed three times with 1-2 ml DMF, and centrifuged for 10 min at 5000  $\times$  g. After repeated washing the SC layers were resuspended in 3 ml of methanol:DMF (1:1, by vol), pelleted again, and finally resuspended in methanol. After evaporation of methanol the SC layers were completely hydrolyzed in 2 ml of 6 N NaOH for 5 h at 150°C. After cooling the hydrolysate was neutralized with 6 N HCl and 1 ml of an aqueous buffer containing 20.2% (by weight) propionic acid, sodium salt, and 9.3% (by vol) of free propionic acid.

Free amino acids were quantified photospectrometrically after derivatization with ninhydrine. The aqueous ninhydrine reagent contained 20.2% (by weight) propionic acid, sodium salt, 9.3% (by vol) free propionic acid, 50% (by vol) ethylene glycol monomethylether, and 2% (by weight) ninhydrine. Fifty microlitres of the SC-hydrolysate was diluted with 450  $\mu$ l deionized water and then supplemented with 25  $\mu$ l of an aqueous solution containing free ascorbic acid (0.4%, by weight) and 500  $\mu$ l of the ninhydrine reagent. The mixture was shaken vigorously and heated to 100°C for about 20 min, cooled down to room temperature, and supplemented by 50 ml of 50% ethanol (by vol). Extinction was measured at 570 nm.

**Lipid analysis by HPTLC** Lipid samples were dissolved in 200  $\mu$ l chloroform:methanol (2:1, by vol). HPTLC silica gel plates, 10  $\times$  20 cm (Merck, Darmstadt, Germany) were cleaned once by development with chloroform:methanol (2:1, by vol) (Wertz *et al*, 1985). Lipid samples and a standard mixture of lipids consisting of appropriate quantities of Cer(NS), Cer(NP), Cer(AS), palmitoleic acid, and cholesterol dissolved in chloroform:methanol (2:1, by vol) were applied in lanes on the silica plates by using a Camag Linomat IV (Camag, Muttenz, Switzerland) (Melnik *et al*, 1992). Every sample was analysed in duplicate. To each TLC plate at least one sample of NSK was applied that served as internal reference to avoid misjudgement of double peak formation due to variations in HPTLC resolution.

**HPTLC developing procedure I for free fatty acids, sterols, and ceramides** Thin layer chromatograms (TLC) were developed twice in solvent saturated developing chambers (Desaga, Heidelberg, Germany) with chloroform:methanol:acetic acid (190:9:1.5, by vol) up to 6 cm migration from the application are in a modified procedure originally described by Wertz *et al*, (1985).

**HPTLC developing procedure II for free fatty acids, sterols, and ceramides** TLC were developed once with chloroform:methanol:acetic acid (190:9:1, by vol) (Wertz *et al*, 1995) and again with diethylether:hexane:acetic acid (80:20:1.5, by vol) (Lampe *et al*, 1983). Using this solvent system SSL were separated into ceramides, free fatty acids, and free sterols.

**Quantitative charring of TLC plates and photodensitometry** TLC plates were air-dried and dipped in 10% CuSO<sub>3</sub> in 8% H<sub>3</sub>PO<sub>4</sub> (by vol) aqueous solution for 20 s. Quantitative charring was done at 180°C for 20 min. When TLC plates were cooled to room temperature, lipid samples on TLC plates were quantified by photodensitometry using a Camag TLC Scanner II (Camag, Muttenz, Switzerland). Analytical data were processed by computer using the Camag Cats Software 3.16 (Camag, Muttenz, Switzerland). Ceramides were numbered according to their position on the TLC plate. Quantification of lipid fractions were based on the known quantities of the co-migrated standards. Reproducibility was ascertained by

duplicate analysis for each sample. Mass calculation for the ceramide fractions was based on the comigrating standards of Cer(NP), Cer(NS), and Cer(AS).

**Molecular mass of ceramides comigrating with Cer(AS) by Matrix assisted laser desorption ionization time of flight (MALDITOF) mass spectrometry (MS)** Eight microlitres of the SSL solution from NEAE (n=2) or NSK (n=2) was applied to one half of a TLC plate as a 3.5 cm line. On the other half, a solution with the Cer(AS) standard was applied. After development, the TLC plate was cut in two halves. The half with the Cer(AS) standard applied to it was charred and used for localization of the corresponding Cer(AS) peak of the SSL sample. The silica gel was scraped off the HPTLC plate at the estimated position and ceramides were twice eluted from the silica gel with 1 ml isopropanol for 10 min using ultrasonication. After centrifugation and separation of the silica gel, isopropanol was evaporated off. For MALDITOF MS (Bruker Biflex, Karlsruhe, Germany) lipids were dissolved in 20 µl isopropanol and mixed with 40 µl of a saturated matrix solution of  $\alpha$ -cyano-4-hydroxycinnamic acid; 0.5 µl of this mixture was applied to the spectrometer for analysis.

## RESULTS

**The composition and levels of barrier lipids in NEAE are significantly different compared with NSK** To first confirm the results of previous studies we determined whether SC lipids are altered in NEAE compared with NSK. Barrier lipids were isolated and analysed from cyanoacrylate strippings taken from long-term untreated skin sites of the shoulder of NEAE and NSK or from whole SC of either skin type. For determination of lipid composition and amount (µg lipid per mg SC protein) we used the classical HPTLC procedure II.

When comparing the mean percentage of the three barrier lipid classes in cyanoacrylate strippings, for NEAE we detected a significant increase of sterols, a significant reduction of free fatty acids, and a significant increase of Cer<sub>total</sub> (Table I). As free fatty acids may have been of sebaceous as well as of epidermal origin, their omission from calculation gives more barrier specific results about the proportion of the lipids. Thereafter, the percentages of Cer<sub>total</sub> and sterols of NSK were similar to those in NEAE. In lipids extracted from whole SC, we found similar mean percentages for NSK and NEAE and each class of barrier lipids.

The quantities of Cer(EOS), Cer(NS), Cer(NP), Cer(AS), Cer(AP), and Cer(AH) were significantly reduced in cyanoacrylate strippings of NEAE (Table II). Additionally, Cer(EOS)/Cer<sub>total</sub> and Cer(NP)/Cer<sub>total</sub> were significantly decreased and Cer(EOH)/Cer<sub>total</sub> was significantly increased (Table III).

Our data demonstrate a decrease in quantity and a change in composition of ceramides in the SC of NEAE compared with NSK. Changes in the overall barrier lipid pattern can only be observed in the outermost SC levels as shown by analysis of cyanoacrylate strippings.

**Increased polarity of the HPTLC solvent system resolves two ceramide subfractions in Cer(AS) position** To increase the resolution of ceramide fractions contained within a single band on HPTLC we slightly increased the polarity of the solvent system used for development by adding a larger quantity of acetic acid (procedure I). This procedure caused a marked shift of the Cer(NP) and Cer(NS) standards to higher reference values if compared with the position of the peaks after development with procedure II (Fig 1). The position of the Cer(AS) standard, which contained long

**Table I. Overall barrier lipid composition in different samples of NSK and NEAE<sup>a</sup>**

Skin type/sample	Percentage (sterols + Cer <sub>total</sub> + fatty acids = 100%)			Percentage (sterols + Cer <sub>total</sub> = 100%)	
	sterols	Cer <sub>total</sub>	fatty acids	sterols	Cer <sub>total</sub>
NSK					
cyanoacrylate strippings (n=10)	10 ± 2	26 ± 10	64 ± 11	67 ± 9	33 ± 9
whole SC (n=6)	33 ± 3	37 ± 3	30 ± 3	46 ± 4	54 ± 4
NEAE					
cyanoacrylate strippings (n=10)	22 ± 2*	47 ± 11*	31 ± 11*	70 ± 9	30 ± 9
whole SC (n=5)	34 ± 8	36 ± 4	29 ± 12	48 ± 5	52 ± 5

<sup>a</sup>Mean ± SD. \*p < 0.01 compared with corresponding value of NSK cyanoacrylate strippings (Student's t-test, normal distribution checked by Kolmogorov-Smirnoff test).

**Table II. Ceramide quantities in cyanoacrylate strippings<sup>a</sup> of NSK and NEAE**

Skin type	Cer(EOS)	Cer(NS)	Cer(NP)	Cer(EOH)	Cer(AS)	Cer(AP)	Cer(AH)
NSK (n=9)	2.2 ± 0.8	4.4 ± 1.1	4.0 ± 1.1	1.9 ± 0.4	3.6 ± 1.0	2.3 ± 1.1	3.0 ± 0.8
NEAE (n=10)	1.0 ± 0.7*	2.5 ± 1.0*	1.9 ± 1.2*	1.5 ± 0.6	1.9 ± 1.1*	1.5 ± 0.9*	1.8 ± 1.0*

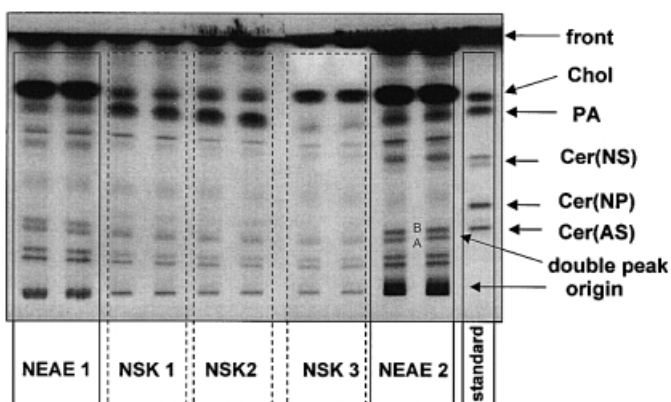
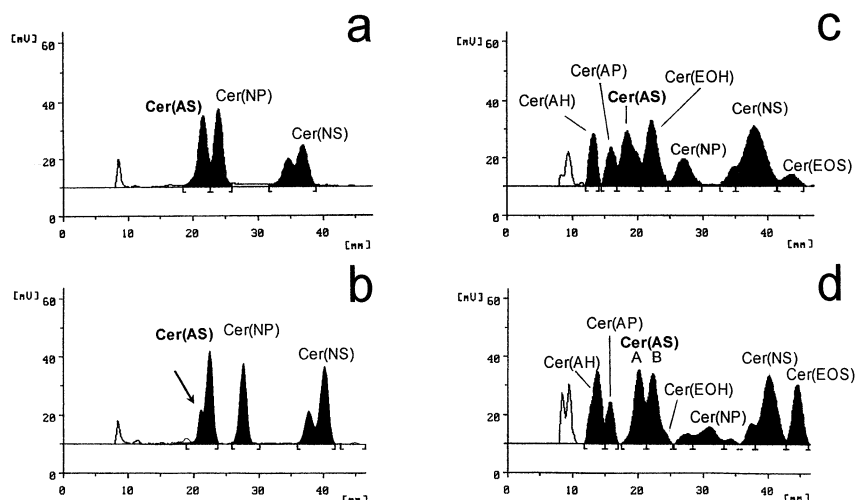
<sup>a</sup>[µg per mg SC protein]; Mean ± SD. \*p < 0.01 compared with NSK (Student's t-test, normal distribution checked by Kolmogorov-Smirnoff test).

**Table III. Ceramide composition<sup>a</sup> in NSK and NEAE after development with different procedures**

Skin type	Cer(EOS)	Cer(NS)	Cer(NP)	Cer(EOH)	Cer(AS)	Cer(AP)	Cer(AH)
NSK (n=14), procedure I	0.18 ± 0.05**	0.12 ± 0.02**	0.14 ± 0.10	0.09 ± 0.05	0.22 ± 0.03**	0.10 ± 0.01	0.15 ± 0.03
NSK (n=10), procedure II	0.10 ± 0.03	0.21 ± 0.05	0.19 ± 0.03	0.09 ± 0.02	0.17 ± 0.04	0.10 ± 0.03	0.14 ± 0.02
NEAE (n=14), procedure I	0.18 ± 0.08**	0.17 ± 0.04*	0.07 ± 0.06**	0.08 ± 0.05**	0.27 ± 0.03** <sup>o</sup>	0.09 ± 0.02	0.15 ± 0.02
NEAE (n=10), procedure II	0.07 ± 0.03''	0.23 ± 0.07	0.15 ± 0.05'	0.14 ± 0.05''	0.15 ± 0.02	0.12 ± 0.04	0.14 ± 0.02

<sup>a</sup>[Cer(XX)/Cer<sub>total</sub>]; mean ± SD; <sup>o</sup>sum of both subfractions; \*\*p < 0.01 if compared with corresponding values of same skin type after procedure II; \* p < 0.05 if compared with corresponding values of same skin type after procedure II; ' p < 0.01 if compared with corresponding values of NSK after procedure II; '' p < 0.05 if compared with corresponding values of NSK after procedure II; (Student's t-test, normal distribution checked by Kolmogorov-Smirnoff test).

**Figure 1. Influence of development procedure on peak position of ceramides in HPTLC.** (a, c) Classical development procedure II; (b, d) more polar procedure I; (a, b) ceramide standards; (c, d) SSL of NEAE. (a, b) Cer(NP) and Cer(NS) are obviously more affected by increase of polarity of solvent system than Cer(AS), but the peak of the latter shows a clear separation (arrow). (c, d) Double peak formation at the Cer(AS) position in SSL of NEAE only with procedure I [d; a small peak, tentatively designated Cer(EOH), obviously converged into the Cer(AS) double peak and formed a small shoulder at its right flank].



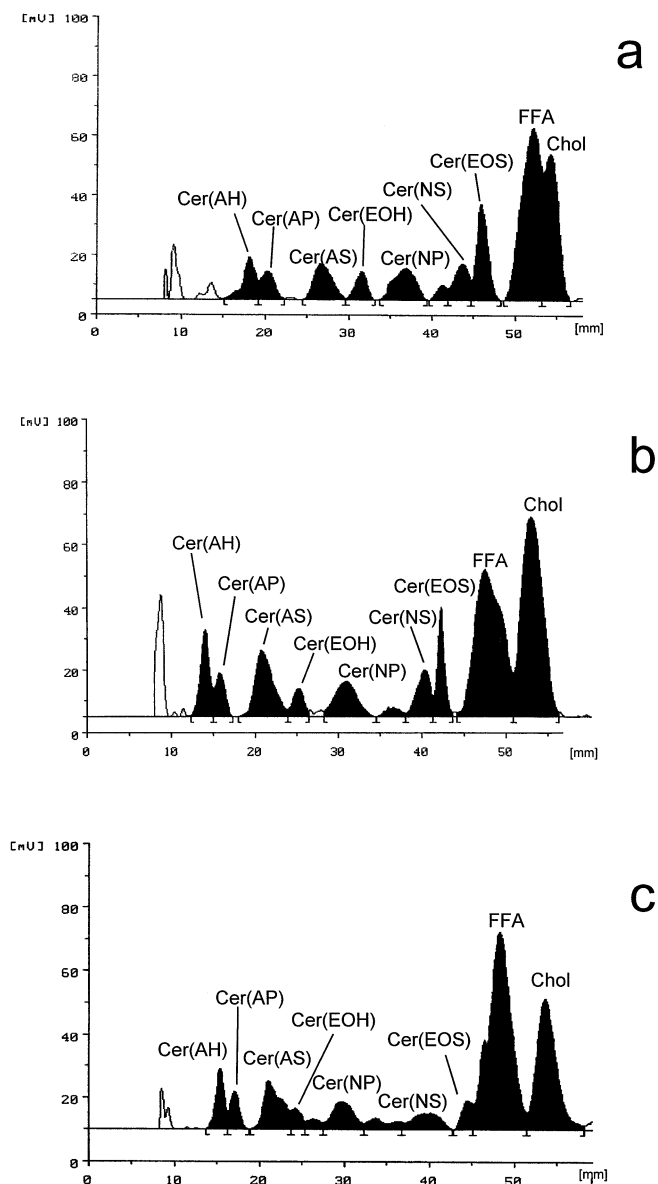
**Figure 2. Representative TLC plate of SSL of NEAE and NSK after development with the more polar solvent system I.** Two different SSL samples of NEAE were developed together with three different SSL samples of NSK. The formation of a double band in the Cer(AS) position with its subpeaks A and B could be demonstrated for NEAE only. Chol, cholesterol; PA, palmitic acid.

chain (e.g., C<sub>24</sub>) and short chain (C<sub>18</sub>)  $\alpha$ -hydroxy fatty acid, was affected less by the change of solvents but showed a clear peak separation (Fig 1).

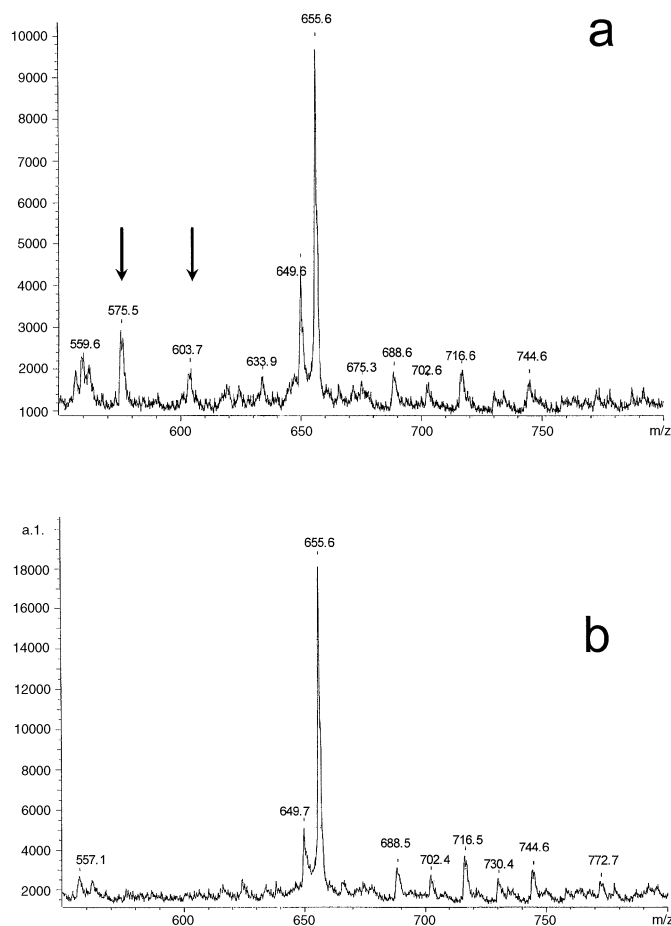
Both procedures (I and II) separated ceramides of human SC into seven main fractions that were designated according to their position on the plate, with Cer(EOS) being the least and Cer(AH) the most polar species (Robson *et al.*, 1994). If procedure II was employed for development of NEAE SSL only a single Cer(AS) peak was present (Fig 1); however, the higher polarity of system I favored the formation of a double peak in the Cer(AS) position in SSL of NEAE (Figs 1, 2). In contrast, SSL of NSK showed no clear peak separation in Cer(AS) position when developed with NEAE samples. In some cases the single peak of NSK displayed a shoulder at its right flank, which seemed to co-localize with subfraction B of the double peak.

In SSL of NEAE, the double peak in the Cer(AS) position comprised  $27\% \pm 3\%$  (mean  $\pm$  SD) of Cer<sub>total</sub> (Table III). The subpeaks comprised  $15\% \pm 3\%$  (A) and  $12\% \pm 2\%$  (B) of Cer<sub>total</sub>. In SSL of NSK the Cer(AS) peak comprised 22% of Cer<sub>total</sub>. Furthermore, when using procedure I the ratios of Cer(EOS)/Cer<sub>total</sub>, Cer(NS)/Cer<sub>total</sub>, Cer(NP)/Cer<sub>total</sub>, Cer(EOS)/Cer<sub>total</sub>, and Cer(AS)/Cer<sub>total</sub> were significantly changed in barrier lipids of NEAE if compared with the values of procedure II (Table III). In NSK Cer(NP) and Cer(EOH) were not affected.

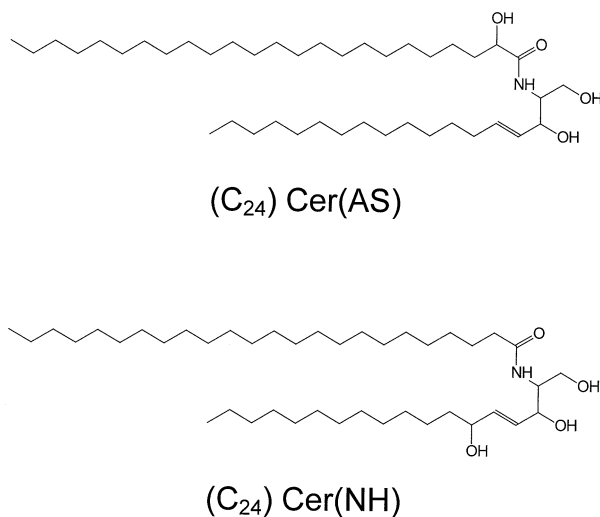
As shown in Fig 2, the double peak in the Cer(AS) position was already visible on the TLC plate in many cases if compared with the corresponding spot of NSK SSL. Again, a separation of the Cer(AS)



**Figure 3. No formation of the Cer(AS) double peak in SSL of seborrheic eczema, psoriasis, or senile xerosis.** SSL from (a) non-eczematous skin of seborrheic eczema, (b) non-eczematous skin of psoriasis, (c) senile xerosis. Chol, cholesterol; FFA, free fatty acids were developed with procedure I for HPTLC as described in Materials and Methods.



**Figure 4. Representative MALDI mass spectra of ceramides in the Cer(AS) position.** Spectra of mass of lipid species extracted from HPTLC at the Cer(AS) position near (a) NEAE, (b) NSK. Arrows indicate two additional peaks (605-23) and (576-23) in SSL of NEAE.



**Figure 5. Molecular structures of (C<sub>24</sub>) Cer(AS) and (C<sub>24</sub>) Cer(NH).**

peak is not detectable in SSL of non-lesional skin of patients with psoriasis (n=2), senile xerosis (n=2), or seborrheic eczema (n=2) (Fig 3). The use of the modified development system I demonstrates that Cer(AS) of NEAE is composed of at least two subfractions of almost equal percentage that have different polarities.

**The double peak in the Cer(AS) position is present in human SC lipids extracted by different methods** To determine whether there are differences in the levels of the Cer(AS) double peak depending on the method of lipid extraction we tested different modes.

In NEAE a clear formation of the double peak could be demonstrated for both SSL (Fig 1) and lipids of whole SC. In whole SC of NEAE, the subpeaks comprised  $13\% \pm 2\%$  (A) and  $11\% \pm 2\%$  (B) of Cer<sub>total</sub> (mean  $\pm$  SD; n=5). In whole SC of NSK, the single peak in Cer(AS) position comprised  $23\% \pm 2\%$  of Cer<sub>total</sub> (n=6) after procedure I; however, in lipids extracted from cyanoacrylate strippings the formation of a double peak was comparatively indistinct.

The non-invasive extraction of epidermal lipids from the surface of the skin seems to be the most reliable method for the detection of Cer(AS) peak separation in NEAE.

**The double peak is formed by mono-hydroxylated and mono-unsaturated ceramides of different chain lengths with either sphingosine or hydroxy sphingenine backbone** To further characterize the ceramides represented by the double peak in NEAE, silica gel in the Cer(AS) position was scraped off the TLC plate. Adhering lipids were extracted and mass spectra of the sodium-adducts (At wt. 23) were performed by MALDI-TOF MS.

Common peaks for both skin types represented a molar mass of 665 (688-23), 679 (702-23), 693 (716-23), 707 (730-23), and 721 (744-23) g per mole (Fig 4). Given that the sphingosine backbone has a constant chain length of C<sub>18</sub>, these peaks might represent Cer(AS) species with amide-linked (C<sub>22,24,26</sub>) $\alpha$ -hydroxy fatty acids (Wertz *et al*, 1985). The 611 (634-23) peak could be explained as contamination by Cer(AP). The 553 (576-23) peak might be interpreted as Cer(AS) with (C<sub>16</sub>)  $\alpha$ -hydroxy fatty acid and was present in both samples of NEAE. In the spectra an additional peak at the 604 g position occurred, representing a molecular weight of the lipid of 581 g per mole (605-23), which might be interpreted as Cer(AS) with (C<sub>18</sub>)  $\alpha$ -hydroxy fatty acid. Alternatively, all peaks in the mass spectra in principle could also represent Cer(NH) molecules, in which non-hydroxy fatty acids were linked to 6-hydroxy-4-sphingenine.<sup>1</sup> The molecular structures of Cer(AS) and Cer(NH) are given in Fig 5. According to Stewart and Downing these ceramide species comigrate on the TLC plate. The large peaks at the 650 and 656 positions were caused by matrix material extracted from the silica gel and did not represent any lipids. The levels of other ceramides, such as Cer(EOH), possibly co-eluted from the silica gel were below detection limits of the system.

## DISCUSSION

In this study we demonstrated the formation of a double peak in thin layer chromatography of barrier lipids of NEAE in the position of Cer(AS) by using a modified procedure. Determination of the molecular weight by MALDI-TOF MS suggested that the double peak could be formed by a homologous series of mono-unsaturated ceramides containing an additional hydroxyl group and (C<sub>16,18</sub>) and (C<sub>22,24,26</sub>) fatty acids linked to (C<sub>18</sub>) sphingosine, probably short and long chain subfractions of Cer(AS) or even Cer(NH) (Fig 5).<sup>1</sup> In contrast, in normal skin a single peak in the Cer(AS) position was mainly formed by the long chain subfraction; however, further analysis is necessary to confirm our assumption, because human SC contains free sphingosine, dihydrosphingosine, phytosphingosine, and 6-hydroxy-6-sphingenine of different chain lengths (Stewart and Downing, 1995).

To determine whether the presence of the double peak in the Cer(AS) position depends on the method of lipid extraction we tested different samples to find out the most suitable method for possible clinical application. Although all ceramide fractions should be detectable in equal ratio in all extracts, in lipids extracted from

<sup>1</sup>Stewart ME, Downing DT: A new ceramide in human stratum corneum. *J Invest Dermatol* 112:122, 1999 (abstr.)

cyanoacrylate strippings of NEAE the formation of the double peak was not always distinct. The reason for this is unknown. In contrast, in extracts from the skin surface and whole SC, Cer(AS) peak separation is optimal. The similarity of the results obtained with the latter methods in respect of the formation of the double peak, its percentage of Cer<sub>total</sub>, and the ratio of subpeaks, showed that the double peak occurred in all SC layers. Additionally, the results suggest that it was not artificially caused by the remains of topical products or sebaceous lipids, which accumulate at the surface of the skin and enrich in SSL (Lavrijsen *et al*, 1994). Despite the high risk of contamination by topical preparation, we proposed that the non-invasive extraction of skin surface lipids is a reliable method suitable for the analysis of ceramide composition for detection of the double peak in the Cer(AS) position.

As different methods of lipid extraction remove lipids from different SC layers, discrepancies in the overall lipid composition can be expected. Cyanoacrylate strippings largely reflect the lipid profiles of the upper SC layers and might be contaminated by sebaceous lipids. In accordance with Lavrijsen *et al* (1994), the free fatty acids content markedly affected the results. Ratios obtained after omission of free fatty acids from the calculation more closely resemble those obtained with whole SC. The latter did not show any differences between the overall lipid profiles of NEAE and NSK. Furthermore, the similarity of the lipid ratios in whole SC implies that the deficiency of free fatty acids in NEAE (**Table I**) is only restricted to the uppermost layer of the SC. This can either be attributed to a reduced amount of fatty acids from sebum or an increased loss of free fatty acids. Nevertheless, even a locally restricted alteration of the barrier lipid ratio may be detrimental to barrier function of the affected SC layer (Mao-Qiang *et al*, 1995). SSL were not included in the calculation of overall lipid ratios, as the origin of different lipids is not well defined.

We could confirm previous observations of Imokawa *et al* (1991) and Di Nardo *et al* (1998) about a ceramide deficiency in SC of NEAE and decreased percentages of Cer(EOS) and Cer(NP); however, in contrast to Di Nardo we could not detect an increase in the amount of free sterols in either whole SC or cyanoacrylate strippings of NEAE (**Table I**). As Di Nardo included cholesterol-sulfate in the calculation of lipid ratios, the discrepancy regarding cholesterol can in part be explained by a decreased percentage of cholesterol-sulfate in NEAE.

Although procedure I for HPTLC favored the formation of the double peak, comparison of individual ceramide ratios of NEAE or NSK with corresponding values of procedure II clearly indicates a significant blending of the less polar ceramide species (**Table III**). This phenomenon principally disqualifies procedure I for analysis of barrier ceramides until all peaks have been characterized by additional analysis.

Some of the alterations in the pattern of ceramides of NEAE separated by procedure II were similar to those described for lipids isolated from psoriatic scale (**Table III**) (Motta *et al*, 1993), which are characterized by an increase of the percentage of Cer(NS) and Cer(AS) and a decrease of the percentage of Cer(EOS) and Cer(NP). Motta, however, did not recognize or mention subfractions in the Cer(AS) position. Nevertheless, in non-lesional skin of psoriasis we could not detect the formation of a double peak. Accordingly, non-lesional skin of psoriasis and atopic dermatitis differ in respect to Cer(AS) composition. Furthermore, the double peak could not be detected in non-lesional skin of seborrheic eczema and in senile xerosis.

The Cer(AS) subfractions, which we assume to contribute to the formation of the double peak in NEAE, were already identified in NSK (Wertz *et al*, 1985) as well; however, according to Wertz in NSK the majority of the  $\alpha$ -hydroxy fatty acids in Cer(AS) subfractions have a chain length of C<sub>24,26</sub> (48%). Only a minor quantity contains (C<sub>16,18</sub>)  $\alpha$ -hydroxy fatty acids (16%). As we could not detect short chain ceramides in NSK lipids at the Cer(AS) position by MALDITOF MS but in NEAE lipids, our results may

reflect either an accumulation of the short chain subfraction or vice versa a reduction of long chain subfraction in NEAE.

As the short chain subfraction of Cer(AS) is more polar than the long chain one, an accumulation of the former should have caused an increase of subpeak A of the double peak in the Cer(AS) position; however, it appears that subpeak B is increased in NEAE (**Fig 2**). Consequently, alterations of the quantity of the recently identified Cer(NH)<sup>1</sup> have to be taken into account in order to explain the formation of the double peak in NEAE. Thereafter, its occurrence can best be explained by a reduction of the quantity of Cer(NH), which is above-average or average, concomitant with a reduction of Cer(AS), which is average or below average if compared with the other ceramide species.

In order to evaluate the importance of ceramide profile changes for barrier properties, Bouwstra *et al* (1998) recently studied the influence of different ceramides on the molecular organization of SC lipids *in vitro*. Lipid organization was measured by the formation of a long periodicity phase (LPP) in small angle X-ray diffraction of lipid mixtures. According to Bouwstra, Cer(EOS) plays a dominant role in the formation of the LPP and short chain Cer(AS) species are thought to be supportive. Therefore, one may speculate that the assumed increase in the level of short chain Cer(AS) in NEAE may compensate for the significantly decreased levels of Cer(EOS); however, the increased TEWL indicates that this compensation is not or is only partly effective.

In conclusion, in SSL and lipids from whole SC from NEAE we could show the formation of a double peak in the Cer(AS) position by using a modified procedure for HPTLC. Comparative MALDITOF MS suggested an increase of a short chain Cer(AS) subfraction in NEAE. But other alterations in composition and chain length distribution of mono-unsaturated, mono-hydroxylated ceramides co-migrating with Cer(AS) may contribute to the double peak formation. This particular alteration of ceramide composition at the Cer(AS) position was found in all SC layers. In contrast, in either non-eczematous skin of psoriasis and seborrheic eczema, or in NSK and senile xerosis, a single peak occurred in the Cer(AS) position. Our finding may not be of pathogenic significance. More likely, the presence of the double peak may turn out to be a disease marker, which can be used for early diagnosis of atopic eczema; however, further investigation should attempt to prove whether the double peak is a general phenomenon in NEAE or depends on season, skin site, subtype of the disease, and/or its severity.

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