

Skin Ceramide Profile in Children With Atopic Dermatitis

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Background: Atopic dermatitis (AD) is a common disease, which involves a disruption of the skin barrier function. Skin ceramide (CER) composition, which plays crucial roles in maintaining the barrier function of the stratum corneum, is changed in patients with AD.

Objective: The aim of this study was to identify and quantify skin CER subclasses in association with disease severity in pediatric patients with AD.

Methods: Two hundred thirteen patients were entered into the observational study. We compared their CER profiles using normal-phase high-performance liquid chromatography coupled with dynamic multiple reaction monitoring mass spectrometry.

Results: In total, 12 subclasses of CERs were identified. We found that 2 subclasses, that is, CER[AS] and CER[NS], were elevated ($P = 0.007$ and 0.012 , respectively) and correlated with Severity Scoring of Atopic Dermatitis ($P = 0.004$ and 0.004 , respectively).

Conclusions: Skin CER abundances are changed in children with AD compared with control subjects.

Pediatric atopic dermatitis (AD) is a common skin disease with a global prevalence of 0.8% to 18.4%.¹ In addition to the high prevalence, AD exerts a great disease burden by impacting the quality of life for both patients and their families.² Atopic dermatitis is a multifactorial disease in which genetics, environmental factors, and immune dysregulation all interplay. The dominant mechanisms are immunological dysregulation and skin barrier dysfunction.¹

The skin barrier is located in the uppermost layer of the skin, the stratum corneum. Its functions are to protect the body from excessive transepidermal water loss (TEWL) and to prevent the penetration of compounds into the body via the epidermis.³ The main penetration pathway crucial for the barrier function is carried out by the intercellular lipid matrix, which consists of ceramides (CERs), cholesterol, and free fatty acids, in an approximately equal molar ratio.³ Previous studies conducted in adults have found that several lipids of the CER class changed, including CER [NS], CER [AS], CER [NP], CER [NH],

and so on.^{4,5} In addition, lipid dysregulation is also considered as a secondary phenomenon associated with inflammation, which integrates the 2 major mechanisms of AD pathogenesis. However, these studies were conducted in adults, and our earlier work showed that CER composition differentiated between adults and children.⁶ As little is known about the CERs profile in pediatric patients with AD, and even less about disease severity, we conducted the current study examining the abundances of CER subclasses in children aged 0 to 12 years in association with Severity Scoring of Atopic Dermatitis (SCORAD) index as a measurement of disease severity. We also explored the TEWL as an objective measurement of the barrier function of the skin.

METHODS

Nomenclature

The nomenclature was used according to previous reports.^{3,5,6} For a fatty acyl chain, N stands for nonhydroxy fatty acids, A stands for α -hydroxy fatty acids, and EO stands for esterified ω -hydroxy fatty acids; for the sphingosine chain, DS means dihydrosphingosines, S means sphingosines, P means phytosphingosine, and H means 6-hydroxysphingosine. The first letters in brackets represent the fatty acyl chain, and the last letters stand for the sphingosine chain. The 12 subclasses are indicated as CER[NDS], CER[NS], CER[NP], CER[NH], CER[ADS], CER[AS], CER[AP], CER[AH], CER[EODS], CER[EOS], CER[EOP], and CER[EOH].

Study Population and SC Lipid Collection

The study was reviewed and approved by the ethics committee of Beijing Children's Hospital, and the methods were carried out in accordance with the Declaration of Helsinki. Informed consent to

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TABLE 1. Patient Demographics

Variables	Patients	Control Subjects	P
Age, mean $\pm$ SD, mo	44.5 $\pm$ 34.0	34.8 $\pm$ 31.4	0.055
Sex (male/female), n	94/64	31/24	0.685
SCORAD, mean $\pm$ SD	40.4 $\pm$ 20.4	—	—
Mild (<25), n (%)	49 (31.0)	—	—
Moderate (25–50), n (%)	61 (38.6)	—	—
Severe (>50), n (%)	48 (30.4)	—	—
TEWL, mean $\pm$ SD	45.3 $\pm$ 21.5	28.6 $\pm$ 14.0	<0.001

participate in this study was obtained from the patients and control subjects. From September 1, 2015, to April 13, 2016, 158 subjects with mild, moderate, and severe levels of AD and 55 noneczematous control subjects (predominantly individuals with infantile hemangiomas and melanotic nevi) from Beijing Children's Hospital were enrolled in this study. Cases were identified according to the UK Working Party's Diagnostic Criteria.⁷ For each subject, samples were collected by tape stripping on the lower part of the inner forearm, just above the wrist for control subjects as previously published,⁶ and on nonoozing borders of lesional sites for patients, by a nurse practitioner who had no knowledge of the outcome data. Briefly, the first layer was not retained, but the second to fourth layers at the same place were gathered separately into 5 mL of methanol in a glass tube. Then 100 μ L of internal standard solution (which composed of equal proportions of CER[A(12)DS(18)], CER[N(8)P(18)], CER[A(17)S(18)], and CER[N(2)DS(18)] and prepared in methanol at target concentrations of 0.4 nmol/mL) was added to each tube, and the mixture was vortexed for 15 minutes and then further extracted with ultrasound for 3 minutes. Next, a 200- μ L aliquot of solution in each tube was removed and mixed for determination of protein content. The rest of the solution was then combined and dried under nitrogen. Samples were redissolved in mobile phase B (*n*-heptane/ethanol/isopropanol = 2:1:1, vol/vol) and centrifuged before quantified using liquid chromatography/mass spectrometry⁶ as previously published.⁶ We recorded TEWL from the same area as where stripping was conducted using a Tewameter TM 210 and a Corneometer CM 825 (Courage + Khazaka Electronic GmbH, Cologne, Germany) and on the forearm skin of control subjects. Severity Scoring of Atopic Dermatitis scores were recorded as another outcome variable to measure intensity and extent of the eczema, itch, and sleep loss. The authors had no information on the CER levels of individual subjects until data analyses.

Liquid Chromatography/Mass Spectrometry Analysis and Quantification

Normal-phase–high-performance liquid chromatography was performed by a binary gradient solvent system of *n*-heptane (mobile phase A) and *n*-heptane/ethanol/isopropanol = 2:1:1 vol/vol (mobile phase B) with a flow rate of 1.0 mL/min under the following program: 0 to 7 minutes, 90% to 85% A; 10.1 to 17 minutes, 50% A; and 17.1 to 20 minutes, 90% A. Separation was performed on a Sunfire Silica column (4.6 $\times$ 150 mm, 5 μ m; Waters Corp, Milford, MA) with temperature maintained at 35°C and injection volume at 5 μ L. Atmospheric

pressure chemical ionization mass spectrometry parameters were as follows: polarity, positive; nebulizer pressure, 60 psi; drying temperature, 200°C; dry gas flow, 16 L/min, dry gas temperature, 350°C; spray temperature, 350°C; and capillary voltage, 4500 V. For dynamic multiple reaction monitoring (dMRM) measurements, 483 ion pairs were set (including internal standard compounds). The accuracy and stability were improved from commonly used common MRM as previously published.⁶

Data Analysis

Statistical analyses were performed using STATA 14.0 (StataCorp, College Station, TX). Figures were generated using GraphPad Prism 6.0. Unadjusted comparisons were made using Wilcoxon signed ranks tests. Differences were considered significant when $P < 0.05$. Linear regression was performed to test the association between CER profiles and measurements of disease severity, that is, SCORAD scale⁸ and TEWL index,⁹ adjusting for age and sex. Severity Scoring of Atopic Dermatitis indices were set to 0 for the control group. Matched *t* tests were performed to compare CER abundances in lesional and nonlesional sites.

RESULTS

In total, 158 children with AD and 55 control subjects were included. The demographic and clinical characteristics were summarized in Table 1. There was no significant difference in sex between the patient and the control groups. Age was of borderline significance ($P = 0.055$), and TEWL was significantly higher for the patient group. Severity Scoring of Atopic Dermatitis indices were set to 0 for control subjects and were therefore not compared.

In total, 12 subclasses of CERs were identified; however, CER[EOP] signals were feeble and unstable and were excluded from analysis. The levels of the remaining 11 CER subclasses were illustrated (Table 2, Fig. 1). CER[NS] and CER[AS] were found to be significantly higher in the lesional sites of children with AD as compared with control subjects (Table 2). Increased levels of CER[NS] and CER[AS] were associated with higher SCORAD index adjusting for age and sex

TABLE 2. Skin CER Concentration by Subclasses

CER Subclasses	Concentration		P
	Patient	Control	
EODS	43.1 $\pm$ 34.4	41.5 $\pm$ 27.9	0.711
NS	387.2 $\pm$ 307.3	265.7 $\pm$ 182.8	0.007
NP	550.2 $\pm$ 418.8	497.1 $\pm$ 306.9	0.238
NH	52,463.5 $\pm$ 41,360.4	43,181.7 $\pm$ 38,986.9	0.419
NDS	5792.1 $\pm$ 4857.5	5078.6 $\pm$ 3820.6	0.253
AS	750.7 $\pm$ 714.8	480.8 $\pm$ 451.0	0.012
AP	282.6 $\pm$ 259.4	233.5 $\pm$ 176.3	0.155
AH	651.5 $\pm$ 507.3	551.5 $\pm$ 373.8	0.121
ADS	207.0 $\pm$ 155.8	195.1 $\pm$ 373.8	0.499
EOS	72.7 $\pm$ 72.1	57 $\pm$ 41	0.09
EOH	70.0 $\pm$ 61	60 $\pm$ 43.7	0.121

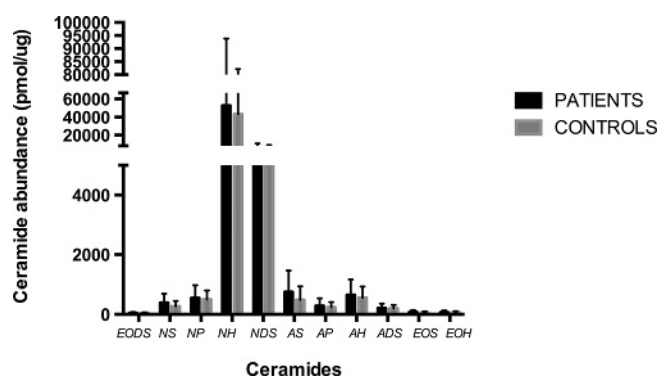


Figure 1. Bar plots showing the concentrations of all CER subclasses in healthy subjects (black column) and AD children (gray column). Bars, SD.

($P < 0.05$ for both) (Table 3). Similarly, CER[NS] level was associated with more TEWL adjusting for age and sex ($P < 0.05$) (Table 3).

We next categorized children with AD into 3 groups with mild (SCORAD <25), moderate (SCORAD ≥ 25 and ≤ 50), and severe (SCORAD >50) disease severity and found no significant difference of the 2 CER subclasses between mild, moderate, and severe patients; however, there seemed to be a trend that children with moderate to severe diseases have elevated levels of CER[NS] and CER[AS] compared with children with mild conditions (Fig. 2).

We further compared the abundances of the 2 classes of CERs in lesional sites with those of the nonlesional sites of the same patient and found a statistically significant difference ($P < 0.001$ for both), but to the opposite direction (abundances mean $\pm$ SD = 387.2 ± 307.3 and 748.4 ± 606.8 ; 750.7 ± 714.8 and 1422.7 ± 1216.3 pmol/ μ g for lesional and nonlesional sites of CER[NS] and CER[AS], respectively).

DISCUSSION

Skin lipids are an integral part for proper skin barrier function. In patients with AD, changes in both lipid composition and organization are noticed.^{5,10–12} When focusing on CER subclasses, AS, AH, AP, ADS, and NS have been reported to be decreased in adult AD skins, whereas NP, NH, and EO were increased in adult AD skins.^{3,5} However, these studies were mainly conducted in adults, and literature is lacking for pediatric patients. In line with the previous study, which indicated age as a component that differentiated children and adult skin CER composition,⁶ our study also demonstrated differential CER composition in pediatric patients. In children, the most abundant

subclasses are NH and NDS, whereas in adults, NH, NP, AH, and AP are concurrently abundant.⁵ These findings demonstrate the necessity of establishing baseline as well as disease-induced changes in CER composition in the population of children. In the main analysis, we found a significant elevation in NS and AS in children with AD ($P < 0.05$). We failed to identify significant changes in other subclasses as were reported previously, which might reflect the discrepancy between study populations. Interestingly, we identified a novel pattern among lesional, nonlesional sites, and controls, with nonlesional sites showing an even more drastic elevation from the control subjects than the lesional sites, which seems to support the idea that AD is a disease that systemically involves the skin of patients with nonlesional skin distinct from normal healthy skin in various aspects.^{5,13,14} It is unclear whether these changes are primary or reflective of a compensatory reaction to other pathogenesis such as epidermal differentiation and immune abnormalities.^{5,13,14} Future studies exploring the upper stream pathway of CER composition are required to provide better clarification.

Previous studies also attempted to identify the correlation between CER composition and disease severity and have identified CER chain length to be an important factor in skin barrier dysfunction.¹⁵ Recent studies have also been focusing on the treatment effect of CER for AD symptoms and have demonstrated that CER-containing cleanser and moisturizer regimen substantially improved skin condition and clinical outcomes related to AD severity, as well as quality-of-life aspects.^{16–18} Although the effectiveness of skin CERs has been acknowledged by several studies, the particular contribution of each individual CER subclass remains to be elucidated. In the present study, we report on a novel correlation between CER compositions, that is, NS and AS levels, and SCORAD index in children.

Our study has several strengths. The major strength is that it is the largest analysis of skin CERs in children to date, allowing characterization of the general profile as well as subgroup comparisons between patients and control subjects. We were also able to improve the sensitivity of our analysis by using a new approach of lipid quantification using normal-phase liquid chromatography coupled with dMRM of mass spectrometry.⁶ As reported previously, our signal response of dMRM was approximately 10-fold higher than that of common MRM used in earlier studies.⁶ Meanwhile, we were able

TABLE 3. CER[NS] and CER[AS] Linear Regression Coefficients and P Values

CER Subclasses	SCORAD		TEWL	
	Coefficient	P	Coefficient	P
NS	0.017	0.004	0.011	0.024
AS	0.007	0.004	0.022	0.305

Comparisons are made adjusting for age and sex.

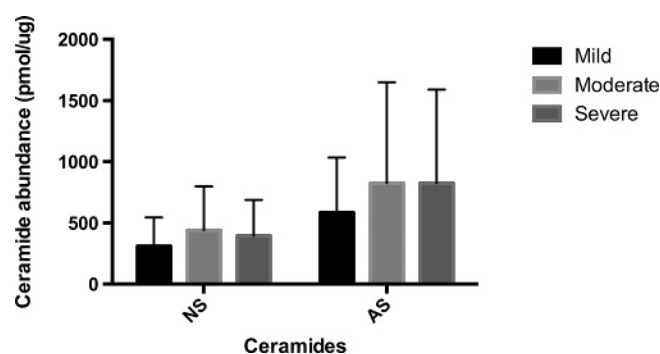


Figure 2. Bar plots of CER[NS] and CER[AS] concentrations in mild (black), moderate (lighter gray), and severe (darker gray) children. Bars, SD.

to control for common confounders (ie, age and sex) and reduced the possibility that our results were biased thereof.

We acknowledge that one of our weaknesses is that age is a differentiating component ($P = 0.055$), which might affect the comparability of the patients and the control subjects. To account for this, we controlled for age as a covariate in our linear and logistic regression models. We were also aware that other factors, including social economic status, diet preferences, and geographic locations, might also be confounders and thus should be taken into account. Larger studies with more data on patient demographics are needed to provide more definitive evidence for the pathologic changes in lipid composition in pediatric patients with AD. Nevertheless, our study, along with previous studies analyzing profiles of skin CERs, which play a key role in impairment of skin barrier function, clearly demonstrates that CER composition is changed in patients with AD. Future studies looking into using CER for treatment purposes could bring promising results.

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