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ORIGINAL PAPER

Age-related and seasonal changes in covalently bound ceramide content in forearm stratum corneum of Japanese subjects: determination of molecular species of ceramides

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Abstract
The stratum corneum (SC) consists of corneocytes surrounded by a neutral lipid-enriched intercellular matrix. Ceramides represent approximately 50% of intercellular lipids, and play important roles in retaining epidermal water. The SC also contains covalently bound ceramides, which are thought to play a crucial role in the formation of lamellar structures, and are involved in maintaining skin barrier function. A previous report showed that levels of free ceramides in human SC changed with the seasons and age, although whether the content of different species of covalently bound ceramides also underwent such temporal changes was unclear. Here, SC samples were taken from 99 healthy individuals of different ages (24–64 years) and during different seasons. The content of different molecular species of covalently bound ceramides in the samples was quantified using HPLC–MS/MS. The levels of total covalently bound ceramides (Total-Cers) significantly decreased approximately 50% in autumn and winter, compared with that of spring and summer. The levels of covalently bound ceramides containing saturated fatty acids (SFA-Cers) in the spring and summer were approximately 2.3-fold higher than that seen in autumn and winter, whereas the level of covalently bound ceramides containing unsaturated fatty acids (USFA-Cers) in spring and summer were approximately 1.6-fold higher than that in autumn and winter. Furthermore, the ratio between SFA-Cers and USFA-Cers was significantly lower in spring and summer than in autumn and winter. The levels of SFA-Cers, but not USFA-Cers, were significantly lower in individuals ≥ 50 years old compared to those who are 30- and 40-years old in the spring. Our study showed for the first time that, similar to free ceramides, the level of covalently bound ceramides changed with the seasons. However, age-related changes in covalently bound ceramide content were limited in that only the amount of SFA-Cers in the spring was lower in older individuals.

Keywords Covalently bound ceramides · Molecular species · Stratum corneum · Seasons · Age · Human

Introduction

Stratum corneum (SC), the outermost layer of the epidermis, consists of several layers of corneocytes surrounded by a neutral lipid-enriched intercellular matrix. Ceramides, which comprise approximately 50% of intercellular lipids, play an important role in retaining epidermal water and, in combination with cholesterol and free fatty acids, influence epidermal barrier permeability [19]. The SC also contains covalently bound ω-hydroxy ceramides that are most frequently bound via an ester linkage to structural proteins in the epidermal cornified envelope (covalently bound ceramides) [23]. Type of four ω-hydroxy ceramides derived from EOS: ceramide consists of esterified ω-hydroxy fatty acid and sphingosine, EOP: ceramide consists of esterified ω-hydroxy fatty acid and phytosphingosine, EOH: ceramide consists of esterified ω-hydroxy fatty acid and 6-hydroxy sphingosine, EOdS: ceramide consists of esterified ω-hydroxy fatty acid and dihydrosphingosine constitute the covalently bound ceramides in human SC [12, 21]. The covalently bound ceramides acts as a scaffolding of lamellar

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structure [22]. Compared to non-bound (free) ceramides, covalently bound ceramides are thought to play important roles in stabilizing lamellar structure and skin barrier function [14]. Previous studies showed that the amount of free ceramides in the human SC changed with the seasons [3, 9, 16]. These levels also decreased with age, and are lower in atopic dermatitis (AD) patients compared with healthy individuals [8]. Although levels of covalently bound ceramides in human SC are also known to be lower in AD patients compared with healthy people [10], whether levels of these ceramide species also exhibit seasonal and age-related changes in humans is unclear.

Conventional analysis of ceramides in SC has been widely performed by thin-layer chromatography (TLC). However, TLC analysis can have insufficient sensitivity and reproducibility, as well as being challenging to quantify. Ceramides comprise various molecular species that contain carbon chains of different lengths constituting sphingoid bases and fatty acids. Masukawa et al. used HPLC–MS/MS to identify 342 molecular species of ceramides in human SC [12]. Recently, we identified 11 molecular species of protein-bound ω-hydroxy ceramides in the epidermis of mice using HPLC–MS/MS, and showed that ratio of unsaturated fatty acids to saturated fatty acids of ω-hydroxy ceramides is associated with skin barrier structure [15]. However, whether molecular species of covalently bound ceramides change with the seasons and age in human SC is unclear. This study examined seasonal and age-related changes in the levels of molecular species of covalently bound ceramides in human SC. We analyzed the covalently bound ceramides derived from EOS which was reported to be the most species of the four [11, 17].

Materials and methods

Subjects

A total of 99 healthy Japanese subjects participated in this study. The subjects were distributed into four age groups: 20s group, 30s group, 40s group, and over 50 group. The characteristics of subjects is shown in Table 1. All participants were free of any skin disease. Informed consent was obtained from all subjects according to the Declaration of Helsinki Principles. The study was approved by the Institutional Review Board of Meiji Co., Ltd. (No. 2014-009).

Sample collection

The examinations were performed four times, in autumn (November) in 2014, winter (February), spring (May), and summer (August) in 2015. The SC sheets were collected from the left ventral forearm (5.0 cm below the antecubital fossa) by stripping three times with a 60 mm × 25 mm piece of PPS tape (Teraoka Seisakusho Co., Ltd., Tokyo, Japan) under room conditions (24 ± 2 °C and 50 ± 10% relative humidity). Subjects were prohibited from using any topical agents on their left forearm on the day of examination. The collected samples were stored at − 40 °C until analysis.

Extraction of covalently bound ceramides

One quarter of the three tapes collected were cut, immersed in hexane, and sonicated for 30 min at cool temperatures to remove the tape adhesive. The SC cells were recovered from the extracted liquid to a piece of filter paper (Kiriyama Roto Filter-paper 124, Nippon Rikagaku Kikai Co., Ltd., Tokyo, Japan) by suction filtration. After drying, the filter paper was immersed in chloroform/methanol (2:1, v/v) and sonicated for 30 min under cooling to remove free ceramides. The protein pellet was recovered to the filter paper, and dried using a centrifugal evaporator. The filter paper was incubated in 1 M KOH in 95% methanol at 60 °C for 2 h to release the lipids that were covalently bound to the SC by ester-like bonds. The solution including filter paper was neutralized with 1 N HCl and centrifuged. The supernatant including ω-hydroxy ceramides was recovered. The sediment was washed using methanol again. The supernatant were recovered, combined, dried, and redissolved in methanol. The pellet including the proteins was immersed in phosphate buffered saline (pH 7.4) containing 1% sodium dodecyl sulfate and incubated at 60 °C for 2 h to solubilize the protein. The protein concentration was assayed using a commercial kit (Micro BCA assay kit, Pierce Biotechnology, Inc., IL, USA).

Table 1 Characteristics of subjects

Group	Age			Male		Female	
	n	Mean	Range	*n*	%	*n*	%
20s	31	26.7	24–29	8	25.8	23	74.2
30s	33	33.1	30–39	12	36.4	21	63.6
40s	25	44.5	40–49	10	40.0	15	60.0
Over 50	10	56.1	51–63	5	50.0	5	50.0
Total	99	36.3	24–63	35	35.4	64	64.6

Analysis of covalently bound ceramides

Covalently bound ceramides in the SC samples were identified by high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC–MS/MS) (Quattro Premier XE, Waters Corporation, Milford, MA, USA) [15]. All analyses were performed on a 2 mm × 100 mm column with a particle size of 1.7 µm (ACQUITY UPLC® BEH C18, Waters Corporation). Mobile phase A consisted of 5 mM ammonium acetate in 95% methanol, whereas mobile phase B consisted of 5 mM ammonium acetate in methanol. The initial eluent composition was 100% A, followed by an increase to 100% B for 30 min, 100% B for 2 min, and then a reduction to 0% B for 3 min. The total running time was 35 min at an eluent flow of 0.4 mL/min and a column temperature of 40 °C. Analytes were detected by electrospray ionization in the positive mode. Multiple-reaction-monitoring (MRM) was carried out using characteristic fragmentation ions (*m/z* 704.7/264.3 for *d*18:1/28:0, *m/z* 732.7/264.3 for *d*18:1/30:0, *m/z* 730.7/264.3 for *d*18:1/30:1, *m/z* 760.8/264.3 for *d*18:1/32:0, *m/z* 758.8/264.3 for *d*18:1/32:1, *m/z* 788.8/264.3 for *d*18:1/34:0, *m/z* 786.8/264.3 for *d*18:1/34:1, *m/z* 814.8/264.3 for *d*18:1/36:1). The parameters for HPLC–MS/MS analysis were: capillary voltage 3000 V, source temperature 120 °C, desolvation temperature 400 °C, desolvation gas flow 850 L/h, cone voltage 40 V, cone gas flow 50 L/h, and collision energy 30 eV. The ceramide molecular species contents were calculated using a d18:1/24:0 ceramide standard (Avanti Polar Lipids, Inc., Alabaster, AL, USA).

Statistical analysis

All data are presented as means ± standard deviation (SD). Data were analyzed by one-way ANOVA with post hoc analysis performed using Holm test (seasonal variation), or Tukey–Kramer test (stratified analysis by age) (SPSS ver. 22.0, SPSS, IL, USA). Differences between groups were considered to be significant at $P < 0.05$.

Results

Identification of covalently bound ceramides in human stratum corneum

HPLC–MS/MS chromatograms of covalently bound ceramides in a human SC sample typically identified eight molecular species of protein-bound ω-hydroxy ceramides, which consisted of sphingosine (*d*18:1) with a long chain ω-hydroxy fatty acid (28:0, 30:0, 30:1, 32:0, 32:1, 34:0, 34:1, or 36:1) (Fig. 1). The highest peak intensity in human SC samples was seen for ω-hydroxy triacontanoate-sphingosine (*d*18:1/30:0).

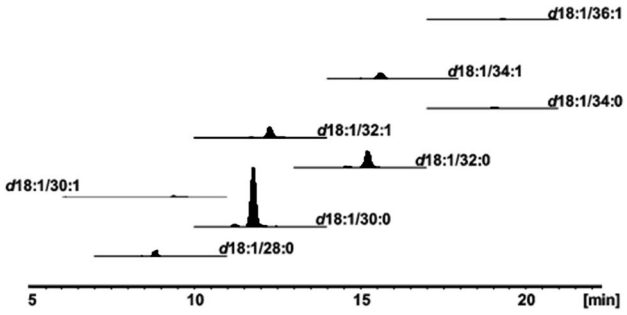

Fig. 1 Representative chromatogram of covalently bound ceramides in a human stratum corneum sample

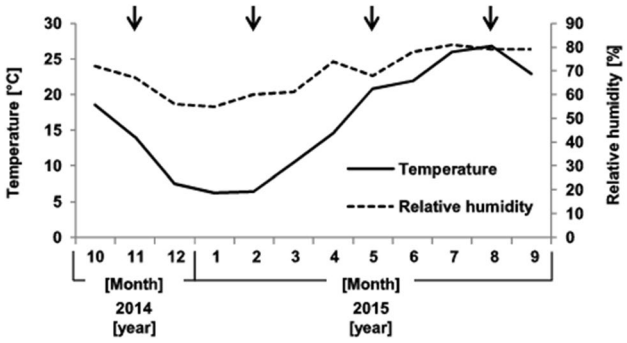

Fig. 2 Annual fluctuations in temperature and relative humidity in Yokohama. The arrows show the collection time points

Seasonal changes

According to data from the Japan Meteorological Agency, the average monthly temperature in Yokohama city, Kanagawa, Japan, is lowest in February and highest in August. Fluctuations in relative humidity were similar to those seen for temperature (Fig. 2).

We next analyzed seasonal changes in the content of total covalently bound ceramides (Total-Cers), ceramides containing saturated fatty acid (SFA-Cers), ceramides containing unsaturated fatty acid (USFA-Cers), and molecular species of covalently bound ceramides. The total-Cers content was significantly decreased in autumn and winter compared with spring and summer (Fig. 3a). Similar changes were observed in the levels of SFA-Cers, USFA-Cers, and for each molecular species (Fig. 3a, b).

The ratio of SFA-Cers to Total-Cers was significantly decreased in autumn and winter compared with spring and summer, whereas the ratio of USFA-Cers to Total-Cers was significantly increased in autumn and winter compared with

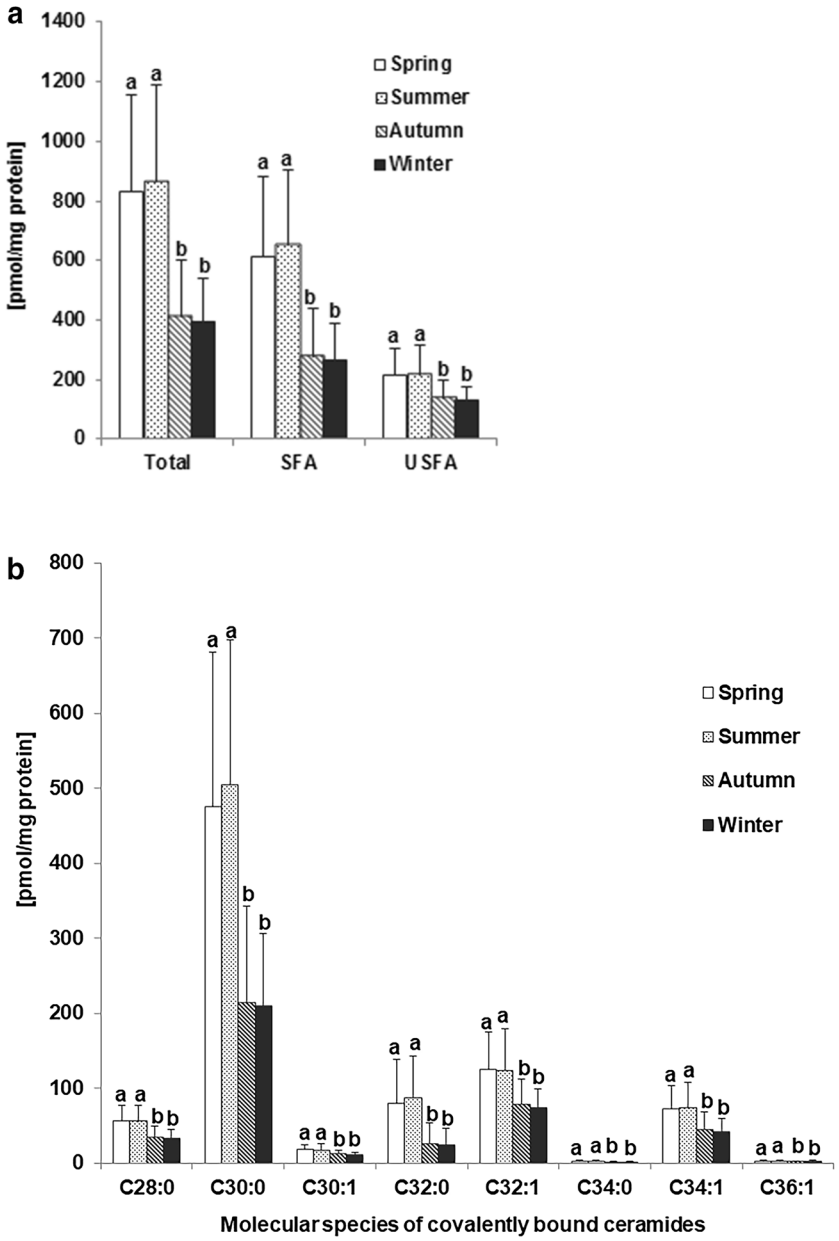

Fig. 3 Seasonal changes in **a** the content of total covalently bound ceramides (Total-Cers) ($n=99$), ceramides containing saturated fatty acid (SFA-Cers) ($n=99$), and ceramides containing unsaturated fatty acid (USFA-Cers) ($n=99$); **b** eight molecular species of covalently bound ceramides ($n=99$). Means without a common letter are significantly different, $P<0.05$

spring and summer (Fig. 4a). Meanwhile, the ratio of SFA-Cers to USFA-Cers was significantly lower in autumn and winter compared with spring and summer (Fig. 4b).

Age-related changes

Analysis of Total-Cers, SFA-Cers, and USFA-Cers among the seasons stratified by age was performed to evaluate age-related changes in covalently bound ceramide content (Table 2). In all age groups, Total-Cers, SFA-Cers, and USFA-Cers levels were significantly decreased in autumn and winter compared with spring and summer. In spring, a significant decrease in Total-Cers was observed for the over 50 group compared with the 30s group. Furthermore, in spring, SFA-Cers levels in the over 50 group were significantly lower relative to the 30s and 40s groups, whereas USFA-Cers contents were not significantly different among the groups.

Discussion

We identified eight molecular species of protein-bound ω-hydroxy ceramides, which consisted of sphingosine (*d*18:1) with a long chain ω-hydroxy fatty acid (28:0, 30:0, 30:1, 32:0, 32:1, 34:0, 34:1, or 36:1) in human SC samples. Among the species, ω-hydroxy triacontanoate-sphingosine (*d*18:1/30:0) had the highest peak intensity. Thus, covalently

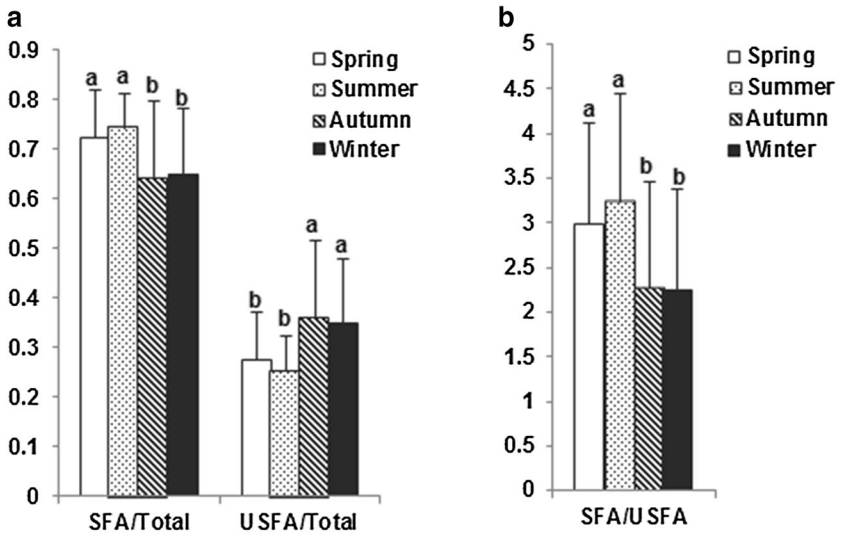

Fig. 4 Seasonal changes in **a** the ratio of ceramides containing saturated fatty acid (SFA-Cers) to total ceramides (Total-Cers) ($n=99$) and the ratio of ceramides containing unsaturated fatty acid (USFA-Cers) to Total-Cers ($n=99$); **b** the ratio of SFA-Cers to USFA-Cers ($n=99$). Means without a common letter are significantly different, $P<0.05$

Table 2 Age-related changes in covalently bound ceramide content

Age	Spring Mean ± SD	Summer Mean ± SD	Autumn Mean ± SD	Winter Mean ± SD
Total-Cers (pmol/mg protein)				
20s	$801.3 \pm 224.4^{A,ab}$	879.7 ± 282.5^{A}	413.6 ± 179.5^{B}	415.1 ± 130.6^{B}
30s	$887.6 \pm 378.3^{A,a}$	922.4 ± 376.9^{A}	405.8 ± 181.1^{B}	403.3 ± 162.9^{B}
40s	$887.8 \pm 362.6^{A,ab}$	849.8 ± 320.0^{A}	442.0 ± 228.7^{B}	381.2 ± 137.7^{B}
Over 50	$582.0 \pm 213.0^{A,b}$	686.6 ± 250.0^{A}	356.9 ± 115.3^{B}	349.9 ± 111.3^{B}
SFA-Cers (pmol/mg protein)				
20s	$583.7 \pm 198.5^{A,ab}$	670.0 ± 233.2^{A}	280.7 ± 160.1^{B}	297.1 ± 123.1^{B}
30s	$673.9 \pm 308.0^{A,a}$	694.7 ± 285.8^{A}	278.3 ± 156.8^{B}	267.9 ± 135.3^{B}
40s	$658.7 \pm 280.8^{A,a}$	625.6 ± 249.3^{A}	293.4 ± 185.9^{B}	251.0 ± 106.0^{B}
Over 50	$383.6 \pm 199.8^{B,b}$	495.4 ± 194.4^{A}	213.9 ± 122.3^{C}	214.1 ± 97.5^{C}
USFA-Cers (pmol/mg protein)				
20s	217.6 ± 64.8^{A}	209.7 ± 83.5^{A}	132.9 ± 58.9^{B}	118.0 ± 37.8^{B}
30s	213.7 ± 90.2^{A}	227.6 ± 112.4^{A}	127.5 ± 53.3^{B}	135.4 ± 51.7^{B}
40s	229.0 ± 114.9^{A}	224.2 ± 98.9^{A}	148.7 ± 79.8^{B}	130.2 ± 45.3^{B}
Over 50	198.4 ± 56.8^{A}	191.3 ± 71.8^{A}	143.0 ± 65.6^{B}	135.8 ± 36.2^{B}

SFA-Cers covalently bound ceramides containing saturated fatty acids, *USFA-Cers* covalently bound ceramides containing unsaturated fatty acids, *Total-Cers* total covalently bound ceramides

Different capital letters indicate significant differences ($P<0.05$) between the seasons

Different small letters indicate significant differences ($P<0.05$) between age groups

bound ceramides in human SC comprised various molecular species containing carbon chains with different lengths and degrees of fatty acid unsaturation.

Our study showed for the first time that covalently bound ceramides (Total-Cers, SFA-Cers, USFA-Cers, and molecular species of covalently bound ceramides) changed with the seasons wherein the content of all molecular species of covalently bound ceramides was higher in spring and summer than in autumn and winter. This result is consistent with previous studies showing that free ceramides also decreased in autumn and winter compared with spring and summer [3, 9, 16]. One possible explanation for this attenuation is the change of skin pH associated with the seasons, with higher pH values seen in winter compared to those in summer [1, 4, 18]. An increase in pH of the SC decreases catalytic activity of both β-glucocerebrosidase and acidic sphingomyelinase, which are important for ceramide synthesis [5, 7, 13]. Furthermore, an increase in the pH of SC inhibited lamellar body secretion in response to serine protease-activated receptor (PAR2) activation that follows activation of serine proteases [6]. Thus, the observed decrease in the covalently bound ceramide content of human SC in autumn and winter

might be related to a reduced rate of ceramide synthesis and inhibition of ceramide secretion in response to increases in skin pH.

This study provides interesting evidence that the ratio of SFA-Cers to USFA-Cers also changed with the seasons. SFA-Cers levels in the spring and summer were approximately 2.3-fold higher than that seen in autumn and winter, whereas USFA-Cers in spring and summer were approximately 1.6-fold higher than that in autumn and winter. These results suggested that USFA-Cers levels were less sensitive to seasonal changes than SFA-Cers. Our previous study in mice demonstrated that conditions that induce dry skin were associated with notably reduced mean percentages of SFA-Cers (30:0, 32:0, and 34:0) ranging from 3.5 to 11.4%, whereas USFA-Cers (32:1, 34:1, and 36:1) ranged from 14.3 to 34.9% relative to normal mice. These findings indicated that the difference in the ratio of unsaturated fatty acids to saturated fatty acids of ω-hydroxy ceramides is also associated with skin barrier structure. Furthermore, Bouwstra et al. demonstrated that the degree of fatty acid chain saturation of ceramide-1 had marked effects on lamellar and lateral lipid organization in vitro [2]. Therefore, both the content of covalently bound ceramides and the ratio of unsaturated fatty acids to saturated fatty acids of ω-hydroxy ceramides are likely associated with seasonal dry skin conditions.

The amount of free ceramides in human SC is known to decrease with age as evidenced by findings of Rogers et al. wherein levels of free ceramides in the SC of the hand gradually decreased between age 20 and 40 [16]. Furthermore, Imokawa et al. reported that free ceramides in SC from the forearm had a negative correlation with age based on the decreased amounts seen in older individuals [8]. However, in this study we found limited age-related changes in covalently bound ceramides in that levels of covalently bound ceramides were significantly lower only for the spring samples taken for the over 50 group. As mentioned above, compared to non-bound (free) ceramides, covalently bound ceramides are thought to play important roles in stabilizing lamellar structures and skin barrier function. Moreover, levels of covalently bound ceramides were shown to be significantly decreased after ultraviolet-B (UV-B) irradiation, tape-stripping, or treatment with sodium dodecyl sulfate, whereas the levels of non-bound ceramides remained unchanged [20]. We consider that covalently bound ceramides likely remain constant with age so as to preserve lamellar structures and attenuate skin barrier dysfunction. Notably, we found that USFA-Cers levels remained unchanged with age, even though SFA-Cers decreased in older individuals. This result may be because USFA-Cers, but not SFA-Cers, are the key molecular species needed to strengthen and maintain epidermal lamellar structures.

Taken together, our study showed for the first time that levels of covalently bound ceramides changed with the seasons. Interestingly, the ratio of SFA-Cers to USFA-Cers also exhibited seasonal variations. However, age-related changes in covalently bound ceramides were limited in that only the level of SFA-Cers in the spring decreased with age. These findings could indicate that the content of covalently bound ceramides and the differences in the degree of unsaturation of fatty acids might be associated with age-related and seasonal skin barrier dysfunction. The limitation of this study is that we analyzed only covalently bound ceramides derived from EOS. Accordingly, an analysis of covalently bound ceramides derived from all esterified ω-hydroxy ceramides needs to be performed in future.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in these studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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