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Abstract

Aging reflects skin appearance drastically, which reduces skin juvenescence. However, the small molecules underlying skin juvenescence have not been well studied. We aimed to explore the molecules potentially responsible for young-looking skin. Eleven healthy women aged 24–59 years were recruited and divided into young and middle-aged groups. Multiple layers of the stratum corneum from the cheek area were taken by tape-stripping, followed by desorption electrospray ionization mass spectrometry imaging (DESI-MSI). Overall, five molecules (*m/z* 284.33, 340.39, 488.39, 628.37, and 863.65) were highly abundant in young subjects. Among them, *m/z* 284.33 and 340.39 were dominantly detected in each layer of all young subjects. Interestingly, *m/z* 488.39 and 628.37 were prominent in young subject 4. All of these molecules were gradually decreased in the successive layers of the stratum corneum in subjects 3 and 4 of the young group. These molecules could be endogenous, co-related with youthful skin, or retained from topical cosmetics. Extensive research is indispensable to characterize them and find the relationship between these molecule's retention capacity in the stratum corneum with different skin parameters. Our findings provide a novel perspective on young skin that could be advantageous in future cosmetic formulations to improve skin juvenescence.

Keywords: Stratum corneum, DESI-MSI, molecules, skin juvenescence.

Introduction

Skin is the most visible external attribute of the human being[1]. It is one of the critical indicators of aging[2,3]. The aging process significantly impacts the skin's young appearance[4,5]. The appearance of the skin is highly correlated to the stratum corneum, which is the outer layer of the skin. Stratum corneum, in turn, consists of multiple layers in it[6]. Improved skin texture and blood flow, prevention of pigmented spot formation, and increased moisture in the stratum corneum are the external parameters for skin to have beautiful, young-looking skin[7]. Keratin is a major component of stratum corneum and constitute more than 85% of the total proteins in it[8]. Keratin and associated proteins are deformed over the aging process[9,10]. However, until now very few studies on the other small molecules in the stratum corneum underlying skin juvenescence have been done. In this connection, we aimed to discover the potential small molecules in the stratum corneum that might be responsible for skin juvenescence. While it is feasible to investigate human skin that has been surgically resected during some diseases, however in healthy individuals, skin sampling is desired to be noninvasive[11]. Several studies have recently been employed in normal conditions to analyze skin[7,12,13]. Recently, several techniques have been employed to analyze skin conditions by imaging the skin based on its external appearance[7,14-16]. Apart from these, mass spectrometry-based studies are a more advanced technique due to providing molecular information as well[17,18]. Among the mass spectrometric methods, the mass spectrometry

imaging (MSI) analysis adds a new dimension to the biomolecular distribution analysis in the skin[19-23]. Currently, multiple MSI modalities are in use, like matrix-assisted laser desorption/ionization (MALDI)-MSI, secondary-ion mass spectrometry (SIMS) imaging, and DESI-MSI. Compared to other MSI modalities, DESI-MSI is a rapidly in-situ molecular imaging technique because it does not require matrix coating on the sample, unlike MALDI-MSI. Moreover, DESI-MSI is used to detect compounds and visualize their spatial distribution in the sample in ambient conditions[11,24-27]. For straightforward, rapidness, and direct surface analysis capacity in ambient condition, in this study, we employed DESI-MSI to detect small molecules of stratum corneum, which are highly abundant in young-aged skin[28].

Materials and Methods

Reagent and chemicals

Acetonitrile, methanol, formic acid, ultrapure water, and isopropanol were purchased from FUJIFILM Wako pure chemical industries (Osaka, Japan). Leucine enkephalin was purchased from Waters Corporation (Milford, MA, USA). Sodium formate was obtained from Sigma-Aldrich Co. LLC (St. Louis, MO, USA).

Ethics

The study was approved by the ethics committee of the Hamamatsu University School of Medicine (Code: 19-123). Study subjects were provided with sufficient information and signed informed consent forms before collecting tape-stripped stratum corneum samples. Each subject's stratum corneum sample was anonymized by assigning a unique numerical subject number.

Study subjects and tape stripped stratum corneum samples collection

In this study, 11 healthy Japanese female volunteers participated. Only individuals who participated in this testing schedule could consent to the stratum corneum sampling and other test items, and not suffering from skin diseases like eczema or other pathological conditions were selected. Subjects who recently received special facial care or medication that may affect the sample collection site (face) were excluded from this study. In addition, only women were chosen as subjects to eliminate sexual differences between men and women. Six subjects were categorized as young, aged between 20-30s. (subject number 1 to 6 in the young group). The other five subjects were between the ages of 40-60s and categorized as middle-aged (subject number 7 to 11 in the middle-aged group) (Table 1). As a sampling area, we selected the cheek area of the face. Redness, pores, and spots were scored by photo evaluation, while skin transparency was scored by visual evaluation. The redness, pores

Table 1. Demographic data of the donors of tape stripped stratum corneum sample

Subject group	Subject ID	Age (years)	Redness (0 to 2 scale)	Pores (0 to 2 scale)	Spots (0 to 2 scale)	Skin transparency (0 to 4 scale)	Skin conditions
Young-aged	1	27	1.5	1.5	0.5	3.5	Redness, many bumps due to acne scars, normal skin tone
	2	30	1.5	1.5	0.5	3.5	Many bumps due to worsening acne, normal skin tone
	3	31	0.5	0.5	0.5	3.5	Many bumps due to acne scars, normal skin tone
	4	25	0.5	0.5	0	0	Brighter skin tone, almost no pigmentation, less uneven skin surface
	5	28	0	0	0.25	1	Lighter skin tone with unevenness due to fine pigmentation, less uneven skin surface
	6	24	0	0	0	1	Lighter skin tone, few bumps on the skin surface, but bumps near the nose
Middle-aged	7	59	0	0	2	3.5	Uneven skin tone due to hyperpigmentation, skin surface irregularity is not worse than that of the same age group, skin tone is darker
	8	55	0	2	2	3.5	Many uneven colors due to pigmentation, uneven skin surface due to flowing texture, dark skin tone
	9	45	0.5	0.5	0.5	2	Less unevenness in skin tone due to pigmentation than the same age group, skin surface irregularity about the same as the same age group, lighter skin tone
	10	41	0.5	0.5	0.5	2	Uneven skin tone due to pigmentation, less uneven skin surface than same age group, lighter skin tone
	11	43	0	0	0.25	2	Lighter skin tone with very little unevenness due to pigmentation

and spots scores were scored on a 5-point scale from 0 to 2 with 0.5 increments. The lower score of redness, pores, and spots indicates better skin condition and is considered to have a youthful appearance of the skin. The skin transparency score was scored on a 9-point scale from 0 to 4 with 0.5 increments. Lower scores indicated higher skin transparency. The skin with more increased skin transparency is typically considered to have a youthful appearance of the skin. These redness, pores, spots and skin transparency score evaluation was constructed based on the "Skin Aging Atlas" exclusive to Asian type[29]. Before stratum corneum sample collection, each subject's face was thoroughly washed with the same (commercial) mild cleanser (Table S1) using tap water. After face washing, the subjects were allowed to acclimate in a constant temperature and humidity-maintained room (room temperature 20 ± 2 °C, humidity 50 ± 5%) for 15 minutes at least. The stratum corneum samples (horny layers of the epidermis) were then collected by 1 cm^2 tape-stripping by cellophane tape (CELLOTAPE™, CT-18, NICHIBAN Co., Ltd., Tokyo, Japan) from the cheek four times from the same spot. Although the thickness of stripped stratum corneum samples was not measured, we tried collecting stratum corneum samples from each subject with a similar method to minimize the removed stratum corneum thickness variation between subjects. The first stripping refers to the stratum corneum's outermost layer, whereas subsequent strippings refer to the stratum corneum's inner layers. In all cases, the tape-stripped samples were preserved at -80 °C until mass spectrometric analysis.

DESI-MSI analysis

For DESI-MSI of the stratum corneum sample, tape-stripped samples were mounted on regular glass slides (Matsunami Glass Ind., Ltd., Kishiwada, Japan). A double-sided tape (Conductive tape assy, 241-08728-92, Shimadzu Corporation, Kyoto, Japan) was attached to the glass slide before attaching the non-adhesive side of the stripped tape. The analysis was

conducted with a quadrupole time-of-flight (Q-TOF) mass spectrometer (Xevo G2-XS Q-TOF, Waters Corporation, Milford, MA, USA) in positive ionization mode. The selected areas on the glass slide were scanned with a scan rate and pixel size of 200 µm/sec and 200 µm × 200 µm, respectively. A solvent pump (ACQUITY UPLC Binary Solvent Manager, Waters Corporation, Milford, MA, USA) was used to supply the solvent (98:2 methanol/water, v/v) at a flow rate of 2 µL/min. Mass resolving power and mass window were set at 20000 and 0.02 Da, respectively. The DESI source conditions were optimized as follows: (i) capillary voltage of 3.0 kV, (ii) nitrogen gas pressure of 0.4 MPa, and (iii) inlet temperature of 120 °C. Analyzer mode was set as "sensitivity." Mass spectra were collected in a mass range of *m/z* 100 to 1000. The sodium formate solution (500 µM) in isopropanol: water (90:10, v/v) was used to calibrate the DESI mass spectra externally, and the detector setup was performed using leucine enkephalin solution (500 µM). The lock mass correction option was used for mass accuracy corrections using *m/z* 309.2036 (Na^+ adduct of diisopropyl sebacate, a typical background peak in mass spectrometry).

Data analysis

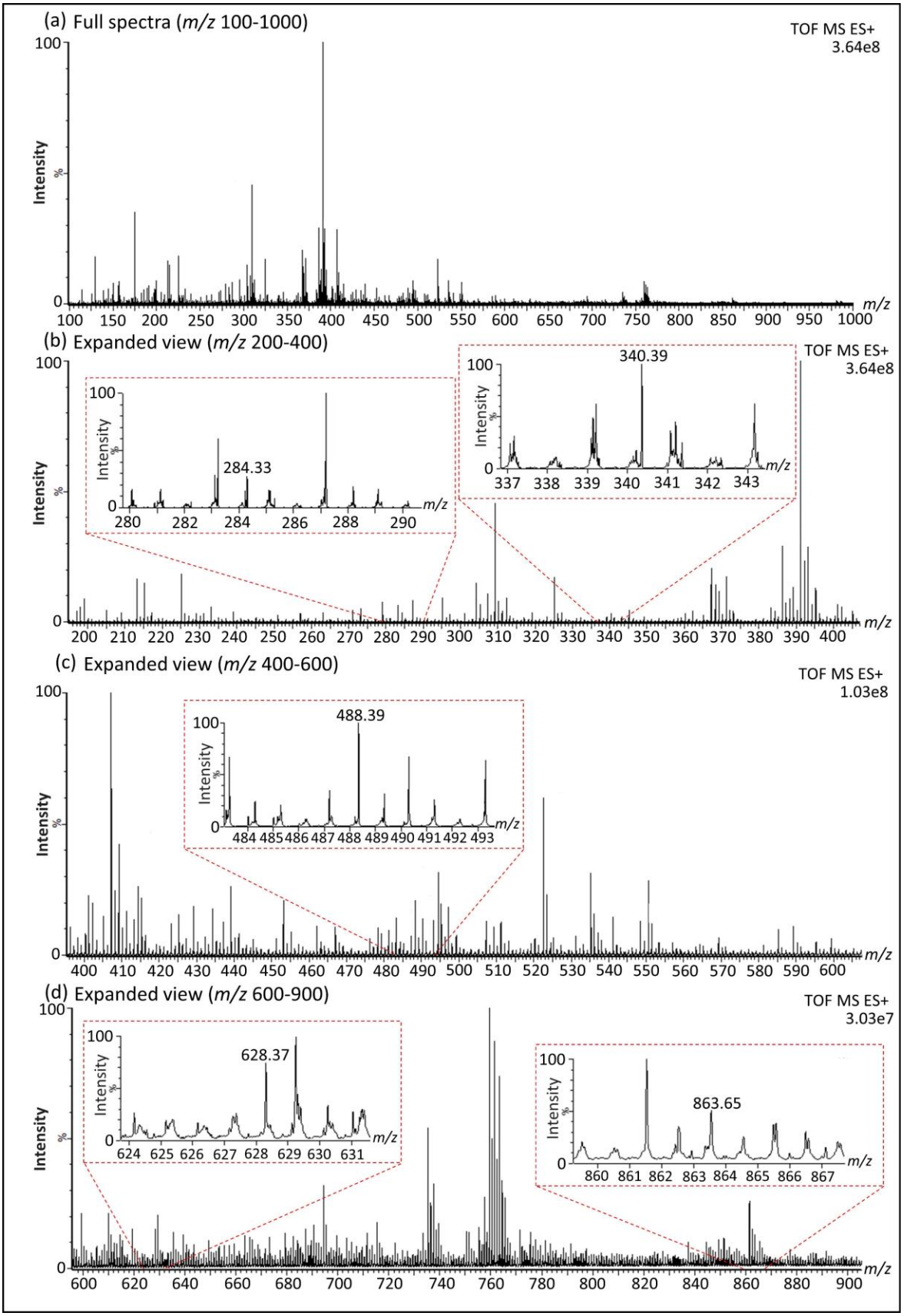
The data acquisition and processing were made using the MassLynx (Version 4.1; Waters Corporation, Milford, MA, USA) program for DESI-MSI experiments. Raw data derived from the DESI-MSI experiment was imported into HDImaging software (Version 1.4; Waters Corporation, Milford, MA, USA). The candidate selection was performed from the list of 300 most abundant *m/z*s generated in HDImaging software and manually compared the DESI-MSI ion image between groups (Fig. S3). Thus, we have considered the average intensity between groups in picking candidates. Background peaks were excluded considering the molecule's distribution in the negative control tape sample. This negative control tape sample was the

148 only tape sample attached to the glass slide, hence free from the stratum corneum sample.
149 Region of interest (ROIs) was manually drawn on each sample area to calculate average
150 intensity using HDImaging software. Average signal intensities of individual pixels of the ROIs
151 (Fig. S1b) were then compared for relative abundance.

152 **Results**

153 *DESI-MSI detected highly abundant molecules in young-aged subjects*

154 In positive ion mode, we analyzed the DESI ion distribution of tape-stripped stratum
155 corneum samples. We explored the most abundant 300 DESI-MSI peaks between *m/z* 100 to
156 *m/z* 1000 and, on average, found five molecules (*m/z* 284.33, *m/z* 340.39, *m/z* 488.39, *m/z*
157 628.37, and *m/z* 863.65) are highly abundant in the stratum corneum of the young-aged
158 subject compared to the middle-aged subjects (Fig. 1b to d and Fig. 2b to k). Among them,
159 *m/z* 284.33 was 122%, *m/z* 340.39 was 196%, *m/z* 488.39 was 345%, *m/z* 628.37 was 265%,
160 and *m/z* 863.65 was 137%, highly abundant in average intensity in young-aged subjects
161 compared to middle-aged subjects. *m/z* 414.43 is a molecule shown here as a control, which
162 was not much different in distribution among the young and middle-aged groups (only 7%
163 higher in the young group) (Fig. 2l, m). Among all detected candidate molecules, *m/z* 284.33
164 and *m/z* 340.39 were prominently detected in each layer of all young subjects (Fig. 2b, d and
165 Fig. S2a to h).



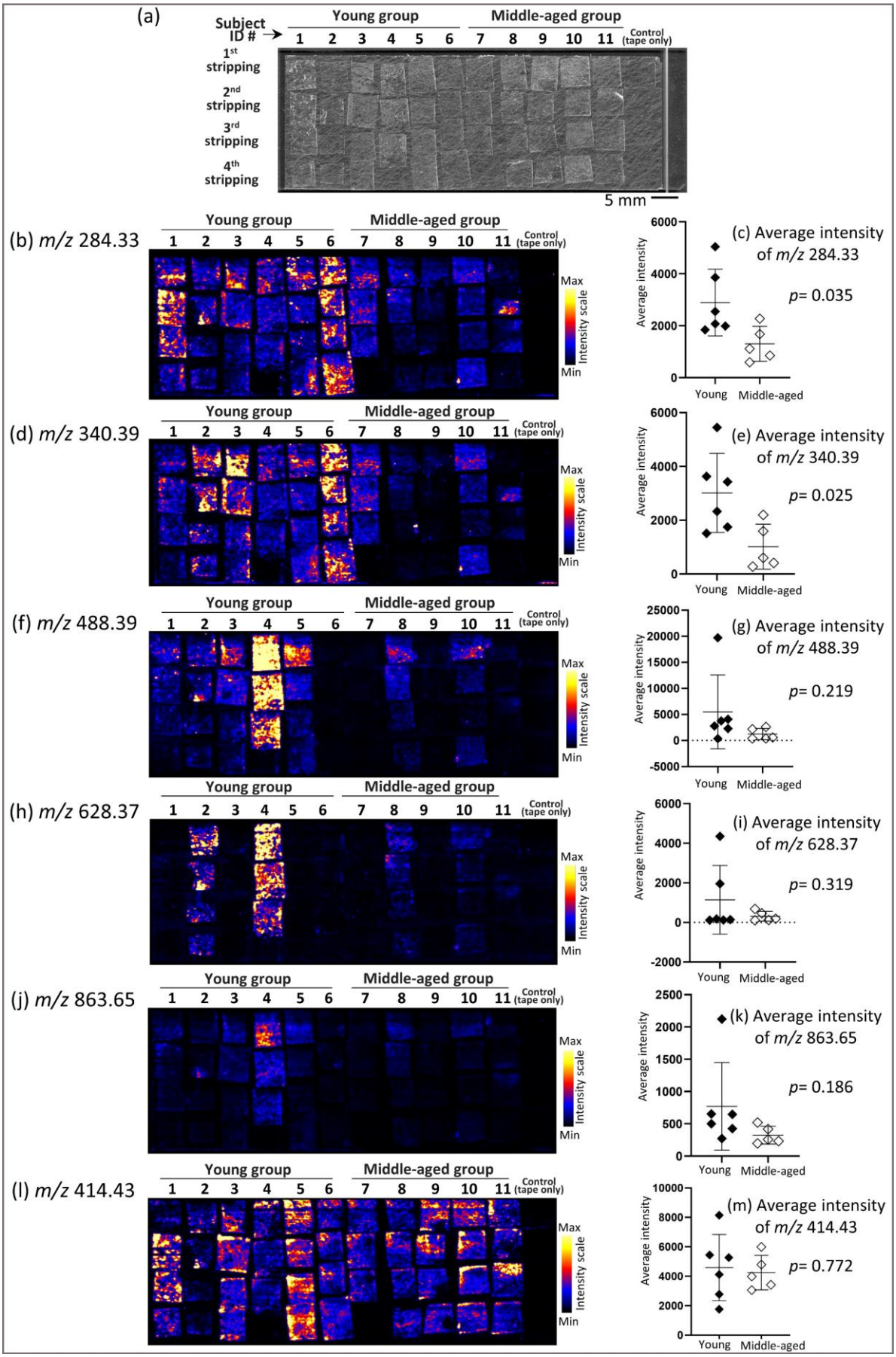
166 **Fig. 1. Average DESI-MSI mass spectrum from tape stripped stratum corneum.**

Young-aged subject 4 showed the most prominent distribution of m/z 488.39 and m/z 628.37

Although the candidate molecules were highly abundant on average in the young subjects, we discovered the most prominent distribution of *m/z* 488.39 (Fig. 3a to c) and *m/z* 628.37 (Fig. 3d to f) in subject number 4 from the young-aged panel. By analyzing the average intensity of each stratum corneum layer, we also discovered that the intensity was gradually decreasing in the deeper layers of the stratum corneum (Fig. 2f, h).

Candidate molecules were gradually decreased in deeper layers of the stratum corneum of young subjects 3 and 4

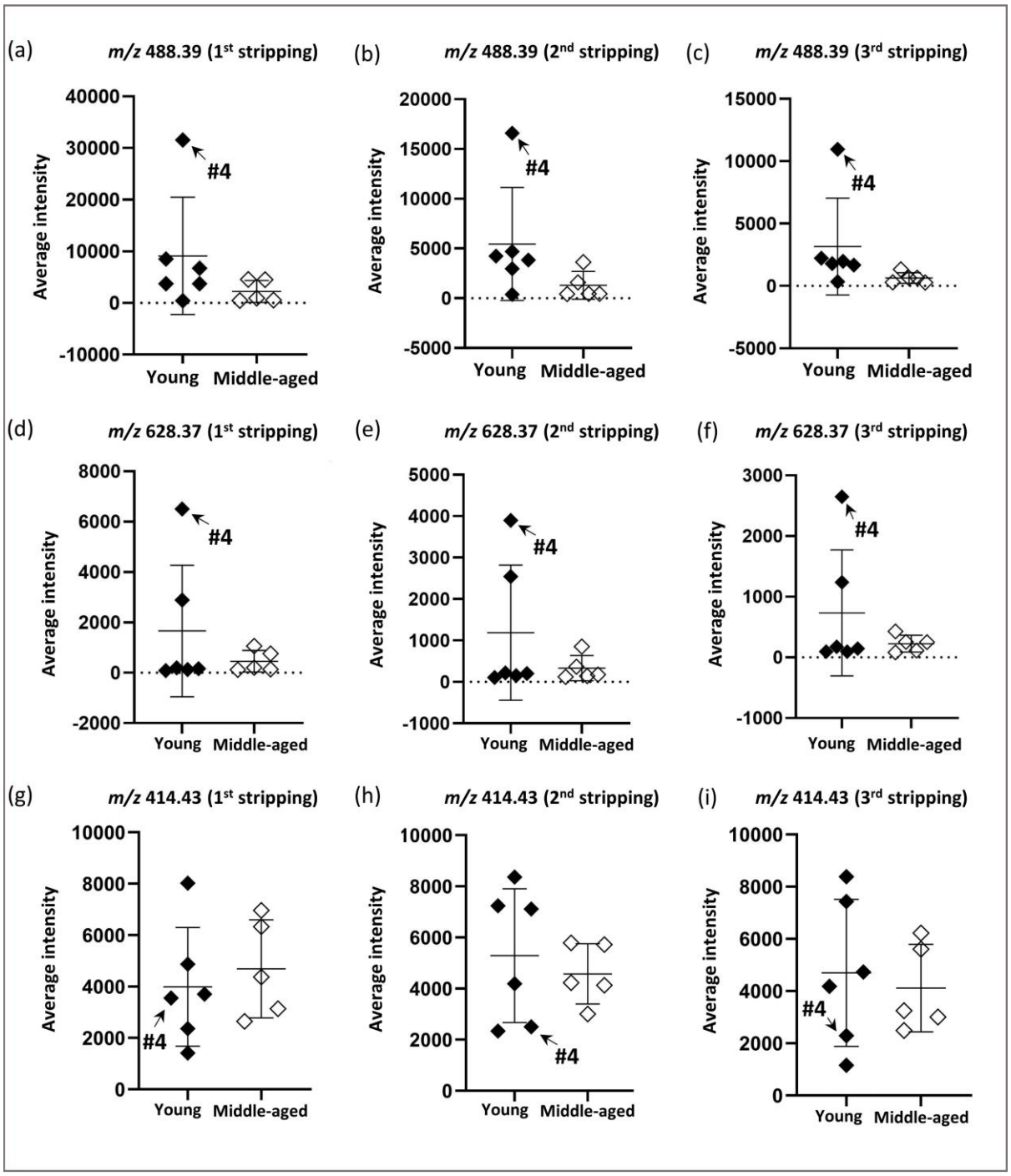
Although, on average, all candidate molecules are rich in young-aged subjects, interestingly, they are most abundant in the first stripped stratum corneum of the young subject 3 (Fig. 4a to e) and 4 (Fig. 4f to j). These molecule's abundance is gradually decreased in the subsequent strippings. That refers to the gradual decrease of the candidate molecules in the deeper layers of the stratum corneum of the young-aged subjects 3 and 4.


(a)
Subject ID #
Young group
Middle-aged group
1 2 3 4 5 6 7 8 9 10 11
Control (tape only)
1st stripping
2nd stripping
3rd stripping
4th stripping
5 mm
(b) m/z 284.33
(c) Average intensity of m/z 284.33
p= 0.035
(d) m/z 340.39
(e) Average intensity of m/z 340.39
p= 0.025
(f) m/z 488.39
(g) Average intensity of m/z 488.39
p= 0.219
(h) m/z 628.37
(i) Average intensity of m/z 628.37
p= 0.319
(j) m/z 863.65
(k) Average intensity of m/z 863.65
p= 0.186
(l) m/z 414.43
(m) Average intensity of m/z 414.43
p= 0.772
Max
Min
Intensity scale
Average intensity
Young
Middle-aged

181

182 **Fig. 2. Prominent distributions of highly abundant molecules in the tape-stripped stratum**

183 **corneum samples from young and middle-aged subjects.**



184

185 **Fig. 3. Average intensity of *m/z* 488.39, *m/z* 628.37, and *m/z* 414.43 in each time of tape**

186 **stripped stratum corneum samples.**

187

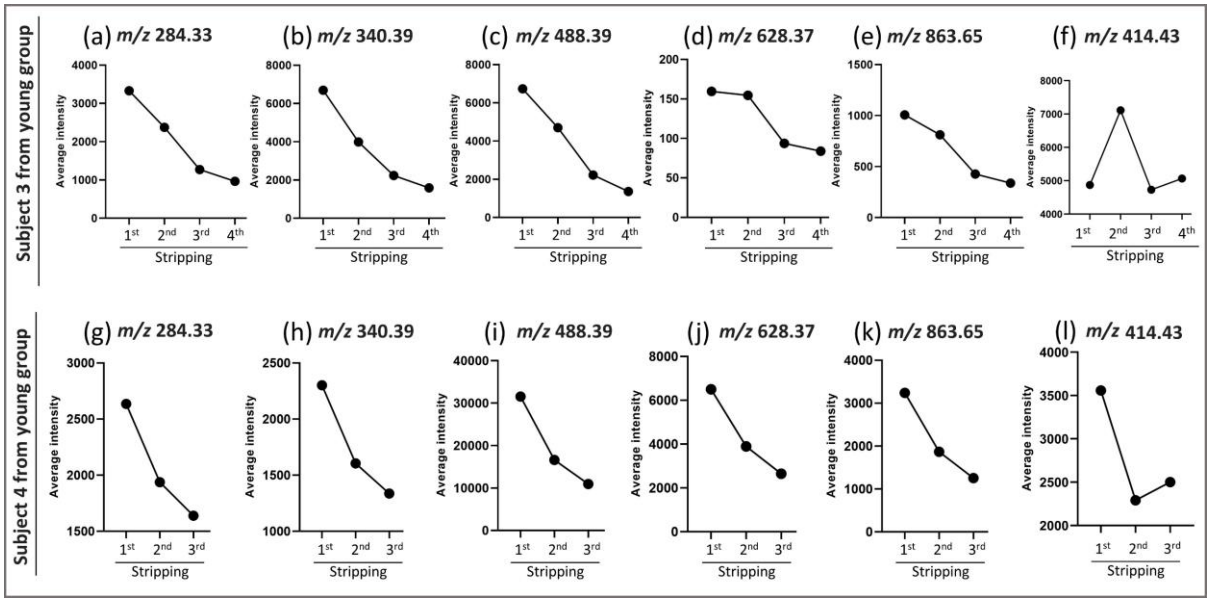


Fig. 4. Stratum corneum's layer-wise average intensity of the candidate molecules in subject 3 and subject 4 from the young group.

Discussion

In this investigation, we could detect abundant small molecules from the stratum corneum of young, healthy individuals using the robust measurement ability of DESI-MSI[30,31]. Unlike earlier studies that employed DESI-MSI to explore stratum corneum molecular abundance, our study focused on the cheek area of the face as a representative part of the body to study age-dependent molecular abundance[22]. Among the candidate molecules, *m/z* 284.33 and *m/z* 340.39 prominently exist in all stratum corneum of all young subjects (Fig. 2b, d and Fig. S2a to h). Our finding surmises that these molecules might be highly related to skin juvenescence. However, our study is limited to positive-ion mode-based detection of the potential candidate molecules highly relevant for young-aged skin. The addition of negative

202 ion mode, molecular identification by comprehensive analysis using liquid chromatography-
203 mass spectrometry, liquid chromatography with tandem mass spectrometry, and further
204 functional studies are required to determine these molecule's relationship with young-aged
205 skin[32]. Interestingly subject 4 of the young-aged subjects showed a most prominent
206 distribution of *m/z* 488.39 and *m/z* 628.37 in her stratum corneum (Fig. 3a to f). This most
207 prominent abundance reflects the unique properties of this young subject, who has a very
208 smooth skin surface with no pigmentation (Table 1). In the future, it would be beneficial to
209 deepen the research on this subject to find out the exact relationship between its skin
210 parameters and the retention capacity of those molecules. Also, we observed a gradual
211 decrease of the candidate molecules only (Fig. S4 a to f) in the subsequent tape strippings of
212 subjects 3 and 4. We surmised that some specialty, like reduced cell numbers in the deeper
213 layers of the stratum corneum of these two subjects, might reflect such distributions[22]. In the
214 future, it would be informative to study the exact relationship between these subject's
215 stratum corneum and the candidate molecule's gradual decrease.
216 This preliminary study shows the differential abundance of some specific molecules in the
217 stratum corneum of young-aged subjects. This is also supported by the previous studies where
218 it was evident that the property of the stratum corneum, like age and skin condition, plays a
219 role in the compositional difference in the skin[9,10]. This study is the groundwork for the
220 relationship between skin condition and underlying molecular distribution in the stratum

corneum. We successfully detected higher distribution of some small molecules in the young subjects. In essence, it is indispensable to identify and characterize the candidate molecules to consider them for inclusion in product development for promoting skin juvenescence. In the future, our findings may support establishing novel candidate molecules for the skin care product industry to promote or retain skin juvenescence.

Acknowledgements

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Conflicts of Interest

The authors declare no conflict of interest. The funders remain neutral in study design, data collection and analysis, manuscript writing, or publication decisions.

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319

320

Figure legends

Fig. 1. Average DESI-MSI mass spectrum from tape stripped stratum corneum.

(a) full positive-ion mass spectrum ranged *m/z* 100-1000. (b) Expanded view of masses ranged 200-400 showing detection of *m/z* 284.33, and *m/z* 340.39. (c) Expanded view of masses ranged *m/z* 400-600 showing detection of *m/z* 488.39, and (d) Expanded view of masses ranged *m/z* 600-800 showing detection of *m/z* 628.37, and *m/z* 863.65.

Fig. 2. Prominent distributions of highly abundant molecules in the tape-stripped stratum corneum samples from young and middle-aged subjects.

(a) Stratum corneum sample orientation in glass slide. (b) DESI-MSI ion images of *m/z* 284.33, (c) average intensity comparison of *m/z* 284.33 between young and middle-aged subjects. (d) DESI-MSI ion images of *m/z* 340.39, (e) average intensity comparison of *m/z* 340.39 between young and middle-aged subjects. (f) DESI-MSI ion images of *m/z* 488.39, (g) average intensity comparison of *m/z* 488.39 between young and middle-aged subjects. (h) DESI-MSI ion images of *m/z* 628.37, (i) average intensity comparison of *m/z* 628.37 between young and middle-aged subjects. (j) DESI-MSI ion images of *m/z* 863.65, (k) average intensity comparison of *m/z* 863.65 between young and middle-aged subjects. (l) DESI-MSI ion images of *m/z* 414.43, a compound showing similar distribution in young and middle-aged subjects, (m) average intensity comparison of *m/z* 414.43 between young and middle-aged subjects. All values are presented as mean ± SD and *p* values were calculated between groups by Student's unpaired two-tailed t-test.

Fig. 3. Average intensity of *m/z* 488.39, *m/z* 628.37, and *m/z* 414.43 in each time of tape stripped stratum corneum samples.

(a) Average intensity of *m/z* 488.39 in first, (b) second, and (c) third stripped stratum corneum. Average intensity of *m/z* 628.37 in first (d), second (e), and third (f) stripped stratum corneum.

Average intensity of *m/z* 414.43 in first (g), second (h), and third (i) stripped stratum corneum as control. All values are presented as mean ± SD.

Fig. 4. Stratum corneum's layer-wise average intensity of the candidate molecules in subject 3 and subject 4 from the young group.

(a) Average intensity of *m/z* 284.33, (b) *m/z* 340.39, (c) *m/z* 488.39, (d) *m/z* 628.37, (e) *m/z* 863.65, and (f) *m/z* 414.43 (as control) in young subject 3. Average intensity of (g) *m/z* 284.33, (h) *m/z* 340.39, (i) *m/z* 488.39, (j) *m/z* 628.37, (k) *m/z* 863.65, and (l) *m/z* 414.43 (as control) in young subject 4.

Supplementary figures

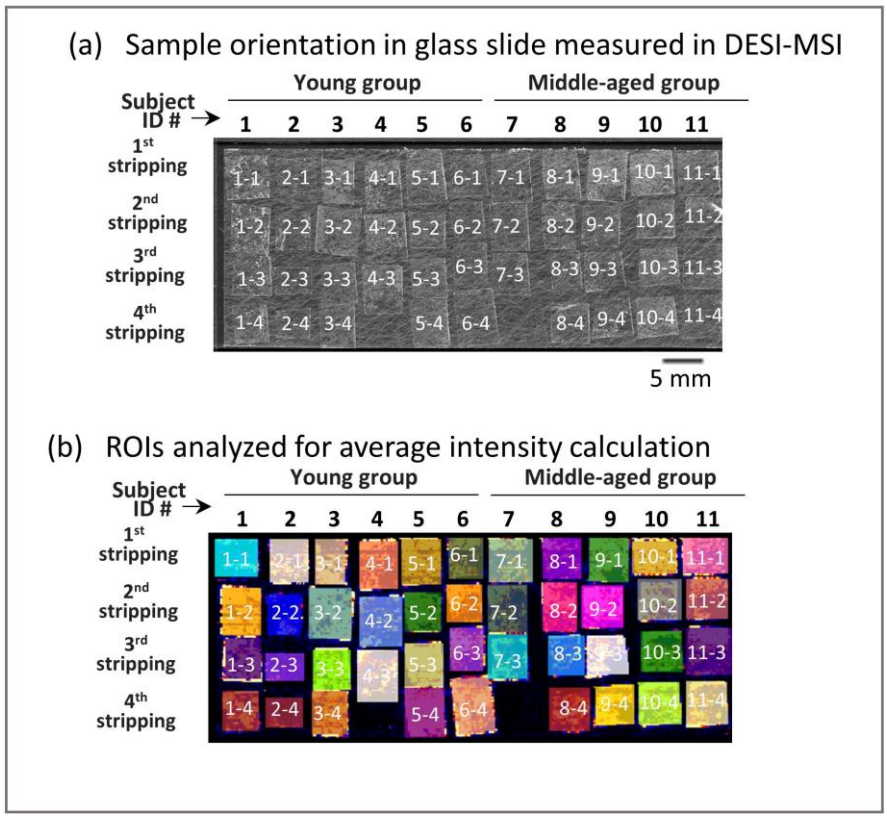

Fig. S1. ROIs drawn to measure average intensity. (a) sample orientation in glass slide measured in DESI-MSI. (b) ROIs drawn.

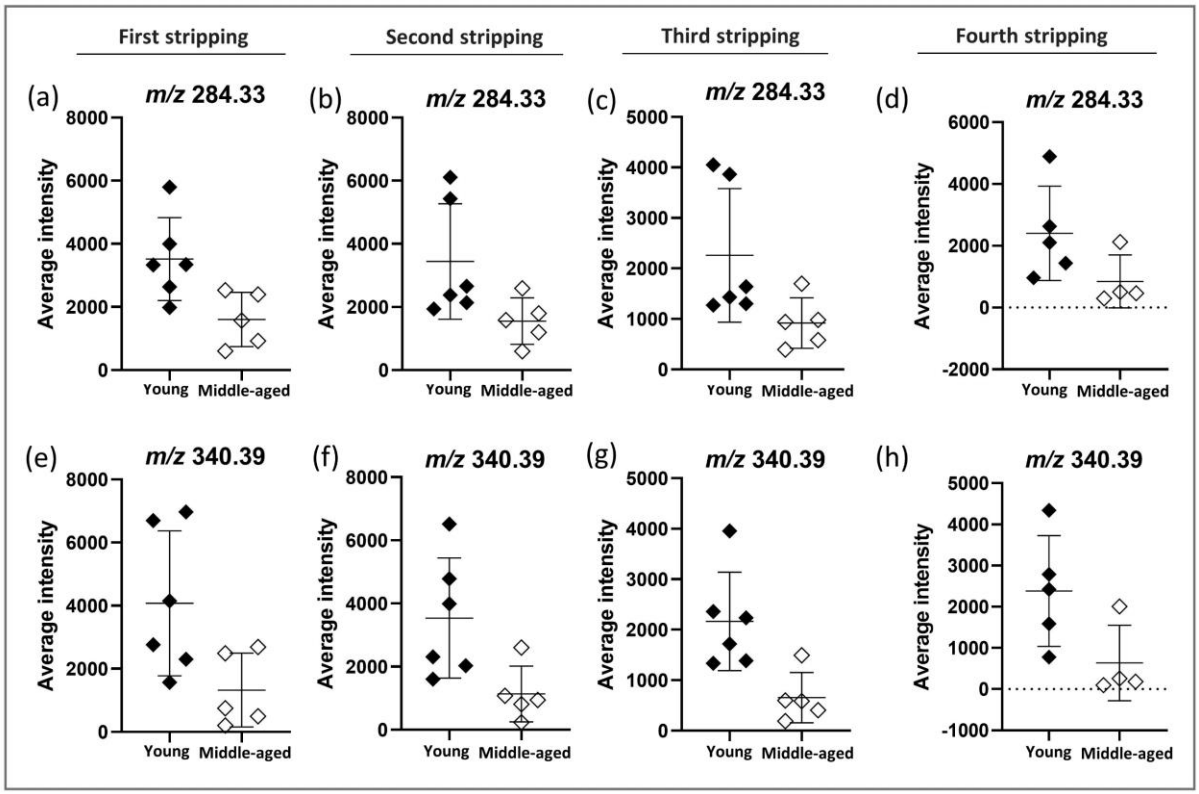

Fig. S2. *m/z* 284.33 and *m/z* 340.39 were prominently detected in each layer of all young subjects. All values are presented as mean ± SD.

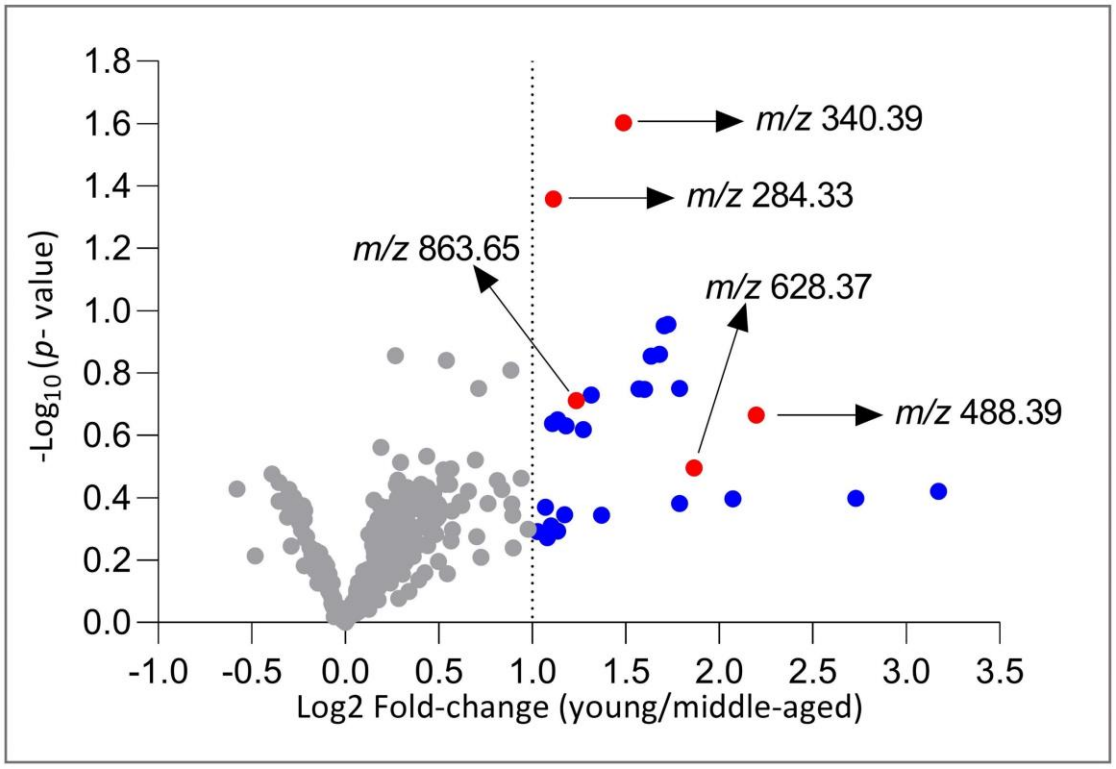

Fig. S3. Volcano plot compares the molecules from young and middle-aged subjects. Log2 fold changes and their corresponding $-Log_{10}$ *p*-values of 300 most abundant molecules generated by HDImaging software were taken for construction of the volcano plot. Molecules increased more than 2-fold are depicted in blue dots, among them which molecules are detected in all samples and average intensity was higher in young groups are depicted in red dots. All other molecules are not found to be relevant to this study and depicted as gray dots. Student's unpaired two-tailed t-test was used for the volcano plot construction.

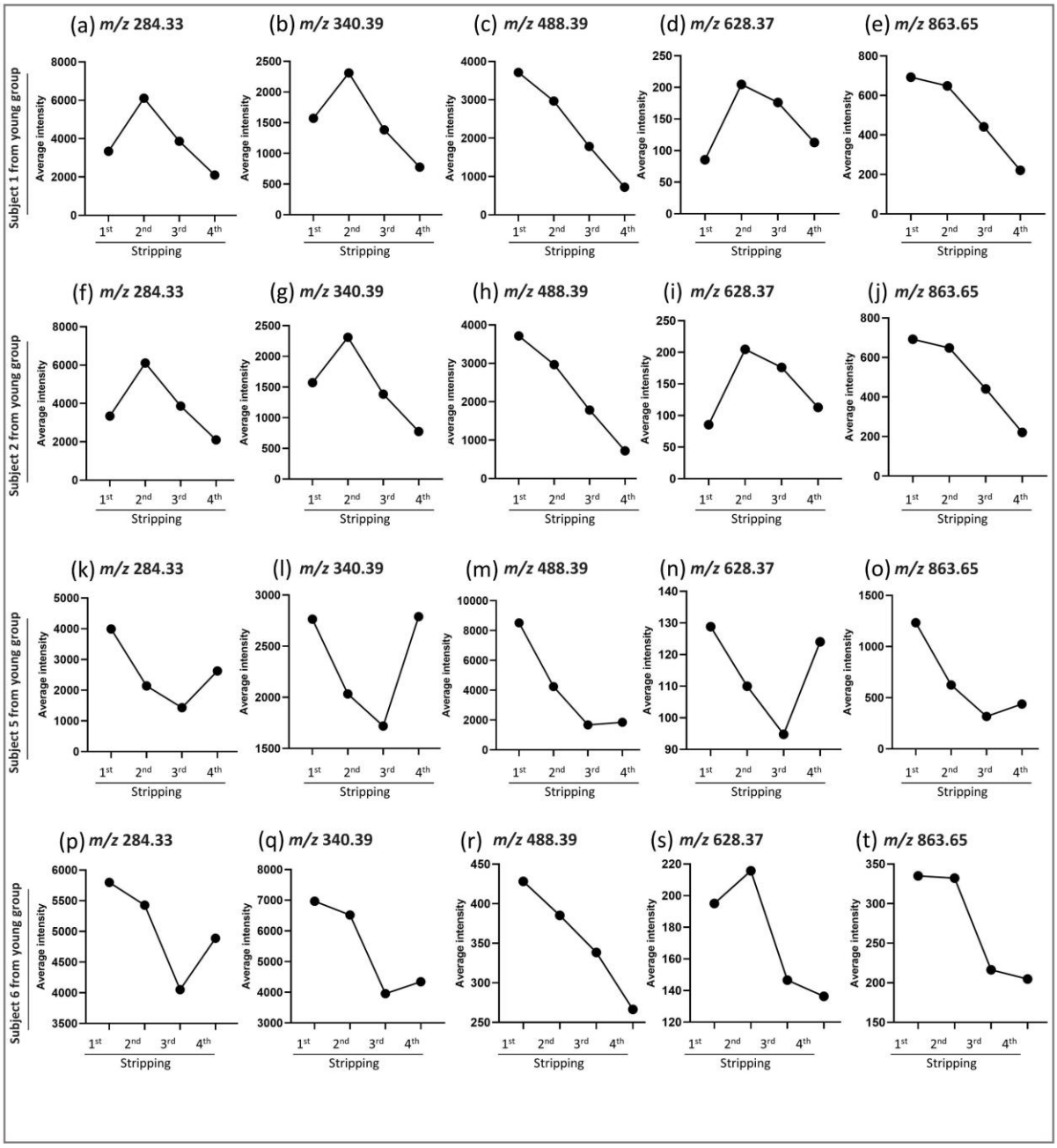

Fig. S4. Stratum corneum's layer-wise average intensity of the candidate molecules in subject 1, 2, 5 and 6 from young group. Average intensity of *m/z* 284.33 (a), *m/z* 340.39 (b), *m/z* 488.39 (c), *m/z* 628.37 (d), and *m/z* 863.65 (e) in young subject 1. Average intensity of *m/z* 284.33 (f), *m/z* 340.39 (g), *m/z* 488.39 (h), *m/z* 628.37 (i), and *m/z* 863.65 (j) in young subject 2. Average intensity of *m/z* 284.33 (k), *m/z* 340.39 (l), *m/z* 488.39 (m), *m/z* 628.37 (n), and *m/z* 863.65 (o) in young subject 5. Average intensity of *m/z* 284.33 (p), *m/z* 340.39 (q), *m/z* 488.39 (r), *m/z* 628.37 (s), and *m/z* 863.65 (t) in young subject 6.

Table S1. Constituents of the cleanser used for the face washing before stratum corneum sample collection.

Cleanser constituents
Water (aqua)
Mineral oil (paraffinum liquidum)
Butylene glycol
Glycerin
Alcohol
Polysorbate 80
Glyceryl stearate
Ascorbyl tetraisopalmitate
Lactobacillus/Soybean extract ferment filtrate
Tocopherol
Behenyl alcohol
Carbomer
Cetearyl alcohol
Dimethicone
Lauroyl lysine
PEG-20 glyceryl triisostearate
Potassium cocoyl glycinate
Potassium hydroxide
Silk powder (Serica powder)
Sodium lauroyl glutamate
Stearic acid
Ethylparaben
Methylparaben
Phenoxyethanol