

# Cyclic Change of Fatty Acid Composition in Meibum During the Menstrual Cycle

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**PURPOSE.** To evaluate the fatty acid (FA) composition in the meibum of pre- and postmenopausal women and age-matched men.

**METHODS.** This prospective study involved 24 healthy subjects; six premenopausal women in their 30s with a regular menstrual cycle (young-female [yF] group), six postmenopausal women in their 60s (elderly-female [eF] group), and 12 age-matched men (i.e., young-male [yM] and elderly-male [eM] groups, respectively). The menstrual cycle was divided into six phases (phase I-VI). Meibum was obtained from the meibomian gland orifices via a Daviel spoon, and its FA composition was then analyzed via gas chromatography mass spectrometry (GC-MS). Principal component analysis (PCA) was performed on the GC-MS results.

**RESULTS.** The mean FA composition of all subjects was 40% saturated FAs (SFA) and 60% unsaturated FAs (UFAs). The PCA results of all groups indicated two categories (PC1 [77.5%] and PC2 [12.4%]); one consisting of yF-group samples of mainly phase II and III and the other consisting of the yF-group samples of the rest of the cycle, as well as from eF-group, yM-group, and eM-group samples. Each group had a distinctive nature. The FAs that most contributed to PC1 were C<sub>14:0</sub>, C<sub>16:0</sub>, and C<sub>18:0</sub> in a positive correlation, and C<sub>18:1n9</sub> in a negative correlation.

**CONCLUSIONS.** FA composition noticeably changes during the menstrual cycle and is somewhat affected by sex and age. The ratio of SFAs (C<sub>16:0</sub>, C<sub>18:0</sub>) to mono-UFAs (C<sub>18:1n9</sub>) in the FA composition might have an impact on the lipid quality of meibum, thus suggesting alteration of its melting temperature and viscosity.

**Keywords:** meibomian gland, meibomian gland dysfunction, meibum, fatty acid, sex steroid hormones, menstrual cycle, aging

Meibomian glands are known to be hormone target tissues. In mice and humans, meibomian glands contain mRNA and proteins of sex steroid hormone receptors such as androgen,<sup>1-3</sup> estrogen,<sup>3-5</sup> and progesterone (P4),<sup>3,5</sup> and their gene expressions are reportedly regulated by sex steroid hormones.<sup>6-8</sup> It has been reported that testosterone regulates more than 1500 genes in the meibomian glands of male mice, and that the genes related to lipid metabolism, lipid transport, and sterol biosynthesis are upregulated, while the genes related to keratinization are downregulated.<sup>6,9</sup> In contrast, it has been reported that 17β-estradiol regulates nearly 200 genes in the meibomian glands of female mice, and that the genes related to lipid catabolism are upregulated and the genes related to lipid synthesis are downregulated.<sup>8</sup> Moreover, it has been reported that the morphology of the meibomian glands in mice apparently change in response to estrogen and P4.<sup>10</sup> Thus, it is reasonable to posit that the effects of sex steroid hormones might have an impact on meibomian gland physiology, and in fact, the findings in our previous study revealed a cyclic change of meibomian gland physiology in premenopausal women.<sup>11</sup>

Meibomian glands reportedly synthesize and secrete a mixture of lipids (meibum)<sup>12</sup> that contain a large quantity of

wax esters (WEs) and sterol esters that spread on the aqueous phase of tear film and form the tear film lipid layer (TFLL). Hence, meibum plays an important role in tear film stabilization,<sup>13-15</sup> protects the ocular surface from microorganisms,<sup>16</sup> and even assists in the maintenance of good visual acuity,<sup>17</sup> thus illustrating that meibum is strongly associated with the overall health of the ocular surface.

It should be noted that upon the onset of meibomian gland dysfunction (MGD), the quality and quantity of meibum can change, thus resulting in the tear film instability and evaporative dry eye,<sup>18-20</sup> which is thought to be the leading cause of dry eye.<sup>21</sup> Furthermore, it has been reported that inspissated meibum at the meibomian gland orifices (MGOs) can be related to meibomian gland inflammation (meibomitis) and subsequent ocular surface inflammation,<sup>22-25</sup> thus explaining why researchers in this field have long placed their focus on the lipid composition of meibum. The findings in several previous studies revealed that meibum is composed of a mixture of lipids of different classes.<sup>12,26-30</sup> In the past decade, meibomian lipidome (i.e., the totality of lipids in cells) has been intensively investigated on the level of individual intact lipid species, which revealed the characteristics of meibum; that is, it



is formed mostly of WEs, cholesteryl esters (Chl-Es), (*O*)-acylated  $\omega$ -hydroxy fatty acids (OAHFA) and their Chl-Es (Chl-OAHFA), diacylated fatty  $\alpha,\omega$ -diols, small amounts of triacylglycerols, free cholesterol (Chl), and possibly other sterol esters.<sup>31-33</sup> Since approximately 95% of meibum is comprised of nonpolar lipids [especially WE (41.0%) and Chl-E (31.0%)],<sup>31</sup> we theorize that the fatty acid (FA) composition of those lipids have a great impact on the fundamental properties of meibum, such as its melting temperature and viscosity. For example, at room temperature, olive oil mainly containing oleic acid (C<sub>18:1</sub>) is a liquid, while lard mainly containing stearic acid (C<sub>18:0</sub>) is a solid.

Thus, in this study, we analyzed the total FA composition of the meibum in normal young and elderly subjects of both sexes, and specifically evaluated the cyclic change of FA composition during the menstrual cycle and the hormonal effect on the FA composition of meibum.

## METHODS

### Subjects

The experimental protocols used in this study were approved by the Institutional Review Board of Kyoto Prefectural University of Medicine and Kyoto City Hospital Organization, Kyoto, Japan, and were in accordance with the tenets set forth in the Declaration of Helsinki. Written informed consents were obtained from all subjects prior to their participation.

This study involved 24 healthy volunteer subjects; six young women (young female [yF] group, mean age:  $32.3 \pm 2.6$  standard deviation [SD] years), six young men (young male [yM] group, mean age:  $29.3 \pm 4.0$  years), six elderly women (elderly female [eF] group, mean age:  $59.2 \pm 3.4$  years), and six elderly men (elderly male [eM] group, mean age:  $60.5 \pm 2.2$  years). All subjects in this study were Japanese (i.e., of Asian ethnicity). The yF group consisted of six healthy premenopausal women with a regular 28- to 30-day menstrual cycle and duration of 6 to 7 days. As in our previously reported method,<sup>11</sup> the premenopausal women were seen once a week for 5 weeks, and the menstrual state of the premenopausal subjects was masked until after completion of the investigation. Moreover, the premenopausal women were seen weekly for additional 5 weeks to confirm their menstrual status. The following subjects were excluded from the study: smokers, contact lens wearers, and subjects with eye and/or general disease or who were taking medication at the time of the study.

### Menstrual Cycle and Basal Body Temperature (BBT)

As in our previously reported method,<sup>11,34</sup> the menstrual cycle was divided into six phases: (1) phase I: the first 2 days of menstruation, (2) phase VI: 2 days before menstruation, and (3) phases II-V: the remaining time being divided into four equal periods (typically, 6-day periods). BBT was measured daily immediately after awakening and prior to any physical activity in order to confirm the menstrual state.

### Saliva Sex Steroid Hormones

In all subjects, saliva samples were obtained between 6:00 and 8:00 AM on each day of meibum collection. The concentrations of saliva hormones (i.e., estradiol [E2], P4, free-testosterone, and dehydroepiandrosterone sulfate [DHEA]) were evaluated via the use of an enzyme-linked immunosorbent assay (ELISA) kit (First Health Japan, Co., Tokyo, Japan).

## Meibum Collection

After a warm compress (40°C, 10 minutes) of both eyes using an electronic device (EH-SW50; Panasonic Corporation, Osaka, Japan), meibum samples were obtained from the subjects' eyes via the use of a Daviel cataract spoon after gently squeezing the eyelid margin with a Yoshitomi MG compressor (T.M.I. Co. Ltd., Saitama, Japan) under a surgical microscope. The samples were then dissolved with organic solvent (chloroform/methanol, 1/1, v/v) immediately after collection and stored at -20°C. To avoid any possible lipid contamination through the Daviel spoon, strict attention was paid to the sample preparation as follows: First, the spoon was washed with a detergent and air-dried. It was then washed again with the following two high-grade organic solvents: (1) acetone (to wash off the polar compounds) and (2) hexane (to wash off the nonpolar compounds). The spoon was then covered with aluminum foil, air-dried, and stored in a polyethylene bag. Just prior to sample collection, the spoon was washed with assay solvent (chloroform/methanol), and then covered with aluminum foil and air-dried. At the time of meibum-sample collection, strict attention was paid to avoid contamination by tear fluid and other lipids from the eyelid around the MGOs.

## Lipid Analysis

For lipid analysis, residual pesticide and polychlorinated biphenyl (PCB)-analysis grade chloroform and methanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan), as well as acetone, hexane, and toluene of the same grade (Kanto Chemical Co. Inc., Tokyo, Japan), were used. For identification of FA and for generation of the calibration curve for each FA in gas chromatography mass spectrometry (GC-MS) analysis, a standard mixture consisting of 37 FA methyl esters (FAMES) (Supelco 37 Component FAME Mix; Sigma-Aldrich Corp., St. Louis, MO, USA) and 14% boron trifluoride-methanol solution (GL Sciences, Inc., Tokyo, Japan) were used to transmethyrate the lipids of meibum as follows.

Each sample solution was evaporated to dryness under a stream of nitrogen. A 0.5-mL toluene and 1-mL 7% boron trifluoride-methanol solution (GL Sciences) were then added to the residue, and the sample was heated at 90°C for 16 hours to transmethyrate the FAs in the sample as described previously.<sup>35</sup> The FAMES were then extracted via liquid-liquid extraction with hexane and analyzed by GC-MS performed using a GCMS-QP2010 ULTRA gas chromatograph-mass spectrometer (Shimadzu Corporation, Kyoto, Japan) at an injector temperature of 280°C. The FAMES were separated via the use of a TC-70 capillary column (60 m length, 0.25 mm i.d., and 0.25  $\mu$ m film thickness; GL Sciences). The oven temperature was programmed to increase from 80°C to 250°C at the rate of 3°C/min, held at 250°C for 5 minutes, and then increased to 280°C at the rate of 10°C/min, with a final temperature of 280°C being maintained for 5 minutes. The injected sample was loaded onto the column with the split ratio of 5:1. Helium was used as the carrier gas at a constant linear velocity of 25 cm/s. The temperatures of the interface and the ion source were set at 280°C and 240°C, respectively. FAMES were detected with the electron impact mode setting, and data acquisition was conducted via a scanning mass range of  $m/z$  35 to 350. FAMES in each sample were identified based upon their standard retention times and MS spectra. Unknown peaks observed in the sample were identified by searching the MS database libraries (NIST11 and NIST11s). Since saturated and unsaturated FAMES have iso- and anteiso-isomers, which generate the fragment ions of  $m/z$  74 and  $m/z$  81, respectively, the peak areas of FAME detected in the sample and the standard solution

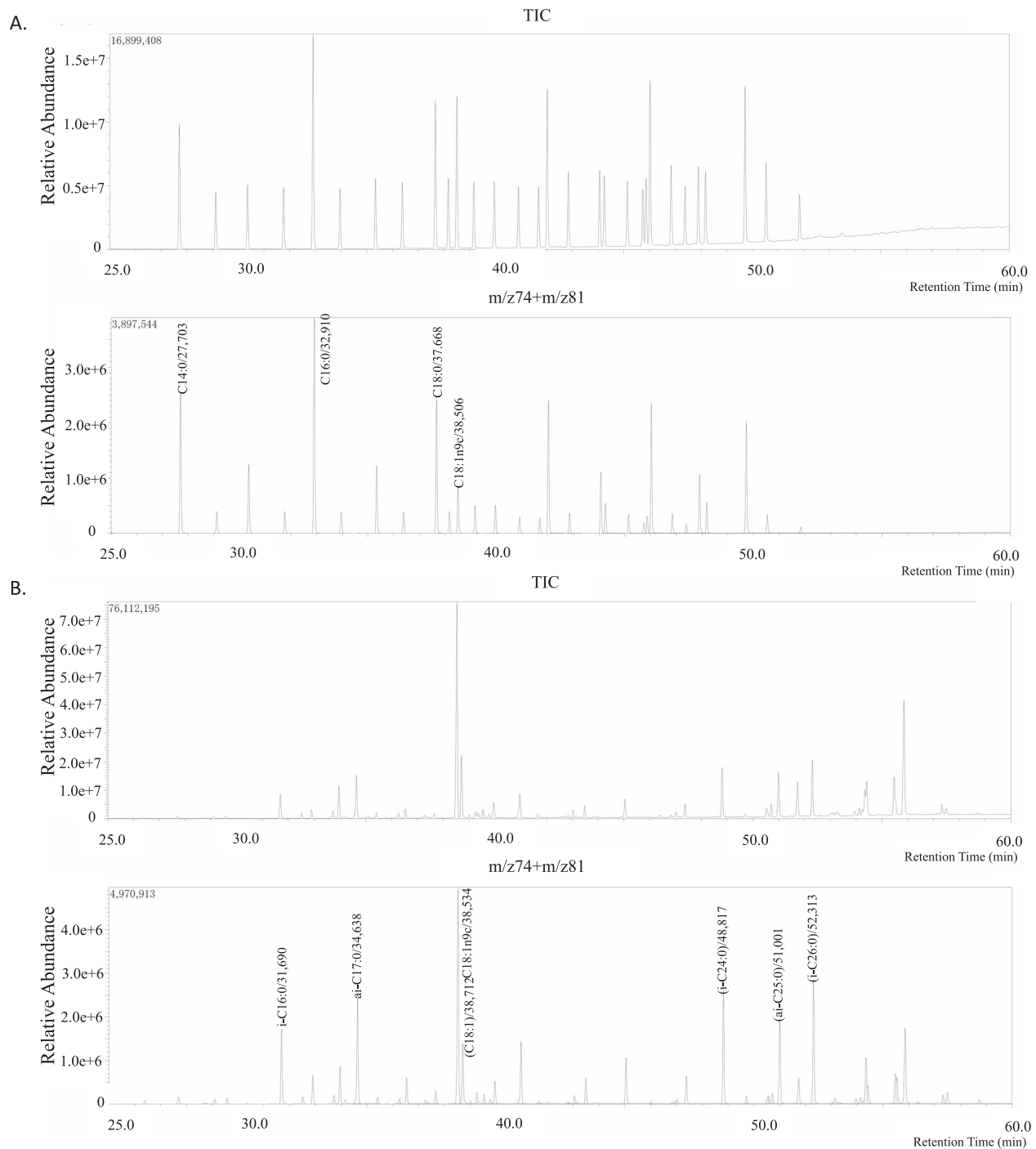
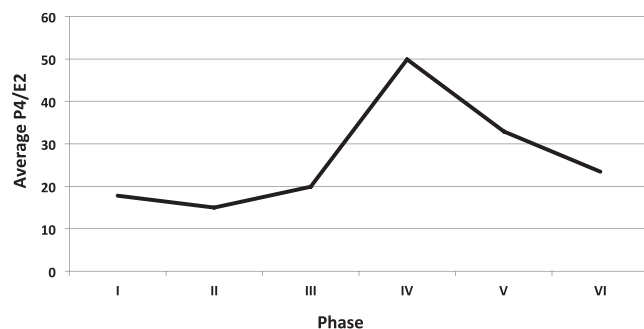


FIGURE 1. Total ion chromatogram (TIC) and extracted ion chromatogram ( $m/z74+m/z81$ ) of standard solution (A) and an example of human meibomian sample (B).

were calculated using the chromatogram of the sum of both fragment ions (Figs. 1A, 1B).

The composition ratio of FAME in the sample was determined as follows: First, the type of FAME was classified into five categories, such as straight-saturated FAs (straight-SFAs), branched-saturated FAs (branched-SFAs), mono-unsaturated FAs (mono-UFAs), di/tri-unsaturated FAs (di/tri-UFAs), and branched-unsaturated FAs (branched-UFAs). The total peak area of each type of FAME was then obtained by summing the peak area of FAMES that belonged to the type. The approximate concentrations of saturated and branched-satu-

rated FAMES were then calculated from the peak areas of those FAMES by comparing with the average peak areas of saturated FAMES in the standard mixture of 37 FAMES, since the branched FAMES were not contained in the standard mixture. Using the same method, the approximate concentrations of unsaturated (mono and branched) and di/tri-unsaturated FAMES in the sample were calculated by comparing with the average peak areas of unsaturated and di/tri-unsaturated FAMES, respectively, in the standard mixture. The total amount of FAME in the sample was then calculated by summing the approximate concentration of each of the five categories, with



**FIGURE 2.** The averaged ratio of saliva concentration of P4 to E2 changes during the menstrual cycle. In this study, it became minimal at phase II of the menstrual cycle.

that total being used to calculate the relative ratio of each category.

### Principal Component Analysis (PCA)

PCA of the raw GC-MS results was performed via the use of Traverse MS software (Reifycs Inc., Tokyo, Japan) equipped with the MRMPROBS algorithm.<sup>36</sup>

## RESULTS

### Menstrual Cycle and Sex Steroid Hormone in Saliva

The phases of the menstrual cycle in the yF group were confirmed by the subjects' BBT and saliva hormone concentration. The mean P4/E2 level in the saliva was found to be correlated well with a standard level at each phase of the cycle, thus confirming that the yF-group subjects had a regular sex hormonal change; the mean P4/E2 levels became minimal at phase II of the cycle (Fig. 2).

### GC-MS Results

GC-MS analyses identified 52 FA species with the carbon-chains C<sub>14</sub> to C<sub>30</sub> in the meibum (Table). The degree of saturation ranged from 0 to 2 double bonds. Among all FAs, C<sub>18:1n9c</sub> (oleic acid) was the most abundant. We also detected many iso-/anteiso(ai)-FAs, as well as odd-numbered carbon-chain FAs in the meibum. Since the poly-UFAs with more than four double bonds, such as C<sub>18:4</sub>-FA-based, are reportedly very minor components (i.e., less than a few percent of their corresponding C<sub>18:1</sub>-based FAs),<sup>37</sup> they were not evaluated in this study.

### FA Composition in the Meibum of Normal Subjects

The average FA composition in the meibum samples obtained from all healthy subjects in this study is shown in Figure 3, and it was found to be comprised of 40% SFA and 60% UFA; mean SFA was comprised of  $3.9 \pm 1.1$  (mean  $\pm$  SD) % straight-SFA and  $37.1 \pm 1.1$ % branched-SFA, and mean UFA was comprised of  $51.8 \pm 0.9$ % mono-UFA,  $3.5 \pm 0.2$ % di/tri-UFA, and  $3.9 \pm 0.4$ % branched-UFA (Fig. 3A). The most abundant FA was C<sub>18:1n9c</sub> ( $29.2 \pm 3.5$ %), followed by C<sub>18:1</sub> ( $8.2 \pm 0.9$ %), i-C<sub>26:0</sub> ( $5.6 \pm 0.7$ %), ai-C<sub>17:0</sub> ( $5.3 \pm 0.8$ %), C<sub>16:1</sub> ( $5.0 \pm 1.0$ %), i-C<sub>24:0</sub> ( $4.2 \pm 0.5$ %), ai-C<sub>25:0</sub> ( $3.8 \pm 0.4$ %), i-C<sub>16:0</sub> ( $3.3 \pm 0.6$ %), i-C<sub>20:0</sub> ( $2.6 \pm 0.3$ %), C<sub>18:2n6c</sub> ( $2.2 \pm 0.3$ %), C<sub>16:0</sub> ( $1.8 \pm 2.0$ %), C<sub>18:0</sub> ( $1.3 \pm 2.2$ %), and others (Fig. 3B). It should be noted that those results were reproducibly observed, and that the

**TABLE.** FA Species With the Carbon-Chains C<sub>14</sub> to C<sub>30</sub> in the Meibum

Carbon Chain Length	Detected Molecular Species of FA
14	C14:0
	i-C14:0
15	i-C15:0
	ai-C15:0
16	C16:0
	C16:1_1
	C16:1_2
	i-C16:0
	i-C16:1
17	C17:1
	i-C17:0
	ai-C17:1
	(-C17:1)
18	C18:0
	C18:1n9c
	(C18:1_1)
	(C18:1_2)
	C18:2n6c
	(C18:2_1)
	(C18:2_2)
	(C18:2_3)
	i_C18:1
	(i_C18:1_1)
	(i_C18:1_2)
	(i_C18:2)
19	ai-C19:0
20	C20:1n9
	(C20:1)
	i-C20:0
	i-C20:1
21	ai-C21:0
22	C22:0
	C22:1n9
	C22:1
	(i-C22:0)
23	(i-C23:0)
	(ai-C23:0)
24	C24:0
	C24:1_1
	C24:1_2
	(i-C24:0)
25	(i-C25:0)
	(ai-C25:0)
26	(C26:0)
	(C26:1)
	(i-C26:0)
27	(ai-C27:0)
28	(C28:0)
	(C28:1)
	(i-C28:0)
29	(ai-C29:0)
30	(i-C30:0)

preliminary experiment revealed that the composition of meibum FAs was not affected, either with or without, a warm compress of the eyelids.

### Cyclic Change of FA Composition in Meibum of Premenopausal Women

The average of each category of FAs of the premenopausal women changed according to the phases of menstrual cycle

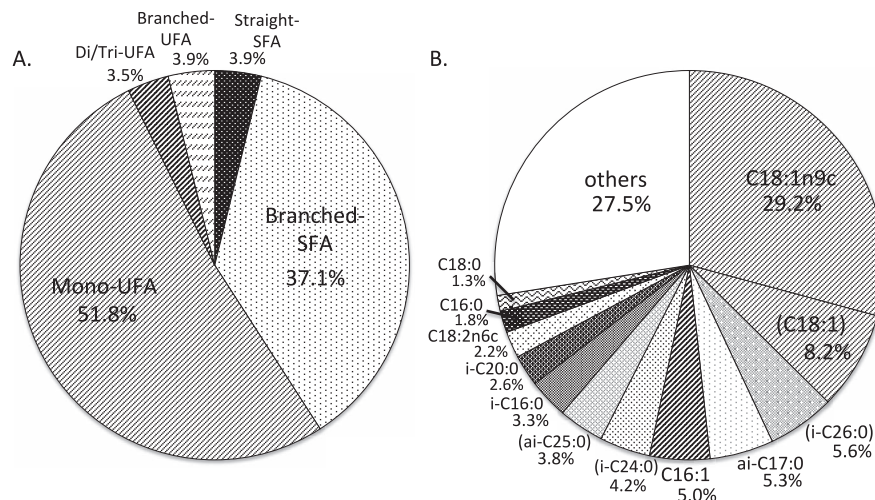


FIGURE 3. The averaged FA composition in meibum from all healthy subjects; the FA categories (A) and the individual FAs (B).

(Fig. 4A). In phase II, the composition of straight-chain SFA significantly increased and the ratio of mono-UFA significantly decreased in comparison to other phases of the cycle ( $P < 0.05$ , 1-way ANOVA). During the menstrual cycle,  $C_{18:1n9c}$  apparently inversely correlated with  $C_{18:0}$ , as did  $C_{16:1}$  with  $C_{16:0}$ . The ratio of both  $C_{18:0}$  and  $C_{16:0}$  significantly increased in phase II ( $P < 0.05$ , 1-way ANOVA) (Figs. 4B, 4C).

### Differences in FA Composition Between the Young and Elderly Subjects

In the yF group, straight-SFA was significantly higher, and mono-, as well as di/tri-, UFA were significantly lower than in those subjects in the yM group ( $t$ -test,  $P < 0.05$ ). On the other hand, no significant difference was observed between the eF group subjects and the eM group subjects in regard to the average of each category of FAs in meibum. In the eF group

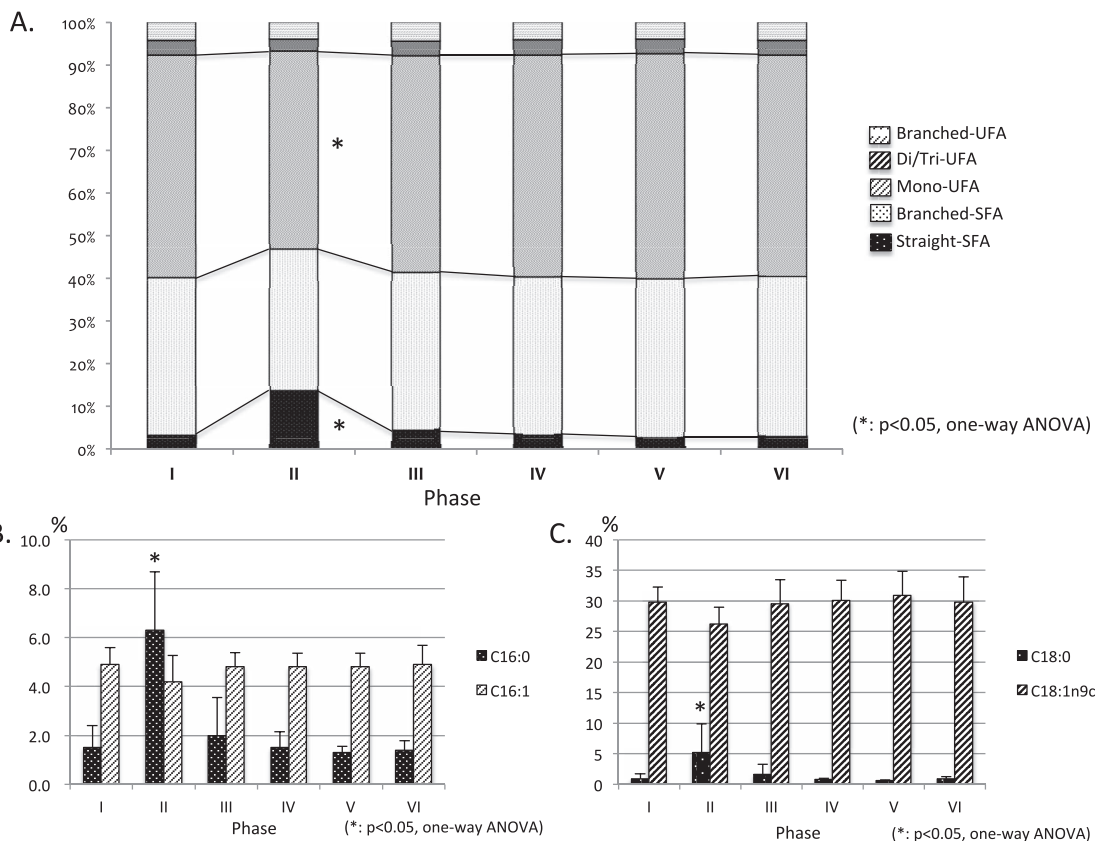
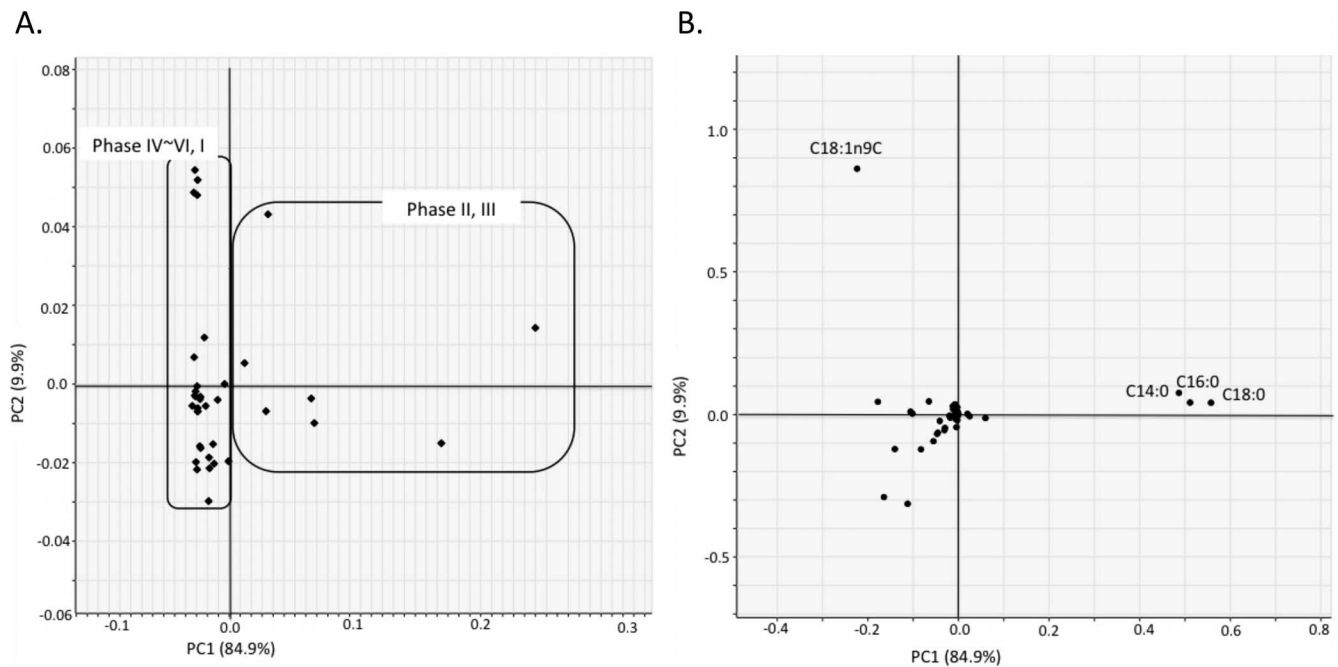


FIGURE 4. Cyclic change of the averaged FA composition of each category in premenopausal women during the menstrual cycle (A). In phase II, the ratio of straight-chain saturated FA (SFA) significantly increased, and the ratio of mono-unsaturated FA (UFA) significantly decreased as compared to other phases of the cycle ( $P < 0.05$ , 1-way ANOVA). The ratio of both  $C_{16:0}$  (B) and  $C_{18:0}$  (C) significantly increased in phase II ( $P < 0.05$ , 1-way ANOVA).



**FIGURE 5.** The PCA results of the healthy premenopausal women. The samples were divided into two categories (A): one consisting of the meibum mainly of phase II and III of the menstrual cycle, and the other consisted of meibum of the rest of the cycle. The FAs that most contributed to PC1 were  $C_{14:0}$ ,  $C_{16:0}$ , and  $C_{18:0}$  in a positive correlation, and  $C_{18:1n9}$  in a negative correlation (B).

meibum,  $C_{18:1n9c}$  was significantly lower than that in the yF group meibum, while  $C_{18:1}$  (which has one double bond, yet not on the ninth carbon), as well as  $C_{16:1}$ , were significantly higher than those in the eF group meibum ( $t$ -test,  $P < 0.05$ ).  $C_{17:0}$  and  $C_{25:0}$ , respectively, were significantly higher in the eM group and eF group meibum than in the yM group and yF group meibum ( $t$ -test,  $P < 0.05$ ).

## PCA Results

**FA Composition in the Healthy Premenopausal Women.** The PCA results indicated the existence of two categories of the healthy premenopausal women (Fig. 5A). The proportion of variance for PC1 (i.e., the ratio of the information amount occupied by the PC1 among the total information amount) was high, 84.9%, and PC2 was 9.9%. One category consisted of the group of mainly phase II and III of the menstrual cycle, in which the lipid component of meibum seemed to have changed, and while the other consisted of the group of the latter half of the cycle (phase VI through IV) and phase I, thus suggesting that each group has a distinctive nature (Fig. 5A). In addition, the FAs that most contributed to PC1 were  $C_{14:0}$ ,  $C_{16:0}$ , and  $C_{18:0}$  in a positive correlation, and  $C_{18:1n9}$  in a negative correlation (Fig. 5B).

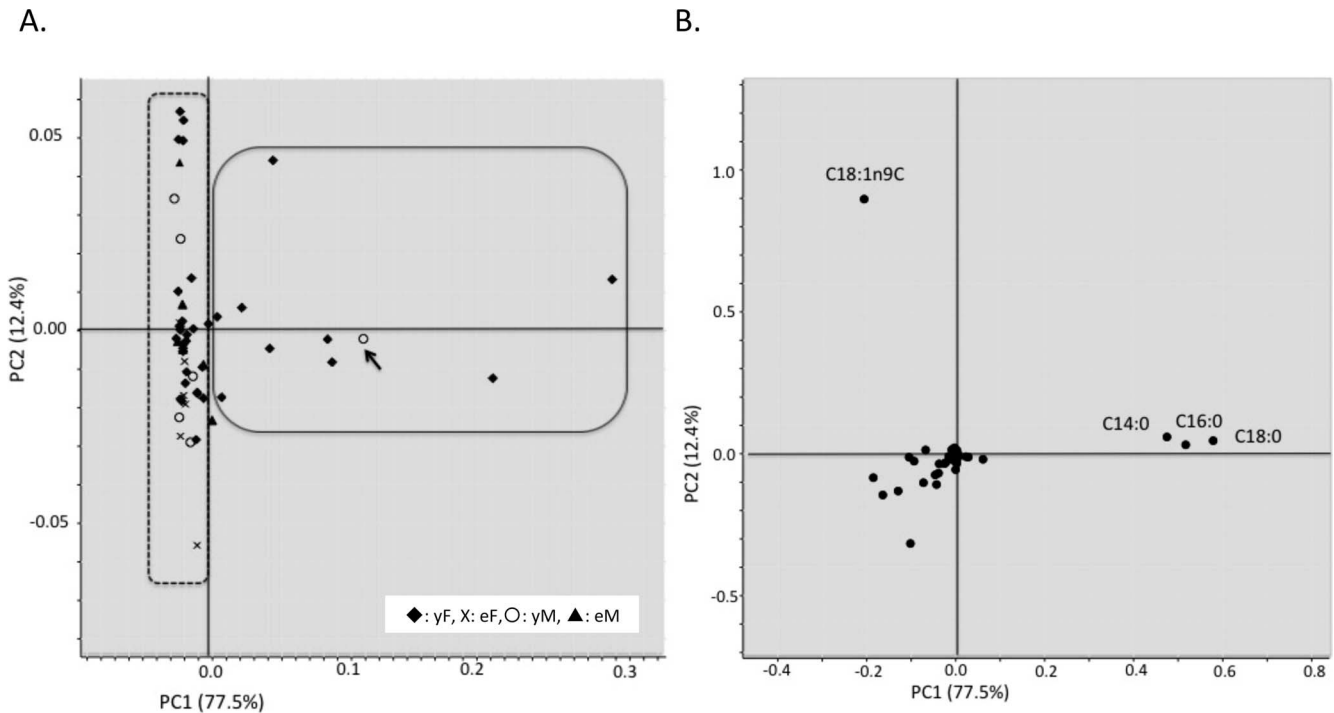
**FA Composition in All Healthy Subjects.** The PCA results indicated the existence of two categories of healthy subjects. The proportion of variance for PC1 was high, 77.5%, and PC2 was 12.4% (Fig. 6A). One consisted of the group of yF subjects in phase II and III of the menstrual cycle, in which the lipid component of meibum seemed to have changed, and another consisted of the yF subjects in the rest of the cycle, as well as the eF group, yM group, and eM group subjects, thus suggesting that each group has a distinctive nature. However, it should be noted that there was one exception, as one subject in the yM group (subject A: yM-A) was included in the same category as yF in the first half of the menstrual cycle (Fig. 5A, arrow). In addition, the FAs that most contributed to PC1 were

$C_{14:0}$ ,  $C_{16:0}$ , and  $C_{18:0}$  in a positive correlation, and  $C_{18:1n9}$  in a negative correlation (Fig. 6B).

**PCA Results of Young and Elderly Subjects.** The PCA results of the yF group and eF group subjects also indicated the existence of two categories. The proportion of variance for PC1 was 80.3%, and PC2 was 10.0% (Fig. 7A). One consisted of the group of yF subjects in the phase II and III of the menstrual cycle, and the other consisted of yF subjects in the rest of the phases of the menstrual cycle, as well as eF subjects. The FAs that most contributed to PC1 were  $C_{14:0}$ ,  $C_{16:0}$ , and  $C_{18:0}$  in a positive correlation, and  $C_{18:1n9}$  in a negative correlation (Fig. 7B). In addition, in the eF group subjects, the FAs  $ai-C_{17:0}$  and  $i-C_{26:0}$  appeared to increase (Fig. 7B). The PCA results of the yM group and eM group subjects failed to indicate any clear differences between the two groups.

## DISCUSSION

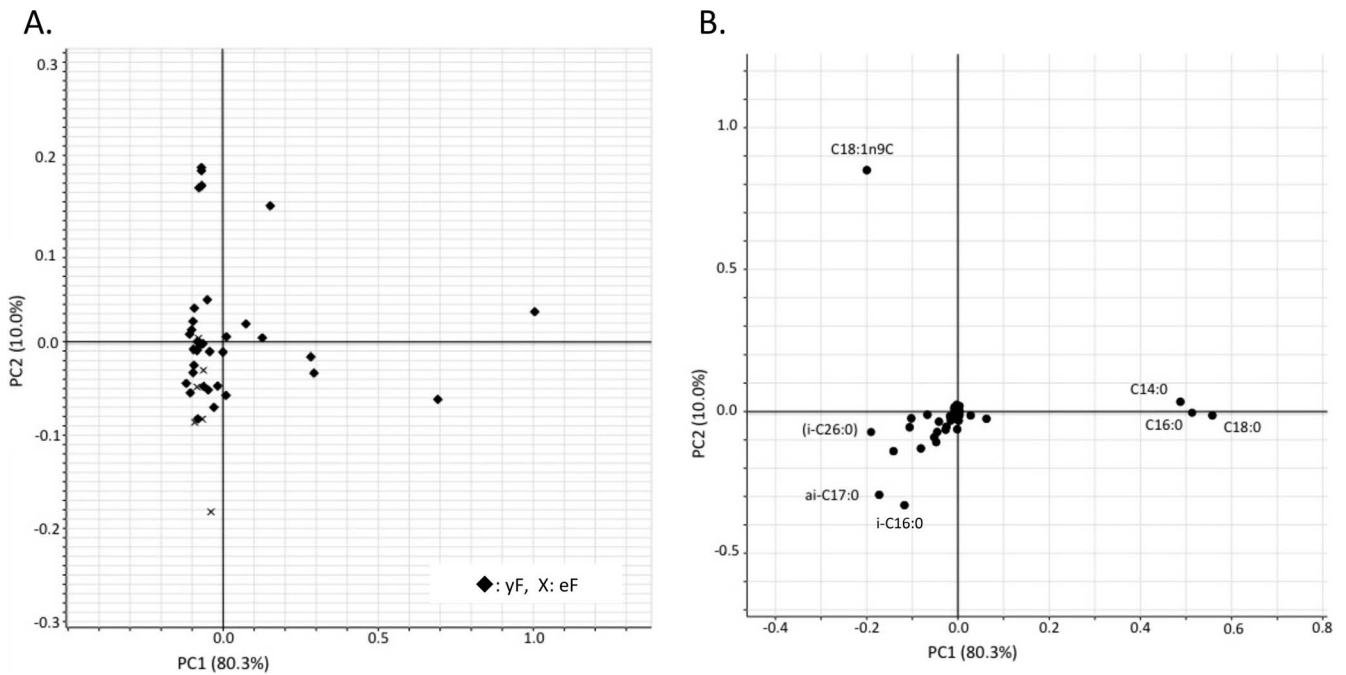
Our results clearly demonstrated that the FA composition of normal subjects is comprised of 60% UFAs and 40% SFAs, in which 90% of the SFAs were branched-SFAs. This finding correlates well with the study by Nicolaidis and Santos,<sup>38</sup> in which it was reported that a large portion of the FA and fatty alcohol (FAI) were comprised of branched and/or unsaturated species, and a large portion of the straight chain FA was unsaturated. This finding indicates that such FA composition would be ideal to keep meibum easily spreading onto the tear film by lowering the melting temperature at the ocular surface temperature, since both branching and an increase in the degree of unsaturation have been shown to lower the melting temperatures of long-chain lipids,<sup>39,40</sup> as previously discussed by Butovich et al.<sup>41</sup> The ratio of the most-abundant mono-UFA in this present study was  $C_{18:1n9}$  (30%), which is consistent with the findings in previous reports.<sup>12,35,41</sup> In this study, the conditions used for the transmethylation of FAs were the same as those previously described by Joffre et al.<sup>35</sup>



**FIGURE 6.** The PCA results of all healthy subjects. The samples were divided into two categories (A): one consisted of yF meibum mainly of phase II and III of the menstrual cycle, and the other consisted of yF meibum of the rest of the cycle, as well as eF, yM, and eM meibum. Note that one subject of the yM group (subject A: yM-A) was included in the same category as yF in the first half of the menstrual cycle (A, arrow). The FAs that most contributed to PC1 were C<sub>14:0</sub>, C<sub>16:0</sub>, and C<sub>18:0</sub> in a positive correlation, and C<sub>18:1n9</sub> in a negative correlation (B).

In both studies, SFA constituted approximately 40% of the total FAs; however, the straight-SFA fraction was much lower and the branched-SFA was the main SFA in our results. However, the reason for the ratio difference between SFA and

branched-SFA is unclear. The difference of the technique used to collect meibum samples (i.e., a Schirmer strip versus a Daviel cataract spoon),<sup>42,43</sup> and the difference of the instrument used for GC-MS and/or even the sample collection



**FIGURE 7.** The PCA results of the yF group and eF group subjects. The samples were divided into two categories (A): one consisted of the group of yF subjects in phase II and III of the menstrual cycle, and the other consisted of yF subjects in the rest of the phases of the menstrual cycle, as well as eF subjects. The FAs that most contributed to PC1 were C<sub>14:0</sub>, C<sub>16:0</sub>, and C<sub>18:0</sub> in a positive correlation, and C<sub>18:1n9</sub> in a negative correlation (B). In addition, in the eF subjects, the FAs ai-C<sub>17:0</sub> and i-C<sub>26:0</sub> appear to increase (B).

from subjects of different ethnicity, might have affected the SFA composition.

As a hormone target tissue, the meibomian gland gene expression levels in mice are known to be upregulated by androgens<sup>6,9</sup> and downregulated by estrogens.<sup>8</sup> We recently reported the cyclic change of human meibomian gland physiology during the menstrual cycle.<sup>11</sup> In the present study, an obvious cyclic change of FA composition during the menstrual cycle was also found in premenopausal women; C<sub>16:0</sub> and C<sub>18:0</sub> increased and C<sub>18:1n9</sub> decreased significantly, especially before ovulation (at the time that P4/E2 became minimal), while an opposite ratio of C<sub>18:1n9</sub> to C<sub>16:0</sub> and C<sub>18:0</sub> was observed in the latter part of the menstrual cycle. In our previous report,<sup>11</sup> the duration of fluorescein break-up time of tear film (F-BUT) was positively correlated with the size of the MGO; that is, the diameter of the MGO decreased in the latter half of the luteal phase of the menstrual cycle until menstruation correlates well with a shortening of F-BUT. Interestingly enough, unsaturated FAs such as C<sub>18:1n9</sub> have been demonstrated to induce differentiation of human epidermal keratinocytes leading to abnormal keratinization of sebaceous glands.<sup>44</sup> Coincidentally observed in this study, C<sub>18:1n9</sub> oleic acid increased in the latter half of the menstrual cycle in normal premenopausal women, which might be a trigger to stimulate keratinization of the MGOs, thus resulting in the smallest MGO size and the shortest BUT at the end of the menstrual cycle.

As previously reported by using GC-MS,<sup>35,45,46</sup> the most abundant UFA was C<sub>18:1n9</sub> and the second most abundant was C<sub>16:1</sub>, while the most abundant SFA was i-C<sub>26:0</sub> and the second most abundant was ai-C<sub>17:0</sub>. Earlier, Butovich et al.<sup>41</sup> reported that meibomian WEs are mostly based on moderately long FAs with C<sub>15</sub> to C<sub>19</sub> carbon chains, with the vast majority of them being of C<sub>16</sub>, C<sub>17</sub>, and C<sub>18</sub> types. In accordance with the findings in the reports by Butovich et al.,<sup>30,37,41</sup> it has been assumed that the majority of WEs (~72%) were based on C<sub>18:1n9</sub>, while the saturated ones had C<sub>16</sub>, C<sub>17</sub>, and C<sub>18</sub> FA residues in the ratio of 22:65:13.<sup>12</sup> The PCA results in this current study revealed that sex steroid hormones and aging apparently have an influence on the ratio of C<sub>16:0</sub>, C<sub>18:0</sub>, and C<sub>18:1n9</sub>, as well as ai-C<sub>17:0</sub>. Therefore, if the composition of those main FAs would continue to change, the characteristics of meibum, such as the viscosity and/or melting temperature, could also change, thus affecting the tear film stability.

It should be noted that our current study did have a limitation, since as Butovich et al.<sup>41</sup> stated in a previous report, it is impossible to decipher the structure of the original intact compounds such as WEs and CEs (i.e., the exact combinations of their FA and FAI composition), as they undergo transesterification when GC-MC is used for meibum lipid analysis.

Theoretically, meibum could either undergo de novo synthesis in the acinar cells or be taken up from the bloodstream, or both. The evidence for de novo synthesis is supported, due to the fact that the synthetic enzymes for the components and transesterases to form the final products have been detected either directly or indirectly (mRNA) in acinar cells.<sup>6,7,47</sup> To date, there has been no report providing direct evidence that lipids are taken up from the bloodstream to meibomian glands. Thus, it is most likely that all meibum lipids are synthesized by the meibomian glands. According to the concept of “meibogenesis,”<sup>48,49</sup> the first step of meibogenesis is that cellular synthesis of mono-UFAs, mainly C<sub>18:1</sub> and C<sub>16:1</sub> from C<sub>18:0</sub> and C<sub>16:0</sub>, by the rate limiting enzyme stearoyl-CoA desaturase 1 (SCD1).<sup>50</sup> In fact, Miyazaki et al.<sup>51</sup> demonstrated that SCD1 was capable of making C<sub>16:1</sub> and C<sub>18:1</sub>, and they also discovered the MG and sebaceous gland atrophy in SCD1 knock-out (KO) mice.<sup>52</sup> It has been reported that high levels of dietary oleate (C<sub>18:1n9</sub>) and palmitate

(C<sub>16:1</sub>) are unable to correct the deficiency of triglyceride, cholesterol ester, and WE in SCD1<sup>-/-</sup> mice.<sup>52</sup> Hence, SCD1 is essential for meibomian gland as well as meibogenesis. SCD1 gene expression is tightly regulated by various parameters, such as insulin, leptin, sex hormones, and growth factors, etc.<sup>53</sup> Research on the estrogen receptor in KO mice has revealed that estrogen is a negative regulator of SCD1,<sup>54</sup> and following on the findings in that study, it has been reported that the administration of estrogen in humans decreases SCD1 in adipose tissues.<sup>55</sup> In this present study, the FA composition of premenopausal women clearly changed in phase II of the cycle when the P4/E2 became minimal. Increased estrogen at ovulation might have a negative influence on SCD1, thus resulting in increased C<sub>16:0</sub> and C<sub>18:0</sub>, as well as decreased C<sub>18:1n9</sub>. This change would lead to MGD and/or meibomitis in young women.<sup>25</sup> Androgen, on the other hand, has been identified as an activator of SCD1 in hamsters<sup>56</sup> and rats,<sup>57</sup> but to date, not in mice. Sullivan et al.<sup>58</sup> reported that anti-androgen use is paralleled by significant changes in the FA profiles of neutral lipid fractions in meibomian gland secretions. However, in this present study, testosterone did not seem to have any influence on mono-UFA composition.

In summary, our findings show that FA composition changes during the menstrual cycle and that the level of estrogen may have a negative influence on the first step of meibogenesis; that is, cellular synthesis of mono-UFAs from SFA by the rate limiting enzyme SCD1. These findings might be related to the MGD and/or meibomitis in young women, and we are currently analyzing the FA composition of meibum in patients with obstructive MGD and meibomitis.

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