# Meibum Color and Free Fatty Acid Composition in Patients With Meibomian Gland Dysfunction

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**Purpose.** We measured the components of meibum in patients with meibomian gland dysfunction (MGD) and control subjects and then examined the relation between meibum composition and clinical parameters.

**Methods.** Thirty-eight patients with MGD (13 men and 25 women; mean age ± SD, 66.9 ± 15.0 years) and 20 control subjects (8 men and 12 women; 64.5 ± 6.7 years) were enrolled. Ocular symptom score, keratoconjunctival staining score, tear film breakup time, and Schirmer's test value were determined. Lid margin abnormalities and meibomian gland morphology were assessed for upper and lower eyelids, and meibum properties were evaluated at temporal, central, and nasal sites of each lid. Free fatty acid (FFA) composition of meibum was analyzed by liquid chromatography–Fourier transform mass spectrometry.

**Results.** Upper meibum color score was significantly correlated with epiphora and sticky sensation in MGD patients. Meibum grade, color, or viscosity did not differ significantly among the sites evaluated. A total of 103 species of FFA—including very long chain (such as $C_{36}$ and $C_{37}$) and odd-numbered chain (such as $C_{17}$, $C_{19}$, and $C_{21}$) FFAs—were detected in meibum. Free fatty acid composition differed between clear and colored (cloudy or yellow) meibum, with unsaturated FFAs tending to be more abundant in colored meibum.

**Conclusions.** Free fatty acid composition of human meibum correlates with meibum color as determined with a slit-lamp microscope. This finding may provide insight into the pathogenesis of MGD.

Keywords: meibomian gland, meibum, meibography, fatty acid

Posterior blepharitis, an inflammatory condition of the posterior lid margin, is encountered relatively often in ophthalmic clinics. It is associated with allergic conjunctivitis, infectious conjunctivitis, and, in particular, meibomian gland dysfunction (MGD).[1] Given that 86% of patients with dry eye have been found to manifest MGD,[2] posterior blepharitis is also associated with dry eye. In normal individuals, meibomian gland secretion (meibum) is a clear oily liquid that spreads readily to become the outermost surface of the tear film. It is a complex mixture of lipids of various classes including wax esters, cholesteryl esters, (O-acyl)-ω-hydroxy fatty acids (OAHFAs) and their esters, acylglycerols, diacylated diols, free fatty acids (FFAs), cholesterol, and, in smaller amounts, other polar and nonpolar lipids.[3] In patients with MGD, however, meibum gradually adopts a yellow color and its consistency changes from liquid to toothpaste-like. In addition, ductal hyperkeratinization may result in blockage of the duct orifice, deterioration of acini clusters,[4] and stagnation of meibum within the gland. Analysis of meibum might therefore be expected to shed light on the pathogenesis and pathophysiology of MGD.

Although the volume of meibum is small, its components and the changes in these components have been examined as possible disease biomarkers.[5–8] The functions of the various types of FFA in meibum remain to be elucidated, but such information might be expected to offer important clues to the causative mechanisms of MGD, to facilitate biomarker discovery, and to influence therapeutic approaches. To pursue this issue, we have now determined changes in the FFA composition of meibum in patients with MGD by our previously described method,[9] and we have examined the relation of such changes to other meibum parameters and clinical characteristics.

## Methods

### Study Design

This cross-sectional observational study was conducted at The University of Tokyo Hospital, Itoh Clinic (Saitama City, Saitama), and Maeda Ophthalmic Clinic (Aizuwakamatsu City, Fukushima). Subjects were enrolled from December 2012 to December 2013. The study adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board of Tokyo University School of Medicine. All subjects provided written informed consent before entry into the study.

## Subjects

The MGD group included 38 patients (13 men and 25 women; mean age ± SD, 66.9 ± 15.0 years) who were diagnosed with MGD on the basis of Japanese diagnostic criteria (age of ≥20 years; at least one symptom such as an uncomfortable sensation in the target eye; and at least one abnormal lid finding). The control group included 20 individuals (8 men and 12 women; 64.5 ± 6.7 years) who had never been diagnosed with blepharitis or MGD, were aged ≥20 years, and had no history of contact lens wear or eye surgery. Subjects with severe systemic illness or with squamous cell debris (collarette) around the base of the eyelashes were excluded.

## Protocol

One eye was selected as the target eye in each subject. Complications, history of contact lens wear or eye surgery, presence of ocular allergy, and concomitant medications were noted as background information. The subjects were also questioned with regard to their experience of 15 subjective symptoms: ocular fatigue, discharge, foreign body sensation, dryness, uncomfortable sensation, sticky sensation, pain, epiphora, itching, redness, heavy sensation, glare, excessive blinking, burning sensation, and ocular discomfort on arising. Each symptom was assessed on a scale of 0 to 3: 0 = none; 1 = sometimes; 2 = frequent; 3 = chronic.

Lid margin findings were assessed for the upper and lower eyelids with the use of a slit-lamp microscope (TOPCON, Tokyo, Japan). Telangiectasia was assessed on a scale from 0 to 3: 0 = no findings; 1 = mild telangiectasia; 2 = moderate telangiectasia or redness; 3 = severe telangiectasia or redness. Mucocutaneous junction was assessed on a scale of 0 to 3: 0 = Marx's line (ML) courses on the skin side of the meibomian orifice (MO) line and does not touch the MO at all; 1 = parts of ML touch the MO; 2 = ML courses through the MO; 3 = ML courses along the eyelid margin side of the MO.[10] Irregularity, plugging, foaming, and thickness were assessed on a scale from 0 to 2: 0 = no findings; 1 = mild findings; 2 = severe findings.

Corneal and conjunctival staining were scored 0 to 9.[11] The fluorescein tear film breakup time (BUT) was measured three times consecutively after the instillation of fluorescein, and the mean value was adopted. Tear fluid production was evaluated by Schirmer's test without anesthesia.

Meibomian glands were evaluated for the upper and lower lids with the use of a noncontact meibography system (TOPCON) attached to a slit-lamp microscope. Partial or complete loss of meibomian glands was scored on a scale of 0 to 3 (meiboscore), and meibomian gland distortion was graded on a scale of 0 to 2, as described previously.[12,13] Meibomian gland dilation, shortening, and dropout were assessed on a scale of 0 to 2: 0 = no findings; 1 = mild findings; 2 = severe findings.

Meibum was collected at six sites for each target eye: three sites in the upper lid (temporal, central, and nasal) and three sites in the lower lid (nasal, central, and temporal). Powder-free gloves were worn by the collector to avoid contamination. The eyelids were carefully cleaned with a cotton swab dipped in saline before expression of meibum. Meibum was extruded with the use of the Arita Meibomian Gland Compressor (Katena Products, Denville, NJ, USA), and it was collected with a separate stainless steel spatula at each site. Collected meibum was immediately transferred to dry ice for storage.

The degree of ease with which meibum was expressed was evaluated semiquantitatively for each of the six collection sites on a scale of 0 to 3: 0 = clear meibum readily expressed; 1 = cloudy meibum expressed with mild pressure; 2 = cloudy meibum expressed with more than moderate pressure; 3 = meibum could not be expressed even with strong pressure.[14] Meibum color was assessed for each site according to three categories (clear, cloudy, yellow) and a scale of 0 or 1: 0 = clear; 1 = colored (cloudy or yellow). Meibum viscosity was also assessed for each site on the basis of three categories (oily, creamy, toothpaste-like) and a scale of 0 or 1: 0 = oily; 1 = nonoily (creamy or toothpaste-like).

## Lipid Analysis

Meibum collection and FFA composition analysis were performed as previously described.[9] Meibum was dissolved in chloroform:methanol (1:1, vol/vol; Wako, Osaka, Japan) and analyzed by liquid chromatography–Fourier transform mass spectrometry (LC-FTMS). Water containing 0.1% ammonium acetate (Sigma-Aldrich Corp., St. Louis, MO, USA) and methanol containing 0.1% ammonium acetate (Sigma-Aldrich Corp.) were used as the mobile phase. We performed a high mass accuracy full scan (*m/z* of 180–550) with a Q Exactive mass spectrometer (Thermo Scientific, Waltham, MA, USA) to identify individual lipid molecular species.

Given that the amount of collected meibum sample was too small to weigh, we corrected for weight differences among samples by normalizing the FFA data by the total peak area before statistical analysis. The percentage peak area was calculated with the following equation: percentage peak area of FFA *X* = (peak area value of FFA *X* in sample *Y*)/(total peak area value of all FFAs in sample *Y*). The percentage peak area was used as a measure of the content of each FFA among total FFAs in meibum.

## Statistical Analysis

Data are presented as means ± SD unless indicated otherwise. The frequency of each ocular symptom was compared between the MGD and control groups with Fisher's exact test. Means of parameters were compared between the MGD and control groups or between upper and lower eyelids with Student's *t*-test. Meibum color and viscosity were compared between the MGD group and the control group, between the upper and lower eyelids, or among temporal, central, and nasal sites with Fisher's exact test. Meibum grade was compared among temporal, central, and nasal sites of each eyelid in the MGD group with one-way analysis of variance. The relations between clinical signs were evaluated with Spearman's correlation analysis. A $P$ value of $<0.05$ was considered statistically significant.

Principal component analysis (PCA) was performed with the use of AI output software[15] (developed at Osaka University) in order to explore the relation between FFA composition of meibum and clinical signs. Principal component analysis is a primary multivariate technique that has been widely adopted in lipidomics, metabolomics, and proteomics.[16–18] It is applied to transform the representation of the original multivariable data set to a set of new orthogonal variables known as principal components (PCs). The content of each FFA species for all meibum samples was entered into the AI output data set. The score plot of PCA calculated from the MGD group and the control group was evaluated. We then performed volcano plot analysis with the use of Microsoft Excel 2010 (Redmond, WA, USA) to investigate the differences in detail. A volcano plot is a type of scatter plot that allows visualization of changes in large data sets.[19] Given that a volcano plot can be used to compare only two data sets, we reclassified meibum color from the original three groups (clear, cloudy, and yellow) to two groups: clear (clear) and colored (cloudy or yellow). The horizontal axis represents $\log_2$(fold

**Table 1.** Comparison of Clinical Signs Evaluated by Slit-Lamp Microscopy Between the MGD and Control Groups and Between Upper and Lower Eyelids

| Sign | MGD, $n = 38$ | Control, $n = 20$ | *P* Value* |
|---|---|---|---|
| Telangiectasia | | | |
| Upper eyelid | 1.7 ± 0.9 | 0.1 ± 0.3 | <0.001 |
| Lower eyelid | 1.3 ± 0.8 | 0.1 ± 0.2 | <0.001 |
| *P* value† | 0.093 | 0.560 | |
| Mucocutaneous junction | | | |
| Upper eyelid | 1.8 ± 1.0 | 0.1 ± 0.2 | <0.001 |
| Lower eyelid | 1.6 ± 0.9 | 0.1 ± 0.2 | <0.001 |
| *P* value† | 0.404 | 1.000 | |
| Irregularity | | | |
| Upper eyelid | 0.5 ± 0.6 | 0.1 ± 0.2 | 0.005 |
| Lower eyelid | 0.6 ± 0.7 | 0.1 ± 0.2 | 0.001 |
| *P* value† | 0.301 | 1.000 | |
| Plugging | | | |
| Upper eyelid | 1.3 ± 0.7 | 0.1 ± 0.2 | <0.001 |
| Lower eyelid | 1.0 ± 0.7 | 0.0 ± 0.0 | <0.001 |
| *P* value† | 0.068 | 0.324 | |
| Foaming | | | |
| Upper eyelid | 0.0 ± 0.2 | 0.0 ± 0.0 | 0.484 |
| Lower eyelid | 0.1 ± 0.4 | 0.0 ± 0.0 | 0.238 |
| *P* value† | 0.241 | | |
| Lid thickness | | | |
| Upper eyelid | 0.3 ± 0.6 | 0.0 ± 0.0 | 0.030 |
| Lower eyelid | 0.2 ± 0.5 | 0.0 ± 0.0 | 0.040 |
| *P* value† | 0.666 | | |
| Meibum grade, temporal | | | |
| Upper eyelid | 1.7 ± 0.8 | 0.2 ± 0.5 | <0.001 |
| Lower eyelid | 1.5 ± 0.8 | 0.1 ± 0.3 | <0.001 |
| *P* value† | 0.335 | 0.740 | |
| Meibum grade, central | | | |
| Upper eyelid | 1.5 ± 0.9 | 0.1 ± 0.4 | <0.001 |
| Lower eyelid | 1.6 ± 0.7 | 0.2 ± 0.5 | <0.001 |
| *P* value† | 0.377 | 0.672 | |
| Meibum grade, nasal | | | |
| Upper eyelid | 1.4 ± 0.7 | 0.1 ± 0.2 | <0.001 |
| Lower eyelid | 1.6 ± 0.7 | 0.1 ± 0.2 | <0.001 |
| *P* value† | 0.181 | 0.909 | |
| BUT, s | 3.4 ± 2.1 | 6.5 ± 1.6 | <0.001 |
| Schirmer's test value, mm | 10.6 ± 7.2 | 16.4 ± 9.9 | 0.015 |
| Keratoconjunctival staining | 1.2 ± 1.6 | 0.0 ± 0.0 | 0.002 |

Data are means ± SD.
* Student's *t*-test for comparison between MGD and control groups.
† Student's *t*-test for comparison between upper and lower eyelids.

change for colored group/clear group), and the vertical axis represents $-\log_{10}$(*P* value from the *t*-test).

## Results

### Clinical Signs and Meibum Properties

Patients were diagnosed with MGD according to the Japanese diagnostic criteria.[20] The frequencies of all subjective symptoms with the exception of pain and burning sensation were significantly higher in the MGD group than in the control group (data not shown). Most lid margin abnormality scores

**Table 2.** Comparison of Clinical Signs Evaluated by Noncontact Meibography Between the MGD and Control Groups and Between Upper and Lower Eyelids

| Sign | MGD, $n = 38$ | Control, $n = 20$ | *P* Value* |
|---|---|---|---|
| Meiboscore | | | |
| Upper eyelid | 1.9 ± 0.8 | 0.4 ± 0.5 | <0.001 |
| Lower eyelid | 1.9 ± 0.9 | 0.2 ± 0.4 | <0.001 |
| *P* value† | 0.779 | 0.176 | |
| Distortion | | | |
| Upper eyelid | 0.7 ± 0.8 | 0.2 ± 0.5 | 0.018 |
| Lower eyelid | 0.3 ± 0.4 | 0.0 ± 0.0 | 0.020 |
| *P* value† | 0.006 | 0.114 | |
| Dilation | | | |
| Upper eyelid | 0.1 ± 0.3 | 0.0 ± 0.0 | 0.185 |
| Lower eyelid | 0.2 ± 0.4 | 0.0 ± 0.0 | 0.037 |
| *P* value† | 0.177 | | |
| Shortening | | | |
| Upper eyelid | 1.5 ± 0.6 | 0.4 ± 0.5 | <0.001 |
| Lower eyelid | 1.5 ± 0.6 | 0.3 ± 0.5 | <0.001 |
| *P* value† | 0.784 | 0.569 | |
| Dropout | | | |
| Upper eyelid | 1.1 ± 0.8 | 0.1 ± 0.3 | <0.001 |
| Lower eyelid | 1.1 ± 0.8 | 0.0 ± 0.0 | <0.001 |
| *P* value† | 0.984 | 0.165 | |

Data are means ± SD.
* Student's *t*-test for comparison between MGD and control groups.
† Student's *t*-test for comparison between upper and lower eyelids.

and meibum grade were also significantly higher in the MGD group than in the control group (Table 1). Meibum grade did not differ significantly between the upper and lower eyelids (Table 1) or among the temporal, central, and nasal sites of the upper ($P = 0.176$) or lower ($P = 0.811$) eyelids in the MGD group. Most meibomian gland finding scores were significantly higher in the MGD group than in the control group (Table 2). The MGD group had a significantly higher keratoconjunctival staining score and a significantly shorter BUT and Schirmer's test value compared with the control group (Table 1).

The distribution of meibum color and viscosity differed significantly between the MGD and control groups at each of the six sites examined. There were no significant differences in meibum color or viscosity among the temporal, central, and nasal sites of the upper or lower eyelids of the MGD group or the control group, or between the upper and lower eyelids at each site in the MGD group (Table 3). Among subjective symptoms, epiphora and sticky sensation were significantly correlated with the sum of the meibum color score for the upper eyelid in the MGD group (epiphora, $r = 0.49$, $P = 0.020$; sticky sensation, $r = 0.45$, $P = 0.038$).

### Lipid Analysis

Liquid chromatography–FTMS was applied to quantify FFAs in meibum and to determine whether their amounts might change in association with ocular symptoms. The high-resolution negative electrospray ionization (ESI) mode was adopted to analyze the FFAs in all six meibum samples from each of the 38 MGD patients and 20 control subjects. We detected 103 FFA species with carbon chains of $C_{12}$ to $C_{37}$ in meibum (Table 4). These molecules included very long chain FFAs such as those with $C_{36}$ or $C_{37}$ carbon chains as well as odd-numbered carbon chain FFAs such as those with $C_{17}$, $C_{19}$,

**Table 3.** Comparison of Meibum Color and Viscosity Between MGD ($n = 38$) and Control ($n = 20$) Groups, Between Upper and Lower Eyelids, and Among Temporal, Central, and Nasal Sites of Each Eyelid

**Upper eyelid**

| | Temporal | | Central | | Nasal | | *P* Value* | |
|---|---|---|---|---|---|---|---|---|
| **Color** | **MGD** | **Control** | **MGD** | **Control** | **MGD** | **Control** | **MGD** | **Control** |
| Clear | 6 | 19 | 7 | 19 | 7 | 19 | 0.461 | 1.000 |
| Cloudy | 13 | 0 | 13 | 1 | 9 | 1 | | |
| Yellow | 6 | 0 | 6 | 0 | 12 | 0 | | |
| *P* value† | <0.001 | | <0.001 | | <0.001 | | | |

**Lower eyelid**

| | Temporal | | Central | | Nasal | | *P* Value* | |
|---|---|---|---|---|---|---|---|---|
| | **MGD** | **Control** | **MGD** | **Control** | **MGD** | **Control** | **MGD** | **Control** |
| Clear | 6 | 15 | 5 | 16 | 6 | 15 | 0.984 | 0.728 |
| Cloudy | 15 | 3 | 16 | 1 | 14 | 1 | | |
| Yellow | 5 | 0 | 6 | 1 | 7 | 1 | | |
| *P* value† | <0.001 | | <0.001 | | <0.001 | | | |
| *P* value‡ | 0.931 | | 0.815 | | 0.298 | | | |

**Upper eyelid**

| | Temporal | | Central | | Nasal | | *P* Value* | |
|---|---|---|---|---|---|---|---|---|
| **Viscosity** | **MGD** | **Control** | **MGD** | **Control** | **MGD** | **Control** | **MGD** | **Control** |
| Oily | 8 | 19 | 11 | 19 | 10 | 20 | 0.491 | 1.000 |
| Creamy | 7 | 0 | 11 | 0 | 14 | 0 | | |
| Toothpaste | 6 | 0 | 3 | 1 | 3 | 0 | | |
| *P* value† | <0.001 | | <0.001 | | <0.001 | | | |

**Lower eyelid**

| | Temporal | | Central | | Nasal | | *P* Value* | |
|---|---|---|---|---|---|---|---|---|
| | **MGD** | **Control** | **MGD** | **Control** | **MGD** | **Control** | **MGD** | **Control** |
| Oily | 13 | 18 | 10 | 18 | 11 | 17 | 0.749 | |
| Creamy | 7 | 0 | 12 | 0 | 11 | 0 | | |
| Toothpaste | 4 | 0 | 4 | 0 | 3 | 0 | | |
| *P* value† | 0.002 | | <0.001 | | <0.001 | | | |
| *P* value‡ | 0.510 | | 1.000 | | 0.923 | | | |

* Fisher's exact test for comparison among temporal, central, and nasal sites in the MGD group or the control group.

† Fisher's exact test for comparison between MGD and control groups.

‡ Fisher's exact test for comparison between upper and lower eyelids at temporal, central, or nasal sites in the MGD group. When the meibum could not be collected or the color and viscosity could not be evaluated, the data at the site were treated as no data.

or $C_{21}$ chains. FFAs 16:0, 18:0, and 26:0 constituted a major portion (~40%) of total FFAs.

We performed PCA to identify hidden variables in the FFA data for each meibum color. Figure 1 shows the PCA score plot calculated from the MGD and control groups. We evaluated PCA plots including PC1 to PC5 and found that the plot of PC3 vs. PC5 resulted in clear separation of the data points into three groups corresponding to meibum color. The clear meibum samples clustered in the PC3-positive area, and the yellow meibum samples clustered in the PC3-negative area. These results indicated that clear meibum and yellow meibum have different FFA compositions.

We also generated a volcano plot comparing FFA species between the clear meibum group and the colored (cloudy or yellow) meibum group (Fig. 2) for the data obtained from MGD patients presented in Supplementary Table S1. Most saturated FFAs were located on the left side of the plot and most unsaturated FFAs on the right, indicating that the amounts of unsaturated FFAs tended to be increased in the colored group. The FFA content for each meibum color group from MGD patients is shown in Table 5 and Figure 3. Free fatty acid content was calculated as percentage peak area and therefore is not necessarily reflective of the actual molar fraction in the meibum samples. The content of unsaturated FFAs with a chain length of $C_{25}$ to $C_{30}$ increased according to the rank order of clear < cloudy < yellow. The amounts of the unsaturated FFAs 30:4, 32:4, and 32:5 increased significantly from clear to cloudy to yellow, with a high $\log_2$(fold change) and high $-\log_{10}$(*P* value). The saturated FFAs 14:0 and 15:0 had a low $\log_2$(fold change) and high $-\log_{10}$(*P* value), and their amounts showed a tendency to decrease from clear to cloudy to yellow.

## Discussion

We have here identified the FFAs in meibum of individuals with MGD and evaluated the relation between the FFA composition of meibum and clinical signs. Our data reveal a previously unrecognized relation between the color and FFA composition of human meibum, with the amounts of unsaturated FFAs being increased in colored meibum. We previously established and confirmed the sensitivity and robustness of the analytic method applied in the present study.[9] This method of lipid

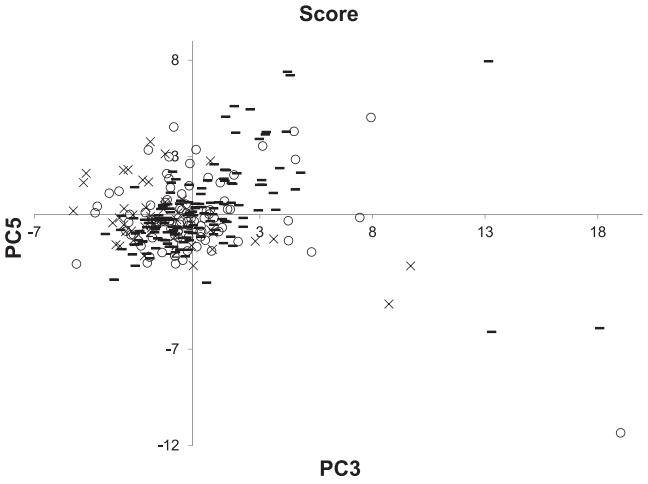


**Figure 1.** Principal component analysis score plot for FFA composition of meibum from subjects in the MGD and control groups. Each data point corresponds to a sample of clear meibum (–), cloudy meibum (○), or yellow meibum (×).

analysis is able to identify and quantify FFAs in meibum with the high sensitivity and precision characteristic of LC-MS technology.

We identified 103 FFA species with carbon chains of $C_{12}$ to $C_{37}$ in human meibum. The degree of saturation ranged from one to six double bonds. Free fatty acids 16:0, 18:0, and 26:0 accounted for a major proportion of total FFAs. Indeed, saturated FFAs constituted the majority (approximately 75%) of total FFAs in meibum, and we also detected unusual FFAs with long saturated carbon chains. Saturated FFAs and their esters play an important role in limitation of tear evaporation.[21] Such evaporation resistance also increases with carbon chain length.[22] Our detection of a variety of saturated FFAs, some with unusually long carbon chains, may thus be related to the function of meibum in limiting evaporation of the tear film. With the use of gas chromatography (GC), Dougherty and McCulley[23] detected FFAs with carbon chains of $C_{12}$ to $C_{29}$ in meibomian gland secretions of patients with chronic blepharitis. They found that the FFAs 18:1, 16:0, and 18:0 were the most abundant species. With the use of GC and GC-MS, Joffre et al.[24] detected FFAs with carbon chains of $C_{14}$ to $C_{26}$ in human meibum and found that the FFA 18:1 was the most

**Table 4.** Molecular Species of FFA Detected in Human Meibum

| Carbon Chain Length | Detected Molecular Species of FFA | | | | | | |
|---|---|---|---|---|---|---|---|
| 12 | 12:0 | | | | | | |
| 14 | 14:0 | | | | | | |
| 15 | 15:0 | 15:1 | | | | | |
| 16 | 16:0 | 16:1 | | | | | |
| 17 | 17:0 | 17:1 | | | | | |
| 18 | 18:0 | 18:1 | 18:2 | 18:3 | | | |
| 19 | 19:0 | 19:1 | 19:2 | 19:3 | | | |
| 20 | 20:0 | 20:1 | 20:2 | 20:3 | 20:4 | | |
| 21 | 21:0 | 21:1 | 21:2 | 21:3 | | | |
| 22 | 22:0 | 22:1 | 22:2 | 22:3 | 22:4 | 22:5 | 22:6 |
| 23 | 23:0 | 23:1 | 23:2 | 23:3 | | | |
| 24 | 24:0 | 24:1 | 24:2 | 24:3 | 24:4 | 24:5 | |
| 25 | 25:0 | 25:1 | 25:2 | 25:3 | | | |
| 26 | 26:0 | 26:1 | 26:2 | 26:3 | 26:4 | 26:5 | |
| 27 | 27:0 | 27:1 | 27:2 | | | | |
| 28 | 28:0 | 28:1 | 28:2 | 28:3 | 28:4 | | |
| 29 | 29:0 | 29:1 | 29:2 | | | | |
| 30 | 30:0 | 30:1 | 30:2 | 30:3 | 30:4 | | |
| 31 | 31:0 | 31:1 | 31:2 | | | | |
| 32 | 32:0 | 32:1 | 32:2 | 32:3 | 32:4 | 32:5 | 32:6 |
| 33 | 33:0 | 33:1 | 33:2 | 33:3 | 33:4 | | |
| 34 | 34:0 | 34:1 | 34:2 | 34:3 | 34:4 | 34:5 | 34:6 |
| 35 | 35:0 | 35:1 | 35:2 | 35:3 | | | |
| 36 | 36:0 | 36:1 | 36:2 | 36:3 | 36:4 | 36:5 | 36:6 |
| 37 | 37:0 | 37:1 | | | | | |

In the *X*:*Y* notation, *X* and *Y* indicate carbon chain length and the number of double bonds, respectively.

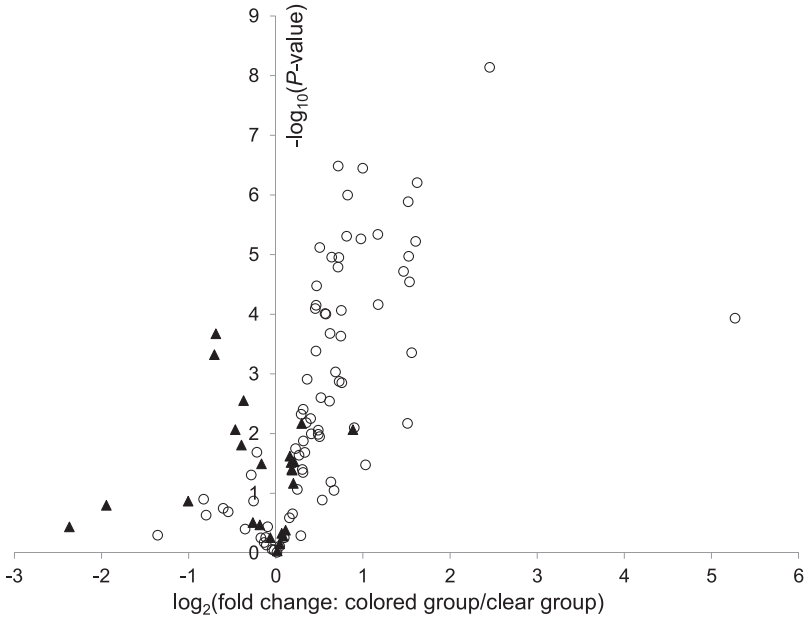


**Figure 2.** Volcano plot comparing the FFA composition of the clear meibum group and the colored (cloudy or yellow) meibum group for MGD patients. The *horizontal axis* is $\log_2$ of the fold change for colored group/clear group, and the *vertical axis* is $-\log_{10}$ of the *P* value calculated from Student's *t*-test. Each data point corresponds to a single species of unsaturated (○) or saturated (▲) FFA (see Supplementary Table S1).

**Table 5.** Free Fatty Acid Content of Human Meibum From MGD Patients According to Color Grade

| FFA | Clear, $n = 40$,* % | Cloudy, $n = 81$,* % | Yellow, $n = 42$,* % | Trend† |
|---|---|---|---|---|
| FFA(12:0) | 0.146 ± 0.132 | 0.126 ± 0.122 | 0.115 ± 0.129 | B |
| FFA(14:0) | 0.415 ± 0.227 | 0.261 ± 0.201 | 0.252 ± 0.174 | B |
| FFA(15:0) | 0.162 ± 0.097 | 0.105 ± 0.082 | 0.0887 ± 0.0623 | B |
| FFA(15:1) | 0.0226 ± 0.0475 | 0.0140 ± 0.0307 | 0.0110 ± 0.0238 | B |
| FFA(16:0) | 10.5 ± 5.8 | 7.76 ± 5.69 | 7.37 ± 6.87 | B |
| FFA(16:1) | 0.402 ± 0.661 | 0.242 ± 0.527 | 0.197 ± 0.321 | B |
| FFA(17:0) | 0.461 ± 0.192 | 0.358 ± 0.166 | 0.354 ± 0.110 | B |
| FFA(17:1) | 0.0987 ± 0.1443 | 0.0671 ± 0.1097 | 0.0609 ± 0.0940 | B |
| FFA(18:0) | 20.2 ± 10.5 | 15.7 ± 10.8 | 14.7 ± 12.2 | B |
| FFA(18:1) | 3.43 ± 1.07 | 2.99 ± 1.28 | 2.88 ± 1.06 | B |
| FFA(18:2) | 1.86 ± 1.99 | 1.60 ± 1.612 | 1.76 ± 1.94 | |
| FFA(18:3) | 0.130 ± 0.150 | 0.115 ± 0.123 | 0.126 ± 0.146 | |
| FFA(19:0) | 0.271 ± 0.106 | 0.246 ± 0.101 | 0.285 ± 0.117 | |
| FFA(19:1) | 0.0448 ± 0.0332 | 0.0425 ± 0.0278 | 0.0391 ± 0.0242 | B |
| FFA(19:2) | 0.0106 ± 0.0111 | 0.0107 ± 0.0099 | 0.0095 ± 0.0059 | |
| FFA(19:3) | 0.0016 ± 0.0027 | 0.0024 ± 0.0025 | 0.0022 ± 0.0019 | |
| FFA(20:0) | 1.21 ± 0.33 | 1.07 ± 0.31 | 1.08 ± 0.266 | |
| FFA(20:1) | 0.375 ± 0.142 | 0.344 ± 0.140 | 0.368 ± 0.134 | |
| FFA(20:2) | 0.165 ± 0.099 | 0.163 ± 0.099 | 0.185 ± 0.095 | |
| FFA(20:3) | 0.257 ± 0.168 | 0.262 ± 0.152 | 0.300 ± 0.185 | A |
| FFA(20:4) | 0.0760 ± 0.1126 | 0.0585 ± 0.0653 | 0.0397 ± 0.0394 | B |
| FFA(21:0) | 0.459 ± 0.215 | 0.454 ± 0.182 | 0.513 ± 0.258 | |
| FFA(21:1) | 0.0357 ± 0.0228 | 0.0314 ± 0.0148 | 0.0272 ± 0.0115 | B |
| FFA(21:2) | 0.0032 ± 0.0050 | 0.0053 ± 0.0064 | 0.0045 ± 0.0048 | |
| FFA(21:3) | 0.0033 ± 0.0030 | 0.0052 ± 0.0040 | 0.0056 ± 0.0036 | A |
| FFA(22:0) | 2.13 ± 0.81 | 2.21 ± 0.68 | 2.29 ± 0.85 | A |
| FFA(22:1) | 1.05 ± 0.53 | 0.874 ± 0.468 | 0.847 ± 0.387 | B |
| FFA(22:2) | 0.0610 ± 0.0346 | 0.0580 ± 0.0298 | 0.0643 ± 0.0358 | |
| FFA(22:3) | 0.0382 ± 0.0467 | 0.0360 ± 0.0426 | 0.0342 ± 0.0259 | B |
| FFA(22:4) | 0.0059 ± 0.0099 | 0.0060 ± 0.0095 | 0.0059 ± 0.0056 | |
| FFA(22:5) | 0.0041 ± 0.0082 | 0.0049 ± 0.0057 | 0.0052 ± 0.0042 | A |
| FFA(22:6) | 0.0351 ± 0.0539 | 0.0308 ± 0.0345 | 0.0212 ± 0.0153 | B |
| FFA(23:0) | 1.60 ± 0.49 | 1.78 ± 0.56 | 1.85 ± 0.67 | A |
| FFA(23:1) | 0.0391 ± 0.0216 | 0.0438 ± 0.0202 | 0.0570 ± 0.0445 | A |
| FFA(23:2) | 0.0009 ± 0.0015 | 0.0016 ± 0.0018 | 0.0020 ± 0.0020 | A |
| FFA(23:3) | 0.0003 ± 0.0005 | 0.0007 ± 0.0008 | 0.0008 ± 0.0009 | A |
| FFA(24:0) | 9.29 ± 2.56 | 10.5 ± 2.9 | 10.2 ± 2.9 | |
| FFA(24:1) | 1.75 ± 0.76 | 1.91 ± 0.80 | 2.46 ± 1.41 | A |
| FFA(24:2) | 0.0937 ± 0.0576 | 0.107 ± 0.0598 | 0.135 ± 0.088 | A |
| FFA(24:3) | 0.0168 ± 0.0125 | 0.0214 ± 0.0157 | 0.0285 ± 0.0278 | A |
| FFA(24:4) | 0.0012 ± 0.0022 | 0.0018 ± 0.0025 | 0.0022 ± 0.0024 | A |
| FFA(24:5) | 0.0001 ± 0.0008 | 0.0001 ± 0.0004 | 0.0000 ± 0.0000 | |
| FFA(25:0) | 6.91 ± 2.52 | 7.75 ± 2.17 | 8.05 ± 2.51 | A |
| FFA(25:1) | 0.140 ± 0.057 | 0.174 ± 0.069 | 0.228 ± 0.141 | A |
| FFA(25:2) | 0.0070 ± 0.0056 | 0.0093 ± 0.0053 | 0.0107 ± 0.0073 | A |
| FFA(25:3) | 0.0005 ± 0.0008 | 0.0011 ± 0.0012 | 0.0015 ± 0.0019 | A |
| FFA(26:0) | 11.5 ± 4.3 | 13.2 ± 4.1 | 12.8 ± 4.2 | |
| FFA(26:1) | 2.62 ± 1.04 | 2.96 ± 1.07 | 3.67 ± 1.61 | A |
| FFA(26:2) | 0.179 ± 0.086 | 0.205 ± 0.093 | 0.257 ± 0.157 | A |
| FFA(26:3) | 0.0328 ± 0.0251 | 0.0452 ± 0.0340 | 0.0603 ± 0.0632 | A |
| FFA(26:4) | 0.0005 ± 0.0009 | 0.0012 ± 0.0020 | 0.0022 ± 0.0037 | A |
| FFA(26:5) | 0.0000 ± 0.0001 | 0.0001 ± 0.0002 | 0.0002 ± 0.0003 | A |
| FFA(27:0) | 5.78 ± 2.29 | 6.68 ± 2.06 | 6.71 ± 2.21 | A |
| FFA(27:1) | 0.113 ± 0.050 | 0.149 ± 0.050 | 0.182 ± 0.085 | A |
| FFA(27:2) | 0.0034 ± 0.0036 | 0.0051 ± 0.0032 | 0.0065 ± 0.0042 | A |
| FFA(28:0) | 3.18 ± 1.39 | 4.00 ± 1.63 | 3.75 ± 1.57 | |
| FFA(28:1) | 2.86 ± 1.07 | 3.24 ± 1.11 | 3.54 ± 1.44 | A |
| FFA(28:2) | 0.193 ± 0.081 | 0.230 ± 0.093 | 0.282 ± 0.138 | A |
| FFA(28:3) | 0.0108 ± 0.0085 | 0.0166 ± 0.0109 | 0.0216 ± 0.0176 | A |
| FFA(28:4) | 0.0002 ± 0.0004 | 0.0007 ± 0.0009 | 0.0009 ± 0.0011 | A |
| FFA(29:0) | 1.68 ± 0.74 | 1.92 ± 0.76 | 1.96 ± 0.84 | A |
| FFA(29:1) | 0.117 ± 0.054 | 0.157 ± 0.062 | 0.168 ± 0.080 | A |
| FFA(29:2) | 0.0075 ± 0.0051 | 0.0117 ± 0.0073 | 0.0134 ± 0.0081 | A |
| FFA(30:0) | 0.744 ± 0.386 | 0.819 ± 0.401 | 0.734 ± 0.441 | |

**Table 5.** Continued

| FFA | Clear, $n = 40$,* % | Cloudy, $n = 81$,* % | Yellow, $n = 42$,* % | Trend† |
|---|---|---|---|---|
| FFA(30:1) | 3.16 ± 1.33 | 3.84 ± 1.60 | 4.09 ± 1.86 | A |
| FFA(30:2) | 0.317 ± 0.138 | 0.422 ± 0.184 | 0.471 ± 0.207 | A |
| FFA(30:3) | 0.0418 ± 0.0225 | 0.0582 ± 0.0345 | 0.0691 ± 0.0449 | A |
| FFA(30:4) | 0.0009 ± 0.0013 | 0.0022 ± 0.0022 | 0.0031 ± 0.0032 | A |
| FFA(31:0) | 0.330 ± 0.178 | 0.358 ± 0.181 | 0.354 ± 0.213 | |
| FFA(31:1) | 0.0981 ± 0.0504 | 0.127 ± 0.058 | 0.121 ± 0.068 | |
| FFA(31:2) | 0.0137 ± 0.0071 | 0.0223 ± 0.0131 | 0.0230 ± 0.0134 | A |
| FFA(32:0) | 0.0926 ± 0.0662 | 0.0893 ± 0.0515 | 0.0669 ± 0.0447 | B |
| FFA(32:1) | 1.41 ± 0.78 | 1.61 ± 0.77 | 1.51 ± 0.79 | |
| FFA(32:2) | 0.336 ± 0.175 | 0.454 ± 0.220 | 0.478 ± 0.242 | A |
| FFA(32:3) | 0.134 ± 0.072 | 0.196 ± 0.129 | 0.205 ± 0.130 | A |
| FFA(32:4) | 0.0109 ± 0.0071 | 0.0185 ± 0.0124 | 0.0208 ± 0.0144 | A |
| FFA(32:5) | 0.0009 ± 0.0016 | 0.0025 ± 0.0027 | 0.0036 ± 0.0035 | A |
| FFA(32:6) | 0.0000 ± 0.0000 | 0.0001 ± 0.0005 | 0.0004 ± 0.0008 | A |
| FFA(33:0) | 0.0396 ± 0.0304 | 0.0410 ± 0.0252 | 0.0383 ± 0.0285 | |
| FFA(33:1) | 0.0313 ± 0.0182 | 0.0410 ± 0.0193 | 0.0367 ± 0.0245 | |
| FFA(33:2) | 0.0057 ± 0.0052 | 0.0100 ± 0.0066 | 0.0087 ± 0.0060 | |
| FFA(33:3) | 0.0078 ± 0.0056 | 0.0162 ± 0.0139 | 0.0144 ± 0.0105 | |
| FFA(33:4) | 0.0003 ± 0.0006 | 0.0015 ± 0.0021 | 0.0015 ± 0.0017 | |
| FFA(34:0) | 0.0271 ± 0.0559 | 0.0158 ± 0.0152 | 0.0091 ± 0.0085 | B |
| FFA(34:1) | 0.436 ± 0.250 | 0.540 ± 0.261 | 0.476 ± 0.315 | |
| FFA(34:2) | 0.162 ± 0.097 | 0.220 ± 0.104 | 0.203 ± 0.115 | |
| FFA(34:3) | 0.150 ± 0.092 | 0.243 ± 0.185 | 0.208 ± 0.140 | |
| FFA(34:4) | 0.0284 ± 0.0153 | 0.0455 ± 0.0279 | 0.0423 ± 0.0276 | |
| FFA(34:5) | 0.0093 ± 0.0061 | 0.0147 ± 0.0097 | 0.0165 ± 0.0108 | A |
| FFA(34:6) | 0.0024 ± 0.0030 | 0.0049 ± 0.0043 | 0.0068 ± 0.0057 | A |
| FFA(35:0) | 0.0027 ± 0.0043 | 0.0051 ± 0.0055 | 0.0045 ± 0.0056 | |
| FFA(35:1) | 0.0051 ± 0.0056 | 0.0091 ± 0.0061 | 0.0080 ± 0.0075 | |
| FFA(35:2) | 0.0007 ± 0.0014 | 0.0021 ± 0.0019 | 0.0018 ± 0.0019 | |
| FFA(35:3) | 0.0020 ± 0.0029 | 0.0050 ± 0.0052 | 0.0038 ± 0.0037 | |
| FFA(36:0) | 0.0047 ± 0.0151 | 0.0014 ± 0.0030 | 0.0008 ± 0.0020 | B |
| FFA(36:1) | 0.123 ± 0.076 | 0.148 ± 0.080 | 0.127 ± 0.095 | |
| FFA(36:2) | 0.0430 ± 0.0285 | 0.0601 ± 0.0316 | 0.0514 ± 0.0318 | |
| FFA(36:3) | 0.0628 ± 0.0446 | 0.0969 ± 0.0821 | 0.0708 ± 0.0495 | |
| FFA(36:4) | 0.0187 ± 0.0129 | 0.0288 ± 0.0184 | 0.0230 ± 0.0166 | |
| FFA(36:5) | 0.0082 ± 0.0057 | 0.0141 ± 0.0090 | 0.0150 ± 0.0118 | A |
| FFA(36:6) | 0.0046 ± 0.0042 | 0.0084 ± 0.0067 | 0.0100 ± 0.0080 | A |
| FFA(37:0) | 0.0012 ± 0.0065 | 0.0001 ± 0.0003 | 0.0004 ± 0.0008 | |
| FFA(37:1) | 0.0005 ± 0.0012 | 0.0001 ± 0.0015 | 0.0010 ± 0.0017 | |

Free fatty acid content was calculated as percentage peak area and, therefore, does not necessarily reflect actual molar fraction in the meibum samples. Data are means ± SD. When more meibum was collected than usual, it was analyzed as duplicate samples.

* $n$ is the sample number (not subject number).

† A, clear < cloudy < yellow; B, clear > cloudy > yellow.

abundant, followed by 16:0 and 18:0. We also detected FFAs 16:0 and 18:0 abundantly, but FFA 18:1 was not as abundant as FFAs 16:0 and 18:0. In contrast, Butovich[25] reported that FFAs 16:0 and 18:0 either were not detected or were relatively minor components. The discrepancy in FFA composition between the studies might be explained by differences in analytical method or treatment of meibum samples. Therefore, the method that allows precise quantification of each FFA should be developed carefully in future work.

With the exception of the highly abundant $C_{16}$ and $C_{18}$ FFAs, we found that the distribution of saturated FFAs peaked in the range $C_{24}$ to $C_{26}$ (Fig. 3). Whereas even-numbered chains tended to be more abundant than odd-numbered chains among shorter-chain saturated FFAs, this trend was not apparent for longer chains. This finding is consistent with the fatty acid distribution in cholesteryl esters of human meibum described by Butovich.[26] Furthermore, we found that FFAs with even-numbered carbon chains tended to be more abundant than those with odd-numbered carbon chains among unsaturated FFAs with one to six double bonds. This finding is also in agreement with the results of Butovich.[26] A possible explanation for these observations is provided by the proposal of Knop et al.[27] that fatty acid synthesis occurs by the addition of $C_2$ units in the form of acetyl-CoA.[27] The use of propionyl-CoA as the carbon source gives rise to fatty acids with odd-numbered carbon chains.

A PCA score plot (PC3 vs. PC5) for FFA composition of meibum from both MGD patients and control subjects revealed clustering of samples according to meibum color (Fig. 1). Further analysis with a volcano plot for FFA composition of meibum from the MGD group revealed that most unsaturated FFAs had a positive $\log_2$(fold change) value and most saturated FFAs had a negative value (Fig. 2). The amounts of the unsaturated FFAs 30:4, 32:4, and 32:5 showed a tendency to increase from clear to cloudy to yellow meibum. Although the unsaturated FFA 32:6 had a high $\log_2$(fold change) value, it was present in extremely small amounts. The discovery of this relation between meibum color and FFA saturation may provide insight into the mechanism of the change in meibum color associated with MGD.

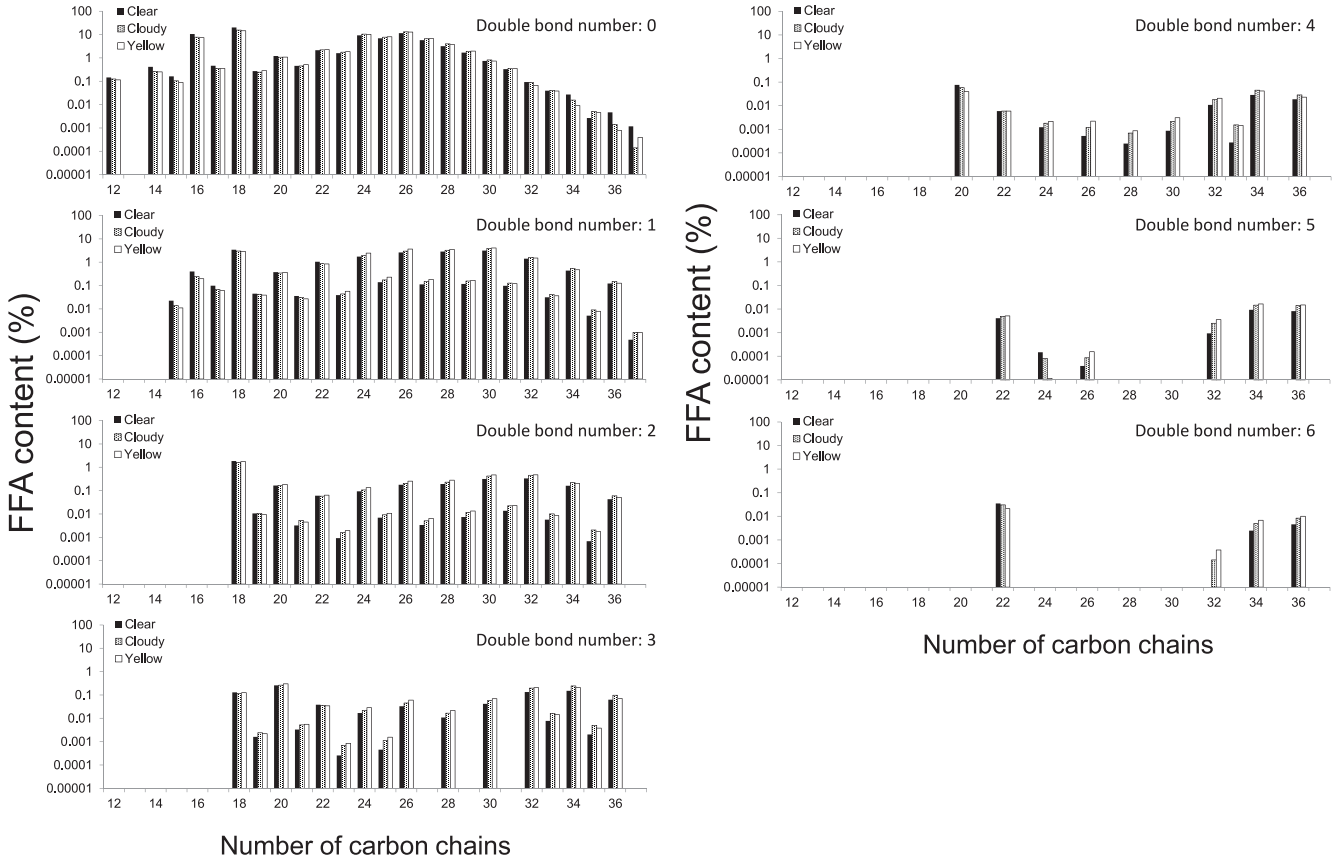


FIGURE 3. Comparison of FFA content among clear, cloudy, and yellow meibum groups for MGD patients according to the number of double bonds in the FFA structure. Free fatty acid content was calculated on the basis of percentage peak area and therefore does not necessarily reflect actual molar fraction.

We found that unsaturated FFAs were more abundant in colored meibum than in clear meibum, suggesting that oxidation of such FFAs might be associated with meibum color. Unsaturated FFAs have one or more double bonds, with such bonds being susceptible to enzymatic or nonenzymatic oxidation.[28] Such oxidation of unsaturated FFAs can generate hydroperoxides through formation of the highly reactive peroxy radical. Human meibum was previously found to contain oxidized fatty acids (Butovich IA, et al. *IOVS* 2005;46:ARVO E-Abstract 4432). The products of lipid peroxidation are toxic and may mediate protein oxidation. Oxidized protein may therefore be present in colored meibum. Indeed, meibum was previously found to contain varying amounts of inclusions (or debris) that did not resemble lipids.[29] These inclusions were insoluble in chloroform and did not melt (i.e., did not become clear) even at temperatures of 50° to 70°C. It is likely that these inclusions contain protein given that protein is insoluble in chloroform. Reactive aldehydes produced as a result of oxidative degradation of unsaturated FFAs in skin give rise to protein carbonylation, and the carbonylated proteins take on a yellowish color.[30] These aldehydes might be formed in skin during inflammation or during the degradation of surface sebum. We therefore suggest that the color of meibum may reflect the amount of inclusions (such as debris comprising carbonylated proteins) produced as a result of lipid peroxidation. A previous study (Obata H, et al. *IOVS* 2002;43:ARVO E-Abstract 60) classified meibum color into three groups—yellow, yellowish white, and white—and proposed that color changes might be caused by lipid peroxidation. Differences in FFA composition between clear meibum and colored (cloudy or yellow) meibum identified in the present study were statistically significant and may therefore serve as biomarkers and provide insight into changes associated with MGD. However, given that FFAs are present in human meibum in only small amounts, functional changes in meibum associated with MGD may depend not only on changes in lipid composition but also on those in the amounts of other components (such as proteins).

We found that the distribution of meibum color and viscosity differed significantly between the MGD and control groups at each of the six sites examined. Clear meibum tended to be oily, and yellow meibum to be toothpaste-like (more viscous). We also found that unsaturated FFAs were more abundant in colored meibum than in clear meibum. Our results thus contrast with the notion that an increased unsaturated lipid content would be expected to render meibum more fluid.[31] The change in meibum viscosity associated with MGD might thus be related to changes in other lipids (such as wax esters or cholesteryl esters) or other substances (such as proteins) rather than to changes in FFA content.

We found that the FFA composition of meibum changes in association with changes in meibum color. We propose that such changes in FFA composition are also associated with inflammation in individuals with MGD. The hydrolysis of phospholipids releases FFAs, some of which may function as inflammatory mediators.[32] In sebum, which is similar to meibum, changes in saturated and unsaturated FFA composition have been considered an initiator of follicular inflammation,[33] and MGD develops as a result of inflammation at the lid margin in posterior blepharitis.

We did not detect differences in meibum color between the upper and lower eyelids or among temporal, central, and nasal sites. Previous studies have evaluated meibum from the upper central eyelid[34,35] or lower central eyelid[2,36] in order to classify meibum condition. Our data indicate that meibum condition is similar for all meibomian gland ducts of a given individual, suggesting that any one part of the eyelid is representative of the entire eyelid.

Our observations demonstrate that the change in meibum color from clear to cloudy or yellow is related to changes in FFA content. The FFA composition of meibum can thus be estimated by observation of meibum color in the clinic. The change in meibum color from clear to cloudy or yellow may also be indicative of the presence of inflammation or oxidative conditions. Further investigations into the relation between meibum color and the biological or biochemical characteristics of meibum are warranted and should provide insight into the most appropriate treatment for MGD.

We evaluated the condition of meibum derived from orifices of meibomian glands. Lesions characterized by meibomian gland dropout were thus not evaluable. In such lesions, meibomian glands are thought to be atrophic and unable to produce meibum. It was therefore difficult to collect meibum from such lesions even though we exerted strong physical pressure with the use of meibomian gland forceps. Patients with severe MGD manifest dropout of most meibomian glands, with the result that little information can be obtained on meibum condition. Our method thus allowed us to evaluate meibum of patients with mild or moderate MGD. Further evaluation of the relation between the volume or FFA composition of meibum and oily layer parameters would be expected to contribute to clinical evaluation of MGD.

In conclusion, we have identified the FFA composition of meibum in MGD patients by LC-FTMS analysis. Furthermore, changes in FFA composition were associated with meibum color. This finding may shed light on the pathogenesis of MGD. Further studies are warranted to elucidate the role and origin of FFAs in human meibum and thereby to identify biomarkers or new treatments for this condition.

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