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Changes in Meibum Lipid Composition With Ocular *Demodex* Infestation

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Purpose: The purpose of this study was to understand the impact of *Demodex* infection in the lipid component of meibum in patients.

Methods: The meibum samples were collected from four groups of subjects: (1) *Demodex*-negative with non-MGD (D−M−; $n = 10$); (2) *Demodex*-positive with non-MGD (D+M−; $n = 10$); (3) *Demodex*-negative with MGD (D−M+; $n = 10$); and (4) *Demodex*-positive with MGD (D+M+; $n = 10$). A liquid chromatography–mass spectrometry (LC-MS) system consisting of ultra-performance liquid chromatography and a Q Exactive high-resolution mass spectrometer was used for lipids separation and detection.

Results: Compared with the D−M− group, the D+M− group had lower levels of phosphatidylcholines (PCs) and lysophosphatidylcholines (LPCs) and higher levels of phosphatidylethanolamines (PEs). Compared with the D−M+ group, the levels of sphingomyelins (SMs) and PCs in the D+M+ group were decreased, whereas the levels of (*O*-acyl)-ω-hydroxy fatty acids (OAHFAs), ceramides (CERs), LPCs, and diacylglycerols (DGs) were significantly increased. Triacylglycerols (TGs), DGs, CERs, and OAHFAs were decreased in D−M+ group, whereas levels of PEs, phosphatidylinositols, and phosphatidylglycerols were increased in meibum obtained from the D−M+ group compared with those in the D−M− group. TGs, SMs, CERs, and PEs were decreased in the D+M+ group, whereas levels of LPCs, LPEs, PCs, and PEs were increased in meibum from the D−M+ group compared with those in the D+M− group.

Conclusions: To the best of our knowledge, this is the first study to assess the changes in meibum from patients with ocular *Demodex* infestation. The significant increase of OAHFAs in the *Demodex*-positive group suggest that OAHFAs may be associated with the progress of ocular *Demodex* infections.

Translational Relevance: OAHFAs could be a potential new therapeutic target for ocular *Demodex* infestation.

Introduction

The meibomian gland is a special type of fully secretory sebaceous gland located in the eyelids that plays a crucial role in maintaining ocular surface health and stability.[1,2] Meibomian gland dysfunction (MGD) is a common eye disorder associated with abnormal secretion of the meibomian glands.[3,4] Epidemiological studies have shown that about 60% of Asians and 20% of Europeans suffer from MGD, and the prevalence of MGD increases with age.[5] However, the etiology and pathogenesis of MGD still remain unclear.

Meibum is a complex mixture of a wide variety of lipids, and it is synthesized and secreted by the meibomian glands.[6–12] Meibum is released from the orifices of the glands to the ocular surface and is the main source of the tear film lipid layer (TFLL).[3,6,13] Meibum can stabilize and delay evaporation of the tear film, can protect the ocular surface from the influence of microorganisms, and is correlated closely with ocular surface health.[14–16] When MGD occurs, the quality or

quantity of meibum changes, leading to reduced tear film stability, loss of lubrication, and ocular surface epithelial damage, resulting in various symptoms.[17–19]

Demodex is the most common ectoparasite in humans, and its infection rate increases with age.[20] Two different species of *Demodex* are known to live on the human body: *Demodex folliculorum* and *D. brevis*. In the eyelids, the former is mainly parasitic to the hair follicles of the eyelashes, whereas the latter resides deeply in the meibomian glands and feeds on the meibum.[20,21] Previous studies have found that *Demodex* mites can cause microstructural changes in the meibomian glands, with more severe structural damage in MGD.[22] In addition, several studies have shown that the *Demodex*-positive rate in MGD patients is higher than that in the control groups, and the *Demodex*-positive patients experienced more severe meibomian gland loss and ocular surface damage.[23–26] However, the role of *Demodex* infection in changes in the meibum is unclear. The goals of the present study were to analyze the lipid component of meibum in patients with ocular *Demodex* infestation and to identify possible biomarkers for disease progression and therapy.

Material and Methods

Study Population

This study was conducted at the Department of Ophthalmology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. Forty subjects were enrolled from October 2019 to October 2020. The study adhered to the tenets of the Declaration of Helsinki and was approved by the Ethics Committee of Tongji Medical College. Informed consent was obtained from all participants after they were fully informed of the specific methods and possible consequences of the study.

Laser scanning in vivo confocal microscopy (IVCM) was performed on all subjects with the Rostock Corneal Module Heidelberg Retina Tomograph 3 (HRT3 Cornea Module; Heidelberg Engineering, Heidelberg, Germany). After adjusting the focusing plane and depth, three eyelashes each were scanned along the temporal side, central side, and nasal side successively. The number of *Demodex* mites in each hair follicle was recorded and the total number of *Demodex* mites in nine hair follicles was calculated. Considering that *Demodex* is also carried in the normal population and is ubiquitous in adult humans, in this study a total number of ≥ 3 was defined as *Demodex* positive, whereas a total number of <3 within nine follicles was defined as *Demodex* negative.[22,27]

Previous studies have shown that the composition of meibum changes in MGD.[7,28–31] In order to avoid this interference, the *Demodex*-positive group and the *Demodex*-negative group were further subdivided into MGD and non-MGD groups. MGD was diagnosed according to the criteria previously summarized by Tomlinson et al.[32] Thus, these participants were divided into four subgroups: (1) *Demodex*-negative with non-MGD (D−M−; $n = 10$); (2) *Demodex*-positive with non-MGD (D+M−; $n = 10$); (3) *Demodex*-negative with MGD (D−M+; $n = 10$); and (4) *Demodex*-positive with MGD (D+M+; $n = 10$). In addition, young adults 18 to 40 years of age were selected to mitigate concerns of the high incidences of *Demodex* infection and MGD in the elderly population. Subjects were excluded if they had acute inflammation of eye or body and other ocular inflammatory disorders, such as eye trauma, eye deformity scar, exophthalmos, eyelid insufficiency, or ocular surface disorders. Subjects with immune system diseases and other serious systemic diseases were also excluded.

After routine histories were obtained, all patients underwent complete eye examinations, as well as photographic documentation of the entire ocular surface, Ocular Surface Disease Index (OSDI) questionnaire, tear breakup time, Schirmer test, and the number of *Demodex* infestation by IVCM. To evaluate meibum quality, eight glands in the center of the upper lid were evaluated on a scale of 0 to 3 for each gland: 0, clear; 1, cloudy; 2 cloudy with debris (granular); and 3, thick, toothpaste-like (average score range, 0–3). Meibomian gland expressibility was evaluated by applying digital pressure on the upper tarsi. We divided the entire lid range into three areas (nasal, central, and temporal sides) and observed five glands in each area, amounting to a total of 15 glands. The degree of expressibility was graded on a scale of 0 to 3 for each area according to the number of glands expressible: 0, all glands; 1, three or four glands; 2, one or two glands; and 3, no glands (average score range, 0–3).

Chemicals

Methanol (A454-4), acetonitrile (A996-4), and isopropanol (A461-4) were of liquid chromatography–mass spectrometry (LC-MS) grade (Thermo Fisher Scientific, Waltham, MA). Additional chemicals included ammonium formate (17843-250G; Honeywell Fluka, Charlotte, NC); chloroform (LC-MS grade; PanReac, Castellar del Valles, Spain); and formic acid (50144-50, Dimka Pure, Richmond Hill,

NY). Water was purified using a Milli-Q Integral apparatus (MilliporeSigma, Burlington, MA).

Meibum Sample Preparation

After gently squeezing the eyelid margin with a cotton swab or finger, meibum samples were obtained via a metal curette and then placed in brown glass bottles. The samples were dissolved in a chloroform–methanol solvent mixture (2:1, v/v), then air-dried and stored immediately at −80°C until being analyzed. All samples were collected within 3 months. During lipid extraction, each sample was weighed to ensure that equal amount was collected. Probabilistic quotient normalization was used to normalize data and to obtain relative peak areas.[33] The batch effect was corrected using quality control sample-based robust LOESS signal correction (QC-RLSC).[34] The samples were thawed slowly at 4°C, then 80 µL of precooled solution (isopropanol/acetonitrile/H_2O; 2:1:1, v/v/v) and 10 µL of SPLASH Lipidomix Quantitative Mass Spec Internal Standard solution (Avanti Polar Lipids, Alabaster, AL) were then added. The mixture was vortexed for 1 minute, then centrifuged at 4°C for 20 minutes with shaking at 4000 rpm. The supernatant was placed in a vial (1.5 mL). For quality control (QC), a mixture of 10 µL of each sample was used to assess the stability and repeatability of the LC-MS analyses.[35] A LC-MS system consisting of a Waters Aquity 2D ultra-performance liquid chromatography (UPLC) column (Waters Corporation, Milford, MA) and Q Exactive high-resolution mass spectrometer (Thermo Fisher Scientific) was used for lipids separation and detection.

UPLC-MS Analysis

LC conditions included an ACQUITY UPLC CSH C18 column (130 Å, 1.7 µm, 2.1 × 100 mm; Waters Corporation). The mobile phase consisted of solvent A (60% acetonitrile aqueous solution + 0.1% formic acid + 10-mM ammonium formate) and solvent B (10% acetonitrile aqueous solution + 90% isopropanol + 0.1% formic acid + 10-mM ammonium formate) under positive ion mode, and solvent A (60% acetonitrile aqueous solution + 10-mM ammonium formate) and solvent B (10% acetonitrile aqueous solution + 90% isopropanol + 10-mM ammonium formate) under negative ion mode. Gradient elution conditions were set as follows: 0 to ∼2 minutes, 40% to 43% solvent B; 2 to ∼2.1 minutes, 43% to 50% solvent B; 2.1 to ∼7 minutes, 50% to 54% solvent B; 7 to ∼7.1 minutes, 54% to 70% solvent B; 7.1 to ∼13 min, 70% to 99% solvent B; 13 to ∼13.1 minutes, 99% to 40% solvent B; and 13.1 to ∼15 minutes, 40% solvent B. The flow rate was 0.35 mL/min. The column oven was maintained at 55°C. The injection volume was 5 µL.

Under the MS conditions, a Q Exactive mass spectrometer was used to obtain MS1 and MS2 data. The MS scan method was in the range of 200 to 2000 m/z. The MS1 resolution was 70,000, the automatic gain control (AGC) target value was 3e6, and the maximum injection time was 100 ms. Based on the precursor ion intensity, the top three ions were selected for MS2 analysis. MS2 resolution was 17,500, AGC was 1e5, maximum injection time was 50 ms, and stepped normalized collision energies were set as 15, 30, and 45 eV. The parameters for electrospray ionization were as follows: sheath gas of 40 L/min, auxiliary gas flow of 10 L/min, spray voltage (|KV|) of 3.80 in positive ion mode and 3.20 in negative ion mode, capillary temperature of 320°C, and auxiliary gas heater temperature of 350°C. In order to provide more reliable experimental results during instrument detection, random sorting of samples was carried out to reduce systematic errors. Every 10 samples were interspersed with one QC sample for testing.

Statistical Analyses

Differences in demographics and clinical features were assessed with the Mann–Whitney U test or χ^2 test. Based on the χ^2 tests, for $n < 40$ Fisher's exact test was used. Multivariate and univariate analyses were used to screen different lipids among groups. The multivariate methods used were principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA).[36–37] PCA is an unsupervised pattern-recognition method. PLS-DA, a supervised pattern-recognition method, was used to test for differences between features with $P < 0.05$. The univariate analyses included fold-change analysis and Student's t-test. The following conditions were considered statistically significant: (1) variable importance in projection (VIP) of the first two principal components of the PLS-DA model ≥ 1, (2) fold change ≥ 1.2 or ≤ 0.83, and (3) Student's t-test $P < 0.05$.

Results

Forty subjects were involved in this study, and each of the four groups had 10 samples. The characteristics of the study population are summarized in Table 1. There was no statistical significance in age, sex, or race among the groups ($P > 0.05$). The average weights of the 10 samples collected from the D−M− group, D−M+ group, D−M+ group, and D+M+ group were

Table 1. Demographic Information

	D−M−	D+M−	D−M+	D+M+
Number	10	10	10	10
Age (y), mean ± SD	25.2 ± 4.9	27.6 ± 4.6	24.7 ± 4.9	30.7 ± 4.8
Sex (male:female)	3:7	3:7	4:6	5:5
Ethnicity	Chinese (100%)	Chinese (100%)	Chinese (100%)	Chinese (100%)

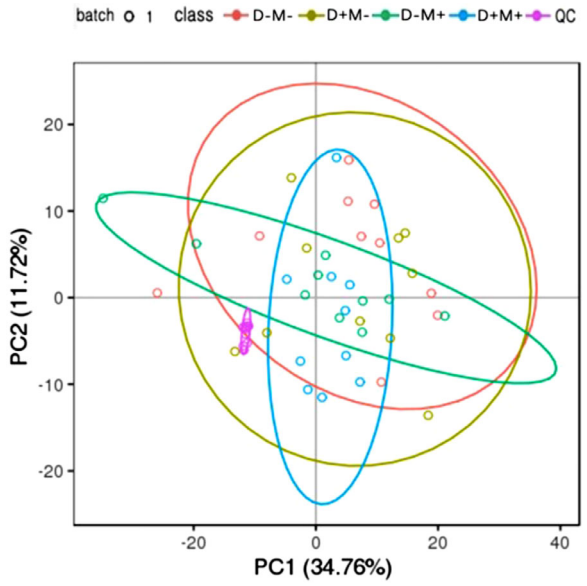

Figure. PCA score plot was obtained from D−M−, D+M−, D−M+, D+M+, and QC samples. The numbers in parentheses show the percentages of the contributions of the first and the second principal components, respectively. PC1, first principal component; PC2, second principal component.

1.2 mg, 1.4 mg, 1.9 mg, and 2.2 mg, respectively. A total of 401 features were detected in the positive and negative modes of the QC samples. In the QC samples with coefficient of variation ≤ 30%, the number of features was 361, accounting for 88.91%. QC samples were used for PCA to evaluate the quality of the experiment. PCA showed that, in the ion model, the clustered QC samples were clustered together, indicating that the LC-MS analysis process satisfied the qualification requirements (Fig.).

There were five significant different features between the D−M− group and the D+M− group (Table 2). The D+M− group had lower levels of phosphatidylcholines (PCs) and lysophosphatidylcholines (LPCs) and higher levels of phosphatidylethanolamines (PEs). The lipids differentially expressed in the D+M− group (LPCs, PCs, and PEs) belong to the class of phospholipids that are present in small proportions in the meibum.

As demonstrated in Table 3, the D+M+ group was associated with a significant increase in the levels of 25 lipids, including (*O*-acyl)-ω-hydroxy fatty acids

Table 2. Lipids Differentially Expressed in Meibum From the D+M− Group Compared With the D−M− Group

Lipid Species	Ratio	*P*
Decreased		
PCs		
42:2	0.16	0.03
LPCs		
16:0	0.77	0.04
18:2	0.69	0.04
Increased		
PEs		
36:0	1.52	0.04
42:2	2.23	0.02

The first number represents the number of C-atoms in the molecule, and the second number represents the number of double bond equivalents in the molecule.

(OAHFAs), ceramides (CERs), diacylglycerols (DGs), and LPCs, as well as significant decreases in the levels of two lipids—including sphingomyelins (SMs) and PCs—compared with the D−M+ group. The most notable difference was an increase of up to 19 different OAHFA species associated with stability of the tear film, but there was no statistical difference in OSDI scores between the two groups.

Fourteen differentially expressed lipids were identified between the D−M+ group and the D−M− group (Table 4). Lower levels of triacylglycerols (TGs), CERs, DGs, and OAHFAs and higher levels of PEs, phosphatidylinositols (PIs), and phosphatidylglycerols (PGs) were observed in the D−M+ group compared with the D−M− group. Meibum quality was poorer in the D−M+ group than in the D−M− group, and OSDI scores were statistically higher in the D−M+ group than in the D−M− group (Supplementary Table S1).

Thirty differentially expressed lipids were identified between the D+M+ group and the D+M− group (Table 5). The D+M+ group had lower levels of TGs, SMs, cholesteryl esters (CEs), and PEs and higher levels of LPCs, LPEs, PCs, and PEs. The meibum quality and meibum expressibility were poorer in the

Table 3. Lipids Differentially Expressed in Meibum From the D+M+ Group Compared With the D−M+ Group

Lipid Species	Ratio	*P*
Decreased		
SMs		
d40:1	0.56	0.03
PCs		
42:2	0.10	0.04
Increased		
OAHFAs		
36:2	1.66	0.03
38:1	1.81	0.04
39:1	1.81	0.01
40:0	2.07	0.16
40:1	1.89	0.01
40:2	1.95	0.02
41:0	1.73	0.03
41:1	1.65	0.03
42:0	1.98	0.01
42:1	1.91	0.01
42:2	1.85	0.03
42:3	1.89	0.01
43:1	1.92	0.05
43:2	1.84	0.01
44:2	1.92	0.04
44:3	1.91	0.01
44:4	1.91	0.01
45:3	2.10	0.01
46:4	2.07	0.01
CERs		
d34:2	1.65	0.04
d37:0	2.37	0.03
d40:1	1.71	0.03
DGs		
32:2	1.65	0.01
38:1	2.21	0.01
LPCs		
30:1	2.84	0.03

"d" in in front of the numbers with colons represents two hydroxyl groups.

Table 4. Lipids Differentially Expressed in Meibum From the D−M+ Group Compared With the D−M− Group

Lipid Species	Ratio	*P*
Decreased		
TGs		
47:0	0.48	0.05
48:0	0.50	0.04
DGs		
32:2	0.35	0.04
CERs		
d32:1	0.46	0.03
d34:2	0.68	0.03
OAHFAs		
34:2	0.13	0.04
36:2	0.48	0.02
38:1	0.57	0.02
48:4	0.53	0.03
Increased		
PEs		
38:4	1.56	0.05
42:2	1.72	0.04
PIs		
38:4	4.51	0.02
PGs		
36:2	3.01	0.03

D+M+ group than in the D+M− group (Supplementary Table S1).

Discussion

In this study, we observed significant differences between the lipid expression in meibum samples from the *Demodex*-positive group and that from controls. This result indicates that *Demodex* exerted a significant effect on meibum composition in the human meibomian gland. In non-MGD groups, including the D−M− group and the D+M− group, normal meibomian gland secretion was found in all subjects, and there were small differences (including five lipids) between the D−M− group and the D+M− group. The lipids differentially expressed in the D+M− group (LPCs, PCs, and PEs) are phospholipids. In past studies, the presence of phospholipids in human meibum has been a matter of debate due to the earlier use of chromatography to report many phospholipid species.[10,11,38] In later studies, they were not reliably detected.[39,40] Recent findings by Saville et al.[41] contributed to the clear identification of phospholipids in this study. In an in vitro study simulating artificial tear film in a model eye, PCs performed better than PEs in maintaining tear film stability[42]; therefore, the decline of PCs and the rise of PEs may reduce the stability of tear film. Based on our grouping criteria, the D+M− group had normal meibum quality and expressibility. Perhaps because of the low proportion of phospholipids in meibum, the changes in the D+M− group had no obvious effect on the physical properties (melting point, fluidity, etc.) of meibum. This is consistent with previous

Table 5. Lipids Differentially Expressed in Meibum From the D+M+ Group Compared With the D+M− Group

Lipid Species	Ratio	*P*
Decreased		
TGs		
54:1	0.51	0.01
55:1	0.36	0.04
56:1	0.25	0.02
57:1	0.36	0.02
57:3	0.28	0.02
58:1	0.27	0.01
58:2	0.37	0.03
58:3	0.27	0.04
59:1	0.30	0.01
59:2	0.36	0.02
60:1	0.24	0.03
60:2	0.25	0.02
60:3	0.26	0.03
61:2	0.30	0.02
SMs		
d40:1	0.51	0.01
d41:1	0.53	0.04
d42:1	0.48	0.01
d42:3	0.51	0.01
CEs		
18:1	0.63	0.01
PEs		
36:0	0.51	0.04
Increased		
LPCs		
16:1	8.69	0.03
18:1	1.58	0.04
18:2	2.18	0.04
LPEs		
18:2	2.45	0.02
PCs		
32:1	3.32	0.05
34:0	2.87	
36:0	1.95	0.02
PEs		
32:1	4.34	0.01

observations that *Demodex* mites are part of the normal skin flora and can be symbiotic with the human body asymptomatically.[43]

However, 27 lipids were significantly different between the D−M+ group and the D+M+ group. It is possible that, after the occurrence of MGD, the meibomian gland is in a pathological state, and its defense ability against bacteria is reduced. Changes in the local environment and secretion characteristics of the meibomian gland also provide a more favorable environment for the reproduction of *Demodex* mites, and the dynamic balance of bacteria flora in the meibomian gland is destroyed, which may aggravate the progression of MGD.

In the present study, the lipids with significant changes in expression were TGs. In the early TFLL model proposed by McCulley et al.,[11] TGs were considered to be transitional lipids, contributing to the bridging between polar and non-polar lipid phases. It has been speculated that, when meibum is exposed to the ocular surface microenvironment, TGs play a similar role in the ocular surface as in skin, releasing lauric acid through the action of bacterial lipase to prevent pathogens and dryness.[44,45] Another study showed that TG levels decreased with increasing severity of dry eye disease.[46] Thus, a decrease in TGs may affect formation of the TFLL and cause ocular surface dryness. In our study, there was a decrease in two kinds of TG species in the D−M+ group compared with the D−M− group. However, up to 14 kinds of TG species were lower in the D+M+ group than in the D+M− group. Therefore, with or without *Demodex* mites, we detected a significant decrease in TG in patients with MGD. TGs might serve as potential biomarkers of MGD.

In a previous computer model of the TFLL, CERs were shown to enhance the surface tension and stability of the TFLL.[47] In sebaceous glands, as a landmark component of intercellular lipids in the stratum corneum, alterations in CER content or some subtypes are closely related to changes in skin barrier function. Zhou et al.[48,49] found that the level of long-chain ceramides decreased significantly in acne patients. In our study, the D−M+ group showed a lower level of CERs than D−M− group, consistent with a previous finding that individuals with poor-quality meibum showed lower levels of CERs than normal individuals. But, there was no statistical difference in the level of CERs between the D+M+ group and the D+M− group. Also, the level of CERs was higher in the D+M+ group than in the D−M+ group. Thus, *Demodex* mites could cause an increase in the levels of CERs.

As an amphiphilic lipid, OAHFA is thought to stabilize the tear film by creating an interface between the non-polar lipid sublayer and the aqueous phase layer.[39,50] An in vitro molecular synthesis study showed that ultra-long-chain OAHFA plays an important role in effective diffusion of the TFLL and has an anti-evaporative effect.[51] OAHFAs may serve as candidate molecular biomarkers of tear film stability in health and disease.[52] Levels of OAHFAs have been found to decrease with increasing severity in patients with dry

eye syndrome.[46] It is possible that changes in the levels of OAHFAs could cause instability of the tear film. In our study, OAHFAs were lower in the D−M+ group than in the D−M− group, which is consistent with past studies, but there was a significant increase in OAHFAs in the D+M+ group compared with the D−M+ group. As in CERs, *Demodex* also could lead to an increase in OAHFAs, and the changes in OAHFAs are more significant than in CERs.

A possible reason for such changes is that when *Demodex* mites damage the meibomian gland and ocular surface the ocular surface function might still be in a compensatory state. To alleviate the instability of the tear film, the meibomian gland makes adaptive changes in the levels of OAHFAs and CERs to relieve ocular surface discomfort. This would explain why there were no statistical differences in ocular surface characteristics between the D+M+ group and the D−M+ group. The new findings also give us ideas for further research.

By comparing the four groups in this study, we found that *Demodex* changed the composition of the meibum. There are several possible mechanisms contributing to this change in composition of the meibum. The movement of bacteria carried by *Demodex* mites and the metabolites of *Demodex* after death can damage the meibomian gland, thereby changing the synthesis of meibum.[53] Also, *Demodex* and bacterial secretions may directly change the composition and proportion of neutral and polar lipids of meibum.[54] *Demodex* mites can carry bacteria into the meibomian gland,[21] and the lipase secreted by the bacteria can degrade the meibum and change its composition, resulting in a release of proinflammatory factor.[55,56]

To our knowledge, this is the first study to examine the effect of *Demodex* on the composition of the meibum. Current investigations on *Demodex* focus mainly on the epidemiological characteristics and the correlation between *Demodex* infestation and ocular surface clinical signs. However, there is a lack of information on the lipidomic changes related to the occurrence of *Demodex*, a topic that is worthy of comprehensive experimental analysis. This study suggests that further investigation of the pathogenic mechanism of *Demodex* in meibomian glands is necessary. The results of this study show that significant changes in the levels of OAHFAs of patients with *Demodex* infestation may provide an important marker for *Demodex* therapy.

There were some deficiencies in our study that should be noted. It is possible that our sample size was too small to identify the significance of this variable. Also, due to the small sample size, we failed to find significant changes in clinical signs of ocular surface simultaneously, but doing so would help to clarify the function of the lipid composition of the ocular surface. In a future study, we will further correlate changes in lipid composition with changes in clinical signs to improve our understanding of the physiological significance of lipid composition, which may help to clarify the role of lipid composition in the ocular surface.

In conclusion, we found changes in lipid composition in patients with *Demodex* infestation. OAHFAs, which increased significantly, could be a potential new therapeutic target for ocular *Demodex* infestation.

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