Longitudinal changes in tear fluid lipidome brought about by eyelid-warming treatment in a cohort of meibomian gland dysfunction[S]

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Abstract Meibomian gland dysfunction (MGD) is a leading cause of evaporative dry eye and ocular discomfort characterized by an unstable tear film principally attributed to afflicted delivery of lipids to the ocular surface. Herein, we elucidated longitudinal tear lipid alterations associated with disease alleviation and symptom improvement in a cohort of MGD patients undergoing eyelid-warming treatment for 12 weeks. Remarkably, eyelid-warming resulted in stark reductions in lysophospholipids ($P < 0.001$ for lyso-plasmalogen phosphatidylethanolamine, lysophosphatidylcholine, and lysophosphatidylinositol), as well as numerous PUFA-containing diacylglyceride species in tears, accompanied by significant increases in several PUFA-containing phospholipids. These changes in tear lipidomes suggest that eyelid-warming leads to diminished activity of tear phospholipases that preferentially target PUFA-containing phospholipids. In addition, treatment led to appreciable increases ($P < 0.001$) in *O*-acyl-ω-hydroxy-FAs (OAHFAs), which are lipid amphiphiles critical to the maintenance of tear film stability. Longitudinal changes in the tear lipids aforementioned also significantly ($P < 0.05$) correlated with reduced rate of ocular evaporation and improvement in ocular symptoms.[JLR] The foregoing data thus indicate that excess ocular surface phospholipase activity detrimental to tear film stability could be alleviated by eyelid warming alone without application of steroids and identify tear OAHFAs as suitable markers to monitor treatment response in MGD.—Man Lam, S., L. Tong, X. Duan, U. R. Acharya, J. H. Tan, A. Petznick, M. R. Wenk, and G. Shui. **Longitudinal changes in tear fluid lipidome brought about by eyelid-warming treatment in a cohort of meibomian gland dysfunction. *J. Lipid Res.* 2014.** 55: **1959–1969.**

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Dry eye syndrome (DES) is a prevalent ophthalmic condition adversely affecting up to 80% of the population over the age of 80, with potential debilitating effects on specific segments of the population such as contact lens wearers, people who have undergone refractive surgeries, postmenopausal women, and patients suffering from a variety of autoimmune disorders (1). DES is a multifactorial disease of the tears and ocular surface caused by a deficiency in tear production or excessive evaporation (2). Regardless of the initiating causes, chronic dryness and the resultant tear film hyperosmolarity leads to inflammation that jeopardizes the structural and functional integrity of the lacrimal gland, meibomian gland, and corneal and conjunctival epithelial tissues. The gradual destruction of

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Abbreviations: CS, cholesteryl sulfate; DAG, diacylglyceride; DES, dry eye syndrome; GIIAPLA$_2$, group IIA phospholipase A$_2$; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; LpPE, lyso-plasmalogen PE; MGD, meibomian gland dysfunction; OAHFA, *O*-acyl-ω-hydroxy-FA; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLA$_2$, phospholipase A$_2$; PLC, phospholipase C; PS, phosphatidylserine; Schir I, Schirmer's I test; TBuT, tear breakup time.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of seven tables and four figures.

these tissues that are major contributors of various tear film components further disturbs tear film homeostasis and results in a vicious cycle of inflammatory events that represents the major pathological mechanism in DES (3). Meibomian gland dysfunction (MGD) is a major cause of dry eye and ocular discomfort (3). MGD is a diffuse condition of the eyelids characterized by progressive obstruction of meibomian gland terminal ducts due to ductal hyperkeratinization or inspissation of secretion (4). In MGD, pathological alterations in the compositions of the meibomian gland secretions, also known as the meibum, the predominant source of lipids for the human tear film, result in the thickening of the meibum, subsequently leading to the blockage of the glandular ducts. The occlusion may also be attributed to excessive colonization by bacterial commensals as well as exfoliated skin materials and crusts as a result of hyperkeratinization of the glandular ducts. These aberrations cumulatively result in a hyposecretion of lipids into the tear reservoir at the lid margins (5).

The currently accepted view of MGD states that the disease is fundamentally a result of (1) a deficiency in meibum and/or (2) abnormal lipids to constitute the tear film lipid layer, either of which will substantially compromise the overall structural integrity of the tear film, leading to reduced tear film stability, loss of lubrication, and damage to the corneal epithelium due to excessive evaporation and the resultant desiccation. In addition, reactive lipid species may elicit inflammation at the ocular surface. For instance, elevated ocular phospholipase A_2 (PLA_2) activity has been shown to exacerbate ocular inflammation in chronic blepharitis and experimental murine dry eye model (6, 7). As the disease progresses, these pathophysiological alterations eventually lead to the emergence of disease symptoms including ocular discomfort and afflicted visual quality (8).

Warm compresses currently represent the most frequently prescribed treatment for patients with MGD or glandular obstruction leading to DES symptoms. The fundamental therapeutic aims of heat therapy are (1) to heat the meibomian gland secretions, thus facilitating their secretion into the tear film; (2) to reduce glandular obstruction; and (3) to increase vascular flow to the tissues surrounding the meibomian glands (9). Eyelid warming is also often used with additional treatment methodologies such as oral (systemic) and topical antibiotics, manual expression of glands, artificial tears, and steroid ointments (5). In the disease state, compositional changes in meibum could lead to elevated melt temperature and enhanced viscosity. Indeed, it has been previously demonstrated that the average lipid order (i.e., stiffness) of meibum for MGD patients is significantly increased compared with that of controls (10). Apart from compromised meibum secretion, the altered composition of the meibum would also lead to considerable changes in viscoelasticity, thus adversely affecting the spreading of the lipid layers after a blink. Warming the eyelid therefore represents one of the earliest therapies for treating MGD, as it is expected that raising the eyelid temperature would lead to conformational changes in the lipid hydrocarbon chains in the meibum, thus increasing the disorder in the packing of these lipids and enhancing the delivery and secretion of meibum out of the glandular ducts. Indeed, numerous studies have demonstrated the temperature-induced elevation in hydrocarbon chain disorder in meibomian lipids (11, 12).

While the biophysical changes in meibum lipids upon warming are well documented, the precise molecular changes in tear lipid composition, if any, that might accompany routine eyelid warming eventually leading to DES symptom alleviation have remained largely unknown. Thus, the primary aim of the current study is to elucidate longitudinal changes in the molecular compositions of tear lipids with eyelid warming over an extended period of routine treatment. A molecular signature associated with disease alleviation and symptom improvement will confer novel insights pertaining to disease pathogenesis and reveal potential markers to monitor disease progression. In addition, longitudinal changes in tear lipid profiles were correlated with improvement in clinical indicators of DES including ocular discomfort and ocular evaporation rate. As the current study focuses on MGD, it would undoubtedly yield a more discerning picture of the contribution by meibomian gland function to the multifactorial syndrome of dry eye per se.

MATERIALS AND METHODS

Clinical cohort and study design

This study involves patients (n = 32) from a 3-month longitudinal study evaluating the effects of eyelid warming in a cohort of MGD patients. Written informed consent was obtained from all participants in the current study. The clinical procedure was specifically approved by the Singhealth Centralised Institutional Review Board (CIRB Ref No.: 2011/197/A) and registered at the ClinicalTrials.gov database (NCT01448369). We adhered to the tenets of the Declaration of Helsinki for all human research conducted in this study. Withdrawal from the study followed the usual good clinical practice in clinical trials. For withdrawn subjects, no data were obtained after the date of the withdrawal. Withdrawn subjects were not replaced.

Patients at the Singapore National Eye Centre dry eye clinic who met the eligibility criteria (supplementary Table I) were invited for screening. Participants were then enrolled with written informed consent obtained by the clinical trial coordinator. Eligible patients (supplementary Table II) were randomly assigned into three respective treatment arms each utilizing a different treatment modality (supplementary Fig. I): traditional method of warm compresses using a hot towel (n = 10); Blephasteam (n = 10); and EyeGiene (n = 12). Blephasteam (Spectrum Théa, France) is an eyelid-warming device available in Europe that can be conveniently used at home (supplementary Fig. IIA). The goggles provide standardized heat of ~38°C to liquefy lipids and humidify the chambers with mineral water to ensure optimal moisture levels. EyeGiene (Eyedetec Medical Inc.) is a self-contained, convenient warm compress system for the eyes (supplementary Fig. IIB). The system is composed of a reusable eye mask and one-time use warmers that are inserted into the eye mask during usage. The warming units are activated by squeezing just prior to usage and deliver

40°C heat for up to 5 min within 30–60 s. The production of heat is based on a sustained thermochemical reaction.

Screening visit was performed at the regular dry eye clinic, and baseline examination was subsequently carried out. Follow-up visits were conducted after 12 weeks of treatment. A window period of ±3 days was permitted for this visit. Tear fluid samples were collected from the right eye of each participant at baseline visit (week 0) and at the end of the treatment period (week 12) using Schirmer's strips as described previously (13). Tear samples collected were frozen immediately and kept at −80°C until further analyses.

Treatment regimes

Patients in each treatment arm carried out routine treatment using the assigned eyelid-warming modality for 10 min each time and for two times a day. All patients were allowed to continue their regular management of MGD in the form of lid scrub with Blephagel. The frequency of use of these measures was monitored in a daily diary, and other types of MGD treatment such as omega-3 tablets, antibiotics or steroid ointments, and the manual expression of meibomian glands were prohibited.

Outcome parameters evaluated

A visual analog scale (VAS) was applied to evaluate DES symptoms as previously described (supplementary Figs. III, IV) (14). The outcome was taken as the change in the global score at week 12 from that at week 0, which was calculated from the discomfort frequency and severity as previously described (14). Other outcome measures include differences in the VAS of visual outcomes (i.e., blurred vision and light sensitivity) (supplementary Figs. III, IV), tear breakup time (TBuT), Schirmer's I test (Schir I), and corneal fluorescent staining. Details on the clinical procedures have been reported previously (15).The severity of MGD was also graded in this study. Microscopic signs of MGD including the presence of misdirected lashes, fragility of lashes, scurf formation, irregularity of meibomian gland orifices, loss of meibomian gland expressibility, formation of plaques, and the number of blocked meibomian gland orifices (i.e., plugs) were recorded. The Yamaguchi grading scheme was used to identify microscopic signs of MGD (16) and essentially indicates the position of the Marx's line relative to the meibomian gland orifices, which has been previously shown to correlate strongly with meibomian gland function (17). Ocular evaporation rate was measured based on infrared thermography in a clinical room setting as reported previously (18).

Lipid extraction and HPLC/multiple-reaction-monitoring analyses

Lipids were extracted from the Schirmer's strips using a modified version of the Bligh and Dyer's method as optimized previously (13). Polar lipids and neutral lipids were analyzed using an Agilent HPLC 1200 system coupled with ABSciex QTRAP 4000 and ABSciex 3200, respectively. Lipidomic analyses were chiefly based on the principle of HPLC/multiple-reaction-monitoring of individual lipid species. The detailed lipidomic platform optimized for human tear fluid analyses have been previously described in details elsewhere (13, 19, 20).

Statistical analysis

One-way ANOVA with post hoc Tukey was first performed to compare the differences in the changes of clinical indices before and after treatment among the three groups (i.e., week 12 − week 0). Following this, paired-*t* comparisons were performed on the tear lipid profiles of a combined group of patients from the three individual treatment arms, obtained at week 0 and week 12 of the study. This would reveal lipid alterations under an extended period of routine eyelid warming. False discovery rate was controlled for based on *q* values calculated using R 3.0.1 (supplementary Table III). Correlation analyses between the changes in individual lipid species/classes with changes in clinical signs following 12-week treatment was performed using Spearman's correlation. Ellipse demarcates 95% confidence region of correlating parameters and lipid species/classes.

RESULTS

No appreciable difference was observed in the clinical outcomes among the three treatment arms at the end of the 12-week period (supplementary Table IV), except for a marginal difference ($P = 0.06$) in the improvement of ocular discomfort using EyeGiene over Blephasteam (supplementary Table IV). The evaluation was based on changes in clinical parameters before and after routine eyelid warming using the respective modality, for a total duration of 12 weeks. The foregoing results thus imply that participants in all three treatment arms essentially received a comparable degree of lid warming throughout the course of treatment considered in terms of clinical outcomes. In other words, while patients in each treatment arm utilized different treatment modalities, the actual treatment received was, in principle, similar among the three groups (i.e., eyelid warming). Therefore, we then grouped together patients from the three treatment arms to evaluate the longitudinal effects of eyelid warming per se on tear lipid profiles over the 12-week treatment period.

Changes in dry eye clinical parameters before and after eyelid-warming treatment

Eyelid warming for 12 weeks resulted in appreciable alleviation of symptoms of ocular discomfort ($P < 0.01$) (**Table 1**, supplementary Table V). The number of plugged orifices were significantly reduced ($P < 0.01$), and there was a noticeable improvement in TBuT ($P < 0.10$), which is in agreement with an earlier study demonstrating that the application of heat to the inner surface of the eyelids on a routine basis leading to steady increases in both TBuT and the number of meibomian glands yielding liquid secretion over a 12-week treatment period (21). On the other hand, no significant changes were observed in Schir I after treatment, which was not surprising because the current MGD cohort did not have discernible lacrimal dysfunction to begin with, even at week 0 (i.e., mean Schir I > 5.5 mm) (supplementary Table II). On another note, eyelid-warming treatment resulted in a reduction in ocular evaporation rate with marginal significance ($P < 0.10$).

Changes in tear lipids before and after eyelid-warming treatment

Routine eyelid warming did not result in a discernible increase in the absolute amount of total lipids in the tear fluid (**Fig. 1A**), which is rather surprising considering the reductions in the number of plugged meibomian glands (see previous discussion) following heat treatment. This

TABLE 1. Changes in ocular symptoms and signs after routine eyelid-warming treatment for 12 weeks

	Week 0	Week 12	*P*
Symptoms			
Ocular discomfort	35.7 ± 3.9	30.7 ± 3.9	[c]
Light sensitivity	23.1 ± 5.3	18.7 ± 4.9	[a]
Blurred vision	25.9 ± 5.2	25.5 ± 5.6	NS
Summed global score	84.7 ± 10.7	74.9 ± 10.7	[a]
Signs			
TBuT	2.5 ± 0.3	4.0 ± 0.8	[a]
Schir I	9.8 ± 1.5	9.6 ± 1.4	NS
Total corneal staining	3.5 ± 0.6	4.1 ± 0.7	NS
Total Yamaguchi's score	5.1 ± 0.6	6.5 ± 0.4	[c]
Blocked glands	18.5 ± 2.0	13.9 ± 2.2	[c]
Ocular evaporation rate	80.6 ± 3.2	71.6 ± 3.6	[a]

Values were presented as means ± SEs. A total of 32 MGD patients successfully completed the entire course of treatment. NS, nonsignificant.

[a] $0.05 < P < 0.10$.

[b] $P < 0.05$.

[c] $P < 0.01$.

[d] $P < 0.001$.

could imply that the relief of meibomian plugs resulted in a restoration of normal lipid turnover, instead of an enhanced amount of lipids at the eyelid margin. In fact, these MGD patients did not have an absolute deficiency in total lipids to begin with because their mean molar concentration of total lipids in tears before treatment (~0.58 μmol ml^{-1}) (Fig. 1A) was comparable to that of a healthy cohort asymptomatic for DES (~0.50 μmol ml^{-1}) as we have previously reported (20).This observation is in good agreement with our previous report that the quality of meibomian lipids, instead of the quantity, might be the actual cause of MGD-associated signs and symptoms of ocular discomfort (20). Indeed, stark changes in the compositions of tear lipids were observed after routine eyelid warming for 12 weeks, as elaborated subsequently.

The most striking changes after lid warming were observed in lysophospholipids (Figs. 1B, **2**). Molar fractions of major lysophospholipid classes, including lyso-plasmalogen phosphatidylethanolamines (LpPEs), lysophosphatidylcholines (LPCs), and lysophosphatidylinositols (LPIs), were reduced by almost half ($P < 0.001$) after treatment, while total lysophosphatidylethanolamine (LPE) also displayed a significant decrease ($P < 0.05$) (Figs. 1B, 2). Remarkably, several individual species of LpPE, LPE, LPI, and LPC were also significantly reduced after treatment (**Fig. 3A–D**), consistent with the overall trends observed for the respective lipid classes. Interestingly, the drastic reduction in total LpPE was paralleled by concomitant increases in total PE (Fig. 1C) and several plasmalogen PE species that possessed a high degree of unsaturation (containing six or more double bonds) (**Fig. 4A**). In addition, numerous diacyl PE, diacyl PC, and diacyl PI species were also significantly increased after the 12-week treatment period (Fig. 4), which corresponded well with the observed reductions in their respective lysophospholipid counterparts. Moreover, similar to plasmalogen PE, various unsaturated PC, PS, and PI species containing two or more double bonds seem preferentially increased after eyelid warming. In addition, while total DAG (Fig. 1D) did not change significantly after the treatment period, levels of individual DAG species containing PUFAs in their structures, namely DAG 16:1/22:5 ($P < 0.05$), DAG 18:0/20:4 ($P < 0.01$), and DAG 18:0/22:6 ($P < 0.05$), were almost halved following eyelid-warming treatment (supplementary Table VI). In addition, DAG 16:0/22:5 and DAG 16:0/22:6 were reduced by ~30% following treatment with marginal significance ($P < 0.10$) (supplementary Table VI). The reductions in the various lysophospholipids and PUFA-containing DAG, together with the concomitant increases in PUFA-containing phospholipid species, indicated attenuation of PLA_2 and PLC activities, respectively, following eyelid-warming treatment. In particular, these phospholipases seem to preferentially target PUFA-containing phospholipid species over their more saturated counterparts.

On another note, molar fractions of major tear film lipid amphiphiles, namely CS ($P < 0.10$) and OAHFA ($P < 0.001$), were noticeably increased following heat treatment (**Fig. 5A**, **B**). Also, the increases in total OAHFA and numerous OAHFA species, including OAHFA 18:1/26:1, OAHFA 18:1/30:2, and OAHFA 18:1/34:1, were significantly correlated ($P < 0.05$) with the reductions in ocular discomfort (Fig. 5C), suggesting that the alleviation of ocular discomfort with treatment was associated with enhanced levels of OAHFA in the tear fluid.

Correlation between changes in the levels of tear lipid amphiphiles with reduction in ocular evaporation rate following heat treatment

The increase in total OAHFA, as well as several other OAHFA species including OAHFA 18:1/25:0, OAHFA 18:1/28:1, OAHFA 16:1/32:1, OAHFA 18:1/30:1, OAHFA 18:1/31:0, OAHFA 18:2/32:1, OAHFA 18:1/32:2, and OAHFA 18:1/34:2, was significantly ($P < 0.05$) correlated with the improvement in ocular evaporation rate following 12-week treatment (Fig. 5D, supplementary Table VII). On the other hand, the reduction in total LpPE, as well as several other LpPE species including LpPE 16:0p, LpPE 18:1p, LpPE 18:0p, and LpPE 20:1p, was significantly correlated ($P < 0.05$) with the improvement in ocular

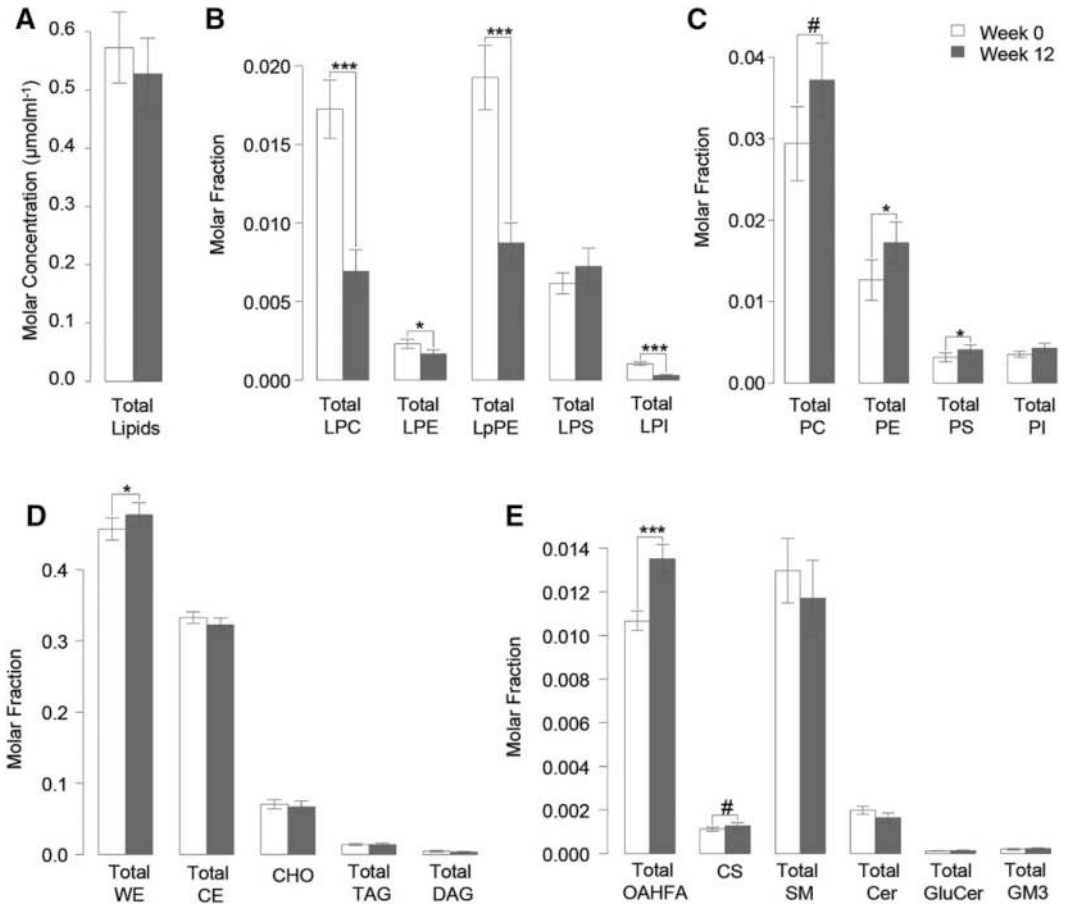


Fig. 1. Changes in tear lipid levels with routine eyelid-warming treatment. Bar plots illustrate the changes in absolute concentration of total lipids (μmol ml^{-1}) (A) and molar fractions of lysophospholipids classes (B), phospholipid classes (C), nonpolar lipid classes (D), and OAHFA, CS, and sphingolipid classes (E) in tears of MGD patients (n = 32) after routine eyelid-warming treatment for a total therapeutic window of 12 weeks. Values were plotted as mean ± SEs. # $0.05 < P < 0.10$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. CE, cholesteryl ester; Cer, ceramide; CHO, free cholesterol; CS, cholesteryl sulfate; DAG, diacylglyceride; GluCer, glucosylceramide; GM3, ganglioside mannoside 3; LPS, lysophosphatidylserines; OAHFA, *O*-acyl-ω-hydroxy-FA; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TAG, triacylglyceride; WE, wax ester.

evaporation rate (Fig. 5E, supplementary Table VII). Similar trends were observed between changes in molar fractions of OAHFA and LpPE with improvement in corneal evaporation rate and scleral (conjunctival) evaporation rates (supplementary Table VII). These observations implied that while OAHFA might have a stabilizing effect on tear film structural integrity, elevated levels of LpPE might compromise tear film stability, leading to an increase in evaporation rate.

DISCUSSION

This is the very first study to investigate longitudinal changes in the tear lipid profiles of MGD patients over a 12-week treatment period. The longitudinal design allows detection of changes in tear lipid profiles corresponding to improvement in ocular symptoms in the same group of individuals over the treatment period, while minimizing confounding factors such as hormonal, genetic, and inter-individual variations inherent in cross-sectional case-control studies. Notably, drastic reductions in various classes of lysophospholipids and appreciable increases in amphiphilic lipids including OAHFA and CS were observed in the tears of patients following eyelid warming.

Involvement of ocular phospholipases in MGD pathogenesis

Group IIA phospholipases A_2 (GIIAPLA_2s) are the most potent mammalian-secreted PLA_2s in terms of antibacterial efficiency against gram-positive bacteria and constitute part of the body's innate immunity as the first-line antimicrobial defense against invading microbes and pathogens (22, 23). The bactericidal properties of GIIAPLA_2 reside in the (1) high positive surface charge of the molecule and (2) its phospholipolytic enzymatic activity (24). In addition, GIIAPLA_2s possess higher binding affinity to anionic phospholipids such as PE compared with zwitterionic PC (the predominant phospholipid constituent of mammalian cell membranes) (23), therefore ensuring the preferential

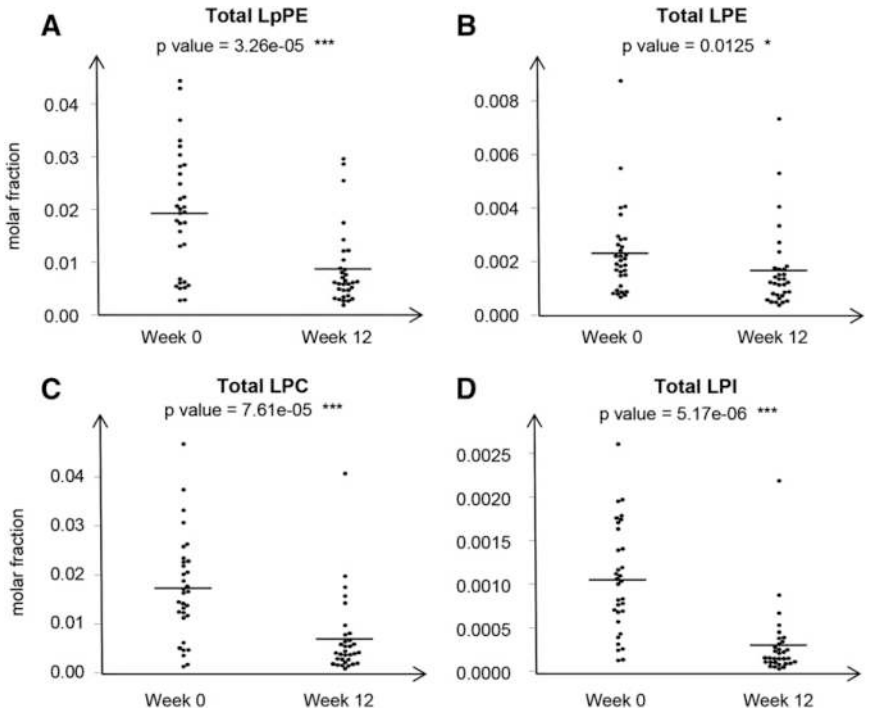


Fig. 2. Changes in lysophospholipid classes in tears with eyelid-warming treatment. Dot plots illustrate the molar fractions of total LpPE (A), LPE (B), LPC (C), and LPI (D) in individual MGD patients (n = 32) before and after the 12-week eyelid-warming treatment. # $0.05 < P < 0.10$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

elimination of bacterial membranes over eukaryotic host tissues (25).

As a consequence of their bactericidal properties, high levels of $GIIAPLA_2$ expression could be found at the various potential routes of pathogenic entry throughout the body, such as the corneal and intestinal mucosal epithelia. The presence of $GIIAPLA_2$ has been previously reported in the human main lacrimal glands and tears (26). In particular, the human tear fluid has been shown to contain one of the highest amounts of $GIIAPLA_2$ among other human secretions, with an estimated concentration of 54.5 ± 33.9 μg ml^{-1} (27). The human lacrimal tissues have been found to contain two distinct acinar cell types each specialized for the production and secretion of lysozyme and $GIIAPLA_2$, respectively (26). The cells expressing $GIIAPLA_2$ have been found in comparatively smaller numbers and localized mainly in central regions of the lobules in main and accessory lacrimal glands (27).

Aho and colleagues (28) have demonstrated an almost 2-fold increase in $GIIAPLA_2$ levels in the tears of dry eye patients compared with age-matched healthy controls. The increase in $GIIAPLA_2$ might serve to compensate for the compromised antibacterial activity in the tear fluid, as a result of concomitant decreases in tear lysozyme and lactoferrin with DES, the major bactericidal proteins normally found in tear fluid (29). In addition, it has been found that the levels of tear $GIIAPLA_2$ were almost doubled in the tear fluid of chronic blepharitis patients compared

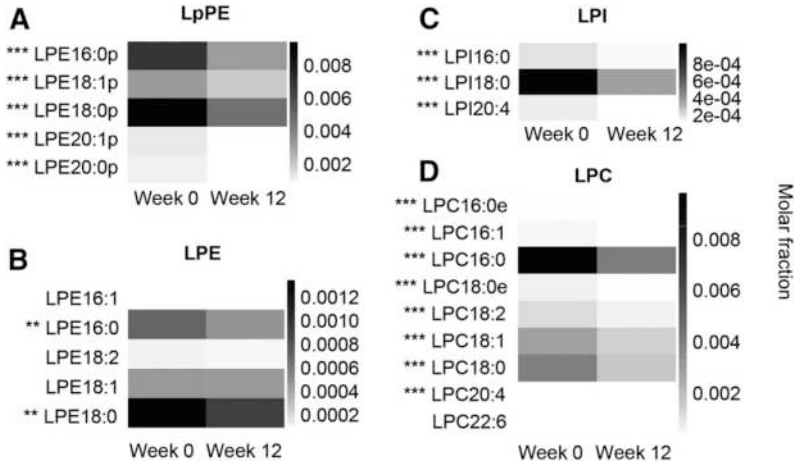


Fig. 3. Changes in individual lysophospholipid species with eyelid-warming treatment. Heat maps illustrate molar fractions of individual species of LpPE (A), LPE (B), LPI (C), and LPC (D) at week 0 and week 12 of the study. # $0.05 < P < 0.10$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

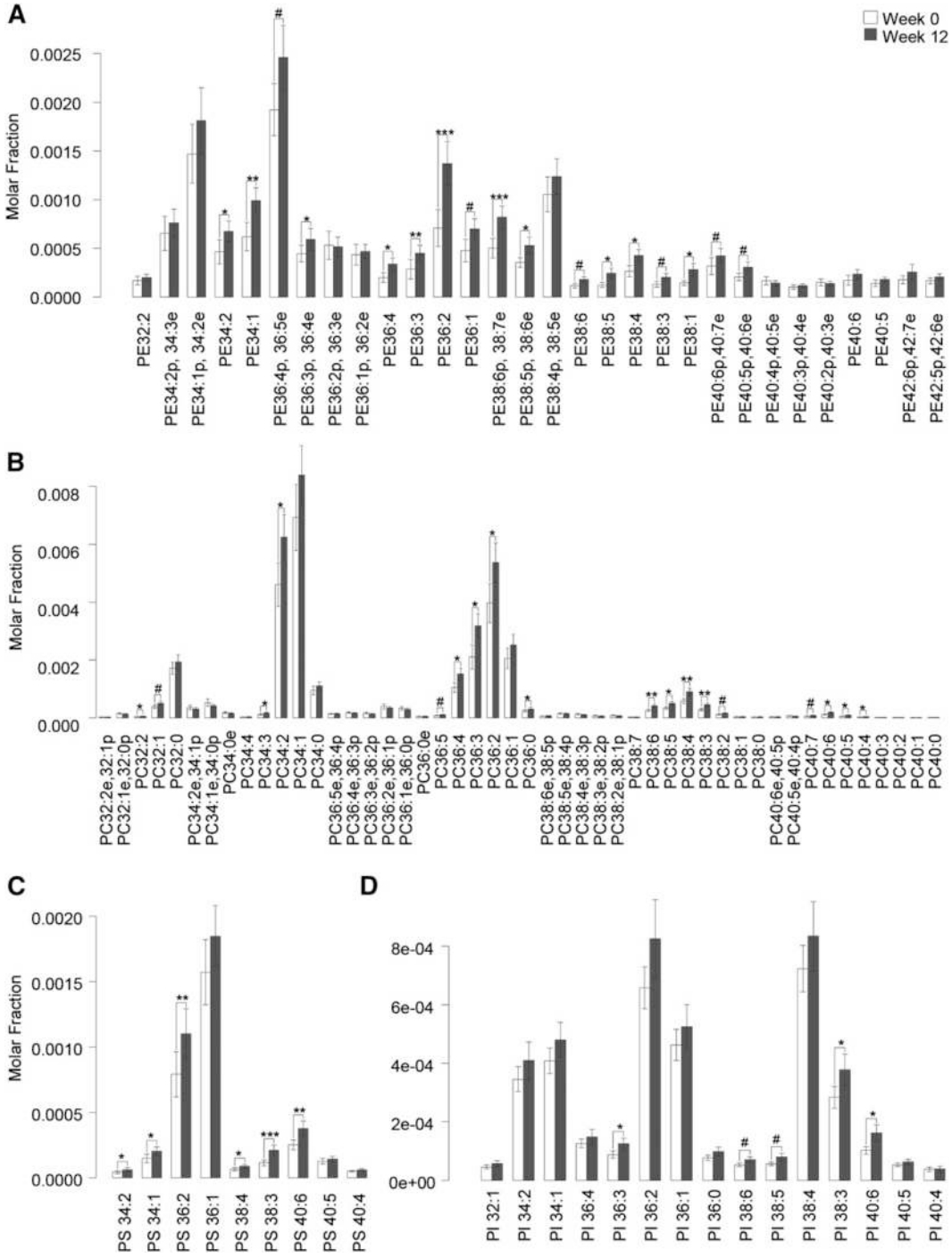
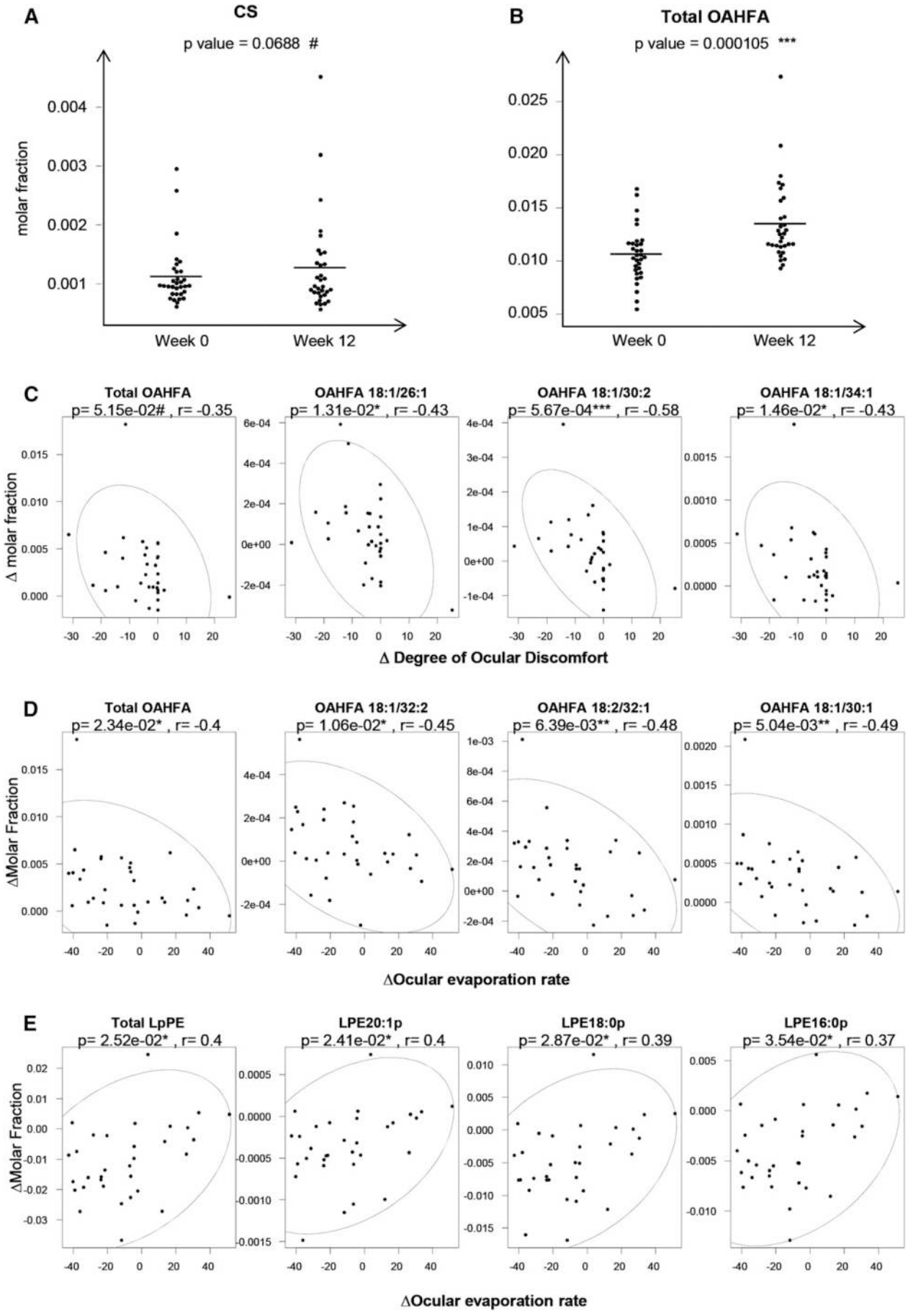


Fig. 4. Changes in individual phospholipid species with eyelid-warming treatment. Bar plots illustrate the molar fractions of individual species of PE (A), PC (B), PS (C), and PI (D) in the tear samples of MGD patients (n = 32) at week 0 and week 12 of the study. Values were plotted as mean ± SEs. # $0.05 < P < 0.10$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

with controls, and the authors have verified that the elevated GIIAPLA$_2$ in tears displayed preferential hydrolysis of PE over PC by an approximated ratio of 3:1 (30).

In corroboration with earlier works on ocular phospholipases (28, 30), we observed that the levels of major lysophospholipids in tears were reduced by almost half after the treatment period, suggesting that ocular PLA$_2$ activity was diminished following routine eyelid warming. The notable reductions in various lysophospholipids following eyelid-warming treatment, without application of other medications such as steroids or antibiotics, are reported for the first time in the current study. We surmise that the diminished PLA$_2$ activity with eyelid warming might be attributed to the relief of intraglandular pressure within meibomian ductal systems via facilitating the melting and outflow of meibum from the obstructed orifices with heating. In obstructive MGD, the elevation in intraglandular pressure imposes considerable mechanical stress on the


A
CS
p value = 0.0688 #
molar fraction
0.004
0.003
0.002
0.001
Week 0
Week 12
B
Total OAHFA
p value = 0.000105 ***
0.025
0.020
0.015
0.010
0.005
Week 0
Week 12
C
Total OAHFA
p= 5.15e-02# , r= -0.35
OAHFA 18:1/26:1
p= 1.31e-02* , r= -0.43
OAHFA 18:1/30:2
p= 5.67e-04*** , r= -0.58
OAHFA 18:1/34:1
p= 1.46e-02* , r= -0.43
Δ molar fraction
Δ Degree of Ocular Discomfort
D
Total OAHFA
p= 2.34e-02* , r= -0.4
OAHFA 18:1/32:2
p= 1.06e-02* , r= -0.45
OAHFA 18:2/32:1
p= 6.39e-03** , r= -0.48
OAHFA 18:1/30:1
p= 5.04e-03** , r= -0.49
ΔMolar Fraction
ΔOcular evaporation rate
E
Total LpPE
p= 2.52e-02* , r= 0.4
LPE20:1p
p= 2.41e-02* , r= 0.4
LPE18:0p
p= 2.87e-02* , r= 0.39
LPE16:0p
p= 3.54e-02* , r= 0.37
ΔMolar Fraction
ΔOcular evaporation rate

ductal and acinar epithelia of gland tissues, which could possibly activate mitogen-activated protein kinase activity leading to the downstream release of chemokines and cytokines, therefore eliciting the ocular inflammatory cascade, as has been previously demonstrated in conjunctival and corneal epithelial tissues (31, 32). The induction of extracellular PLA_2 secretion from various cell types under an inflammatory milieu has also been extensively documented (33–37). Therefore, the release of PLA_2 from meibomian gland acinar and ductal epithelia as a result of elevated intraglandular pressure might represent a plausible mechanistic pathway for the enhanced levels of lysophospholipids observed in the tears of MGD patients. Consequentially, the relief of plugged orifices with eyelid warming could remove the stimulus for the increased PLA_2 secretion, leading to normalization of lysophospholipids levels. Nonetheless, it remains to be determined whether the meibomian gland tissues could secrete PLA_2 in response to mechanical induction.

Alternatively, the mechanical stress could be communicated from the meibomian gland acinar epithelia to the corneal and conjunctival epithelial tissues, as well as the lacrimal gland epithelia, which collectively lead to an enhanced release of PLA_2. The expression and production of PLA_2 in the cornea (6), conjunctiva, and lacrimal glands (37) has been well documented. In fact, the ocular epithelial tissues could be regarded as a continuum, and the lacrimal gland and meibomian gland simply denote invaginations of this continuous epithelium (38). Swartz and colleagues (39) have previously shown that mechanical stress could be communicated between different cell types to bring about comprehensive matrix remodeling in the airway system. It remains to be elucidated, however, whether the mechanical stress imposed on meibomian epithelial tissues in obstructive MGD could be conveyed to lacrimal epithelia that subsequently leads to the production of aqueous tears with elevated levels of PLA_2, as has been observed in tear samples from dry eye (28) and chronic blepharitis patients (30).

Regardless of the precise mechanistic details, the increased release of PLA_2 enzymatic products (i.e., lysophospholipids and FFAs) to the ocular surface could substantially compromise tear film stability, in view of the detergent-like properties of the highly polar lysophospholipids (40). Indeed, we observed significant correlation between reductions in the levels of LpPE with improvement in ocular (both corneal and scleral) evaporation rate, indicating the detrimental effects that LpPE might exert on tear film structural integrity. In addition, the specific increases in highly unsaturated plasmalogen PE after the treatment period implied that such species might be preferentially targeted during MGD pathogenesis. Such highly unsaturated plasmalogen PE species contain PUFAs, such as arachidonic acid (FA20:4) and docosahexaenoic acid (FA22:6) at their *sn*-2 positions. Furthermore, our data also indicated that PLC might exhibit the same trend as PLA_2 in selectively targeting PUFA-containing phospholipids to produce DAGs during MGD pathogenesis, as shown by the appreciable decreases in PUFA-containing DAGs such as DAG 18:0/20:4 and DAG 18:0/22:6 following heat treatment. Upon release, arachidonic acids could be converted to downstream inflammatory mediators including prostaglandins and leukotrienes that further fuel the sustained inflammatory cycle commonly observed in DES (41). Also, the presence of leukotriene B_4 and platelet-activating factor has been reported in human tears (41). Accordingly, the reductions in these proinflammatory metabolite precursors with eyelid warming would be expected to alleviate symptoms of ocular discomfort.

Nonetheless, the consistent decreases in LPE, LpPE, LPC, and LPI with heat treatment suggest that PLA_2 other than $GIIAPLA_2$, which has a specific preference for PE over PC, might participate in MGD pathogenesis. Indeed, cytosolic PLA_2 with high selectivity for phospholipids that possess an *sn*-2 arachidonic fatty acyl group, which hydrolyze PE and PC with equal efficiency (6), have also been found to be expressed in the corneal and conjunctival tissues (6). Moreover, the contribution by bacterial phospholipases to the disease phenotype (i.e., elevated lysophospholipids) observed at the ocular surface should also be considered. The clearance of stagnant meibum would remove potential sources of nutrients for ocular commensals. Furthermore, increased lipid turnover would also promote the elimination of commensal bacteria, which are known to proliferate in obstructed gland ducts (42), via drainage through lacrimal puncta or onto peripheral lid skin. Therefore, a diminished level of commensal bacteria (and bacterial phospholipase activity) might also partially account for the observed reductions in lysophospholipids following eyelid-warming treatment in relieving meibomian plugs.

Meibum-derived amphiphilic lipids in maintaining tear film stability

The relief of obstructed meibomian orifices would release the stagnated meibomian oils onto the eyelid margin, thereby facilitating lipid removal by bulk flow and increasing lipid turnover at the eyelid margins. In our previous work, we have postulated that the lipid anomalies associated with MGD and DES might have resulted primarily from diminished lipid turnover, instead of an absolute deficiency in lipids per se (20), which is in accordance

Fig. 5. Clinical relevance of amphiphilic lipids to MGD. Dot plots illustrate the molar fractions of total CS (A) and OAHFA (B) in individual MGD patients (n = 32) before and after the 12-week eyelid-warming treatment. Following 12-week eyelid-warming treatment, increases in the levels of total OAHFA and numerous individual OAHFA species were significantly and negatively correlated with the alleviation of ocular discomfort (C); increases in total OAHFA and numerous OAHFA species were significantly and negatively correlated with improvement in ocular evaporation rate (D); and reductions in total LpPE and numerous LpPE species were significantly correlated with decreasing rate of ocular evaporation (E). Ellipse indicates region within 95% confidence interval of the correlating parameters. # $0.05 < P < 0.10$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

with our current observation that eyelid warming and disease symptom alleviation were not associated with enhanced absolute amounts of lipids in tears. The increased lipid turnover would facilitate delivery of fresh meibomian and lacrimal secretions onto the ocular surface, while removing toxic contaminants and other metabolized products and reactive lipid species (43). Thus, the appreciable increases in amphiphilic lipids, such as CS and OAHFA, in the tears of MGD patients might be associated with increased delivery of fresh meibomian and lacrimal secretions onto lid margins following eyelid warming.

The higher levels of lipid amphiphiles brought about by routine lid warming would exert positive effects on tear film structural integrity and stability, as indicated by the increase in TBuT following treatment. Interestingly, significant correlations were found between the increases in total OAHFA, as well as numerous OAHFA species, with alleviation in the degree of ocular discomfort after treatment. In addition, increases in the levels of several OAHFA species were also significantly correlated with improvement in ocular evaporation rates after treatment. These implied that OAHFAs could serve as suitable indicators of treatment response and ocular symptom improvement. This finding is in good agreement with our previously reported observation that OAHFAs displayed consistent decreases with increasing DES severity (15) and further strengthens the validity of OAHFAs as appropriate indicators of DES/MGD pathogenesis and severity.

Limitations and future work

While the foregoing lipidomic data indicate attenuated activities of various ocular phospholipases (i.e., PLA_2, PLC) brought about by eyelid warming in MGD patients, enzymatic assays to verify the activities of these ocular phospholipases in human tears were not performed due to the limiting amounts of tear fluids (typically in the range of < 10 µl per sample) that can be obtained from each patient. Nonetheless, elevated activities or levels of ocular phospholipases have been extensively demonstrated in human patients and animal models of dry eye (6, 7, 28, 30, 35, 37, 44). Suppressing the release of lipid mediators liberated from membrane phospholipids via topical application of steroids represents a common treatment for alleviating ocular inflammatory diseases such as the dry eye. Nonetheless, the inhibitory effect of steroids is nonspecific, and patients may suffer from undesirable side effects resulting from perturbed activity of ocular phospholipases that partake in normal phospholipid metabolism (6). In the current study, eyelid-warming therapy has been shown to curtail excess phospholipase activity and disease symptoms on its own without application of additional therapeutic agents. In addition, based on the changes in tear lipidome profiles, the phospholipases implicated seem to preferentially target PUFA-containing phospholipids. It will be interesting to delineate in future the origin (mammalian or bacterial) and identities of the phospholipases involved in MGD pathogenesis, as well as to decipher how elevated intraglandular pressure serves to enhance the activity of these phospholipases at the ocular surface in an appropriate animal model of MGD, which is beyond the scope of the current study.

CONCLUSION

This study reports, for the first time, a comprehensive tear lipid signature associated with alleviation of meibomian gland obstruction and ocular symptom improvement for a relatively homogenous cohort of MGD patients receiving eyelid-warming treatment over a period of 12 weeks. Remarkably, we observed stark reductions in tear lysophospholipids and PUFA-containing DAG at the end of the treatment period, which were associated with improvement in ocular evaporation rate. This observation points to the plausible involvement of ocular phospholipases in the pathogenesis of MGD and indicates that eyelid warming could itself serve to curtail excess phospholipase activity in MGD without external application of steroids, possibly by relieving intraglandular pressure and facilitating lipid turnover at the eyelid reservoir. In addition, the levels of numerous OAHFA species, deemed as critical amphiphilic components in maintaining tear film stability (13, 45), were significantly increased at the end of the treatment period. Importantly, the increase in OAHFA was associated with reductions in both the ocular evaporation rate and the degree of ocular discomfort. Thus, monitoring changes in the levels of tear fluid OAHFA could be effective indicators of MGD progression. Furthermore, the positive association between OAHFA and ocular symptom alleviation suggests that OAHFA might be suitable therapeutics for treatment of DES.

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