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Changes of tear lipid mediators after eyelid warming or thermopulsation treatment for meibomian gland dysfunction

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ABSTRACT

Meibomian gland dysfunction (MGD) represents a major cause of dry eye and ocular discomfort. Lipid mediators, often termed oxylipins, can be produced enzymatically or non-enzymatically, and may modulate inflammatory processes in MGD. Here, we aimed to assess the longitudinal changes of lipid mediators after various eyelid treatments (eyelid warming and thermopulsation) over 12 weeks. Secondly, we aimed to assess the chirality of mono-hydroxyl lipid mediators from tears of MGD and healthy participants.

Tears lipid mediators were extracted from Schirmer's strips and levels were quantified by liquid chromatography mass spectrometry (LC–MS) techniques. We quantified 33 lipid mediators in the tear, 18 of which (including 11-HETE, 20 − OH-LTB_4, and 15-oxoETE) were reduced significantly after treatment. Changes in concentrations of 10-HDoHE ($r = 0.54$) and 15-oxoETE ($r = 0.54$) were correlated to the number of meibomian gland plugs at baseline, so increased severity of MGD was associated with treatment-induced change in lipid mediators.

The chiral analysis demonstrated that 5(*S*)-HETE, 12(*S*)-HETE, 15(*S*)-HETE, 14(*S*)-HDoHE, 17(*S*)-HDoHE and 11(*R*)-HETE were produced with significant enantiomeric excess (ee %) in controls compared to patients, due to enantiomer selective enzymatic action, whereas most lipid mediators were racemates in patients, due to dominance of oxidative effects which have no enantiomeric preference. Treatment of MGD restored the concentrations of 15(*S*)-HETE, 14(*S*)-HDoHE and 17(*S*)-HDoHE with significant ee values, suggesting reduction in oxidative action. Overall, MGD therapy reduced pro-inflammatory molecules generated by lipoxygenase and oxidative stress.

1. Introduction

Dry eye syndrome (DES) is a common chronic inflammatory disease that causes ocular discomfort, fatigue, and visual disturbances, interfering with reading, computer use, driving, and other aspects of quality of life [1–3]. DES affects up to approximately one-third of the population, and Asian populations may have greater predisposition [4,5].

Meibomian gland dysfunction (MGD) represents a major cause of dry eye and ocular discomfort [6]. MGD is defined as "a commonly characterized by terminal duct obstruction and/or qualitative/quantitative changes in the meibomian glandular secretion." Deficient meibum and abnormal lipids in the tear film lipid layer lead to reduced tear film stability, excess tear evaporation, loss of lubrication, and damage to the corneal epithelium [7,8,9]. Our previous study has reported the detection and quantification of tear lipid mediators, and their dysregulation in MGD and dry eye [10]. Another previous study

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identified of pro-inflammatory (Prostaglandins and Leukotriene B_4) and pro-resolving lipid mediators (D-series Resolvins, Protectin D1, and Lipoxin A_4) in human emotional tears [11]. Many of the inflammatory mediators are synthesized through lipoxygenase (LOX), cyclooxygenase (COX) and cytochrome-p450 (CYP-450) activity, however, besides the enzymatic route of biosynthesis, oxidized lipids can also be formed non-enzymatically through the interaction of polyunsaturated fatty acid (PUFA) with reactive oxygen species (ROS) [12]. The oxidation mechanism can involve radical or non-radical species, such as singlet molecular oxygen. This excited species can be produced under stress and inflammatory conditions by reactions that involve biological peroxides [13].

Oxidative stress has also been reported to play an important role in ocular diseases including ocular surface inflammation and dry eye disease [14,15]. Oxidized lipids such as lipid mediators may be mediators of oxidative stress in these conditions. In a biological system like the ocular surface, both enzymatic and nonenzymatic oxidations will contribute to enantiomer formation to variable degrees [16–21]. In the tearfilm, previous studies have not separated enzymatic from non-enzymatic contribution to the tear lipid mediators detected.

Warming the eyelid represents the most basic clinical therapy for MGD by reducing glandular obstruction and reducing tear evaporation [22]. A recent clinical trial has found effectiveness of eyelid warming [23] for the treatment of MGD in an Asian population, including the clinical efficacy of lipiflow, a form of thermopulsation [24,25]. However, the effect of eyelid warming and lipiflow treatments on biosynthesis of lipid mediators upon DE has not been reported thus far. It is possible with treatment, less inflammatory lipid mediators may accumulate in the tears, and this may explain the improved symptoms or other benefits of treatment.

Here, we aimed to assess the longitudinal changes of lipid mediators with eyelid warming and lipiflow treatments over 12 weeks. Secondly, we aimed to assess the chirality of monohydroxyl lipid mediators from healthy and dry patients tear and consequently the proportion of enzymatic and non-enzymatic lipid mediators.

2. Materials and methods

2.1. Chemicals

All 48 non-chiral and 7 chiral (*S* & *R*) endogenous lipid mediator standards and 25 internal standards were purchased from Cayman Chemical (Ann Arbor, MI). Deionized water was obtained from a MilliQ purification (Millipore, USA). Acetonitrile (ACN), methanol (MeOH), and Isopropanol (IPA) were obtained from Fisher Scientific (Hampton, USA). Acetic was obtained from Fisher Scientific (Waltham, MA). Standard curves and internal standard solutions were prepared for different two analytical methods: for general analysis of lipid mediators and chiral analysis of selected monohydroxy fatty acid compounds as reported in detail previously [10,26,27].

2.2. Study design and participants

This study was approved by the SingHealth Centralised Institutional Review Board, and it adhered to the tenets of the Declaration of Helsinki. The single-center investigator-masked interventional study was registered at the Clinicaltrials.gov database (NCT01683318 and NCT01448369). The samples were obtained from 14 healthy and 53 patients that participated in the clinical trials evaluating the effects of eyelid warming (n = 18) or thermopulsation treatment (lipiflow) (n = 35) a cohort of MGD patients diagnosed at the Singapore National Eye Center. Patients who met the eligibility criteria were recruited and written informed consent was obtained from all subjects before the examination. Follow-up visits were conducted after 12 weeks of treatment.

2.3. Treatment regimes

Participants were treated with eyelid warming involves one of 3 kinds of heating done daily for 3 months (warm towel, blephasteam or eyegiene) [23] or a single 12-minute session of lipiflow [24]. All participants were allowed to use eye lubricants. The use of steroids, antibiotics, and anti-inflammatory eye drops such as Restasis were not allowed as specified under the inclusion criteria.

2.4. Outcome parameters and procedures

The following clinical parameters were measured in a standardized way: the change in the clinical irritation scores, the non-invasive keratograph tear breakup time (NIKBUT), Schirmer's I test, the change in corneal fluorescein staining, and the number of plugged meibomian gland openings after 3 months of treatment from baseline values. The NIKBUT was evaluated using the Keratograph K5M (Wetzlar, Germany) and these outcome parameters and procedures of DES evaluation had been previously described [28]. Patients' irritation symptoms were recorded using the Symptom Assessment iN Dry Eye (SANDE) questionnaire as previously described [29]. Tear evaporation rate was measured based on infrared thermography in a clinical room setting as reported previously [30]. The severity of MGD was also graded using microscopic signs of MGD; to show loss of Meibomian gland expressibility, formation of plaques, and the number of blocked meibomian gland orifices (i.e., plugs) were recorded as previously described [31].

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 [32]. Tear samples collected were frozen immediately and kept at −80 °C until further analyses.

2.5. Lipid mediators extraction and HPLC/multiple-reaction-monitoring analyses

Lipid mediators extraction from Schirmer's strips was performed using the method optimized previously [10]. The detailed validation for lipid mediators distribution along the wetted length of the strips, background noise of the Schirmer's strip and comparing different tear collection techniques were reported previously [10]. Lipid mediators were analyzed using an Agilent high-performance liquid chromatography (HPLC) 1290 (Agilent, Santa Clara, USA) system coupled with Agilent 6495 triple-quad mass spectrometer (Agilent), respectively. Lipid mediators analyses were chiefly based on the principle of HPLC/multiple-reaction-monitoring of individual lipid species.

Quantification of lipid mediator concentration was performed with the aid of various commercial endogenous lipid standards. Standard curves and internal standard solutions were prepared; first stock solutions were diluted in methanol to a final concentration of 2 μg/mL. From these stock solutions, a combined calibration curve solution for was diluted to obtain calibration curve range, 0.002 − 834 ng/mL. Lower limit of quantification (LLOQ), signal to noise (S/N) ratio and extraction recovery were also determined for profiling of lipid mediators. For chiral lipid mediator analysis, 18 chiral oxylipin standards were pooled into a single stock solution with an individual analyte concentration of 1.2 μg/mL and a final diluted calibration curve range of 0.17–342.9 ng/mL having a minimum of 6 calibration curve points (**Table S-2**). In addition, before data acquisition, a system suitability test was performed to ascertain instrument performance. The details of human tear lipid mediators analyses have been previously described elsewhere [10].

2.6. Targeted chiral analysis of lipid mediators using Liquid-Chromatography-Mass Spectrometry

The chiral liquid chromatography was performed on CHIRALPAK AD-RH column (2.1 mm × 150 mm, 5 μm, Daicel Cooperation, France). The mobile phase was a gradient of solvents A (water with 0.1 % of acetic acid) and B (acetonitrile/isopropanol 90:10) with flow rate of 0.6 mL min-1. The gradient was initiated with 40 % of A, and changed linearly to 2 % in min 6.3, and to 0 % in min 6.4. The column was then washed with solvent B for 1 min and equilibrated to initial conditions for another 1 min. A XevoTQ-S mass spectrometer (Waters, Milford, MA) was used to detect all compounds using electrospray ionization (ESI) in negative mode. The general MS parameters were set as follows: desolvation temperature: 600 °C; capillary voltage: -2,3 kV; desolvation gas (L/hr): 1000.

2.7. Statistical analysis

The main outcome of the study was the concentration of each lipid mediator (or the ratio of enantiomers) in the tear. 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. Enantiomeric excess (ee) was calculated using the formula: where S and R are the peak areas of the respective enantiomers.

$$ee = \frac{(S - R)}{(S + R)} \times 100$$

One-way ANOVA with post hoc Tukey was first performed to compare the differences in the changes of clinical indices before and after treatment.

3. Results

No appreciable difference was observed in the change in all the tear levels of lipid mediators among the different modalities of eyelid warming and lipflow treatments at the end of the 12-week period. Therefore, we combined samples from all the treatments to evaluate the longitudinal effects of treatment on tear lipid mediators profiles over the 12-week treatment period.

3.1. Dry eye clinical parameters before and after treatment

Treatments for 12 weeks reduced the number of plugged orifices significantly, and resulted in appreciable alleviation of symptoms of ocular discomfort ($P < 0.01$) (Table 1). On the other hand, there were no significant changes observed in NIKBUT and corneal staining after treatment, with marginal reduction in tear evaporation rate ($P < 0.1$).

Table 1
Changes in ocular symptoms and signs after routine eyelid-warming treatment for 12 weeks (n = 37).

	Before	After	p value
Non-invasive keratography break up time (s)	2.32 ± 0.13	2.48 ± 0.12	0.32
No. of plugged glands of upper eyelid	4.83 ± 6.02	1.75 ± 2.62	*0.002*
Total corneal staining	2.22 ± 0.48	2.51 ± 0.61	0.52
Global symptom score (VAS)	46.2 ± 3.277	35.45 ± 3.41	*< 0.001*
Ocular evaporation rate (W/m^2)	56.8 ± 3.99	45.28 ± 3.24	*0.08*

Values were presented as means ± SEs.

Table 2
Tear lipid mediator levels before and after treatments (n = 37).

PUFA[1]	Lipid mediators[2] [ng/mL]	Before treatment (Mean ± SD)	After treatment (Mean ± SD)	Fold change (%)	Paired p value (Before vs After)[3]
AA	15-HETE	15.41 ± 15.98	4.72 ± 3.39	−69.3	*
	12-HETE	7.69 ± 5.24	3.35 ± 4.53	−56.4	*
	5-HETE	5.43 ± 5.83	2.11 ± 2.21	−43.1	*
	11-HETE	10.24 ± 15.45	3.32 ± 8.36	−67.5	**
	8-HETE	5.08 ± 6.48	3.61 ± 1.71	−28.9	0.23
	9-HETE	3.76 ± 3.52	3.84 ± 5.43	2.39	0.91
	LTB_4	1.79 ± 2.41	0.66 ± 0.80	−62	*
	20-OH-LTB_4	1.61 ± 2.52	0.42 ± 0.43	−73.9	**
	6-*trans*-LTB_4	1.05 ± 4.40	0.91 ± 1.15	−13.3	0.32
	PGE_2	2.45 ± 10.70	1.86 ± 8.34	−24.1	0.33
	$PGF_{2\alpha}$	0.73 ± 1.43	0.79 ± 1.29	8.21	0.21
	TxB_2	0.66 ± 1.03	0.36 ± 0.49	−45.4	0.07
	15-oxoETE	6.59 ± 6.99	3.12 ± 3.99	−52.6	**
	1112-DiHETrE	0.25 ± 0.40	0.12 ± 0.14	−52	*
	1415-DiHETrE	0.33 ± 0.46	0.18 ± 0.17	−45.4	*
	5,6-DiHETrE	0.23 ± 0.29	0.19 ± 0.20	−17.3	0.33
	515-DiHETE	1.09 ± 1.43	0.96 ± 0.94	−11.9	0.82
DHA	20-HDoHE	9.13 ± 9.84	4.03 ± 2.75	−55.8	**
	17-HDoHE	1.90 ± 2.97	0.71 ± 0.71	−62.6	*
	16-HDoHE	5.01 ± 5.38	1.69 ± 1.41	−66.2	**
	8-HDoHE	2.14 ± 3.68	0.65 ± 0.75	−69.6	*
	14-HDoHE	2.29 ± 2.67	0.96 ± 0.63	−58	*
	10-HDoHE	2.14 ± 2.85	0.66 ± 0.63	−69.1	*
	11-HDoHE	1.31 ± 2.02	0.93 ± 0.24	−34.8	0.13
	4-HDoHE	0.84 ± 1.50	0.57 ± 0.36	−32.1	0.08
	13-HDoHE	0.94 ± 1.43	0.71 ± 0.47	−24.4	0.23
EPA	12-HEPE	0.79 ± 1.17	0.54 ± 0.34	−31.6	0.12
	18-HEPE	1.11 ± 1.98	0.78 ± 0.44	−29.7	0.12
DGLA	8-HETrE	1.81 ± 2.58	0.59 ± 0.60	−67.4	**
	15-HETrE	13.05 ± 14.34	6.11 ± 5.96	−53.1	*
	EPA	88.10 ± 1003.17	94.12 ± 612.51	6.83	0.6
	DHA	359.99 ± 340.25	569.09 ± 435.44	16.6	0.9
	AA	246.69 ± 399.52	123.16 ± 242.39	−27.6	0.07

[1]Compounds are categorized by their parent polyunsaturated fatty acid (PUFA): arachidonic acid (AA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and dihomo-γ-linolenic acid (DGLA). [2] Concentrations of all lipids in ng/mL. The data were presented as means ± SD. n; number of subjects. [3] * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. diHETrE: Dihydroxyeicosatrienoic Acid, HDoHE: Hydroxydocosahexaenoic Acid, HEPE: Hydroxypentaenoic Acid, HETE: Hydroxyeicosatetranoic Acid, Acid, LT: Leukotriene, PG: Prostaglandin, and Tx: Thromboxane.

3.2. Changes in tear lipid mediators after treatment

Using a comprehensive LC–MS/MS-based targeted lipid mediator profiling approach, we analyzed the changes in the tear lipid mediators changes in patients with DES before and after treatment. The tear concentrations of lipid mediators at baseline and after treatments are shown in Table 2. Tear lipid mediators are identified with help of identical endogenous standards for comparing full product ion spectra and retention time. Thirty three lipid mediators were detected and successfully quantified based on the analytical merits; such as linearity, LLOQ and extraction efficiency (Table S**1, Fig. S1**). The lower limit of quantitation for each analyte was defined as the lowest signal obtained with S/N ≥ 7 (0.01–1.5 ng/mL) and the method offers good linearity for average all analytes (R^2 value = 0.98) (Table S**1 and S2**).

Changes in tear concentrations of 10-HDoHE (r = 0.54) and 15-oxoETE (r = 0.54) were correlated to the number of Meibomian gland

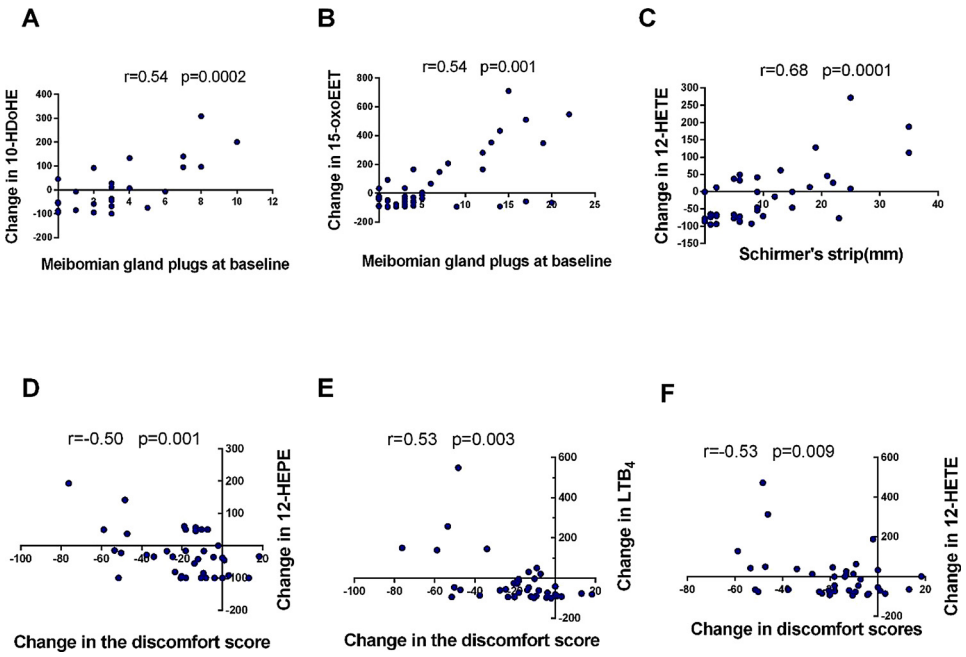

Fig. 1. Scatter diagram showing relationship between tear lipid mediators and discomfort score and Meibomian gland plugs. **A** and **B:** changes of 10-HDoHE and 15-oxoETE were directly correlated to Meibomian gland plugs at baseline. **C:** tear level of 12-HETE was correlated to Schirmer's test. **D-F:** change in the tear concentration of the 12-HEPE, LTB_4 and 12-HETE were inversely correlated to change in discomfort scores. r; correlation coefficient. p; values indicated significance of the correlation. Changes of lipids calculated as, (after treatment-before treatment*100/before treatment). Change in the discomfort score calculated as irritation score (after treatment-before treatment).

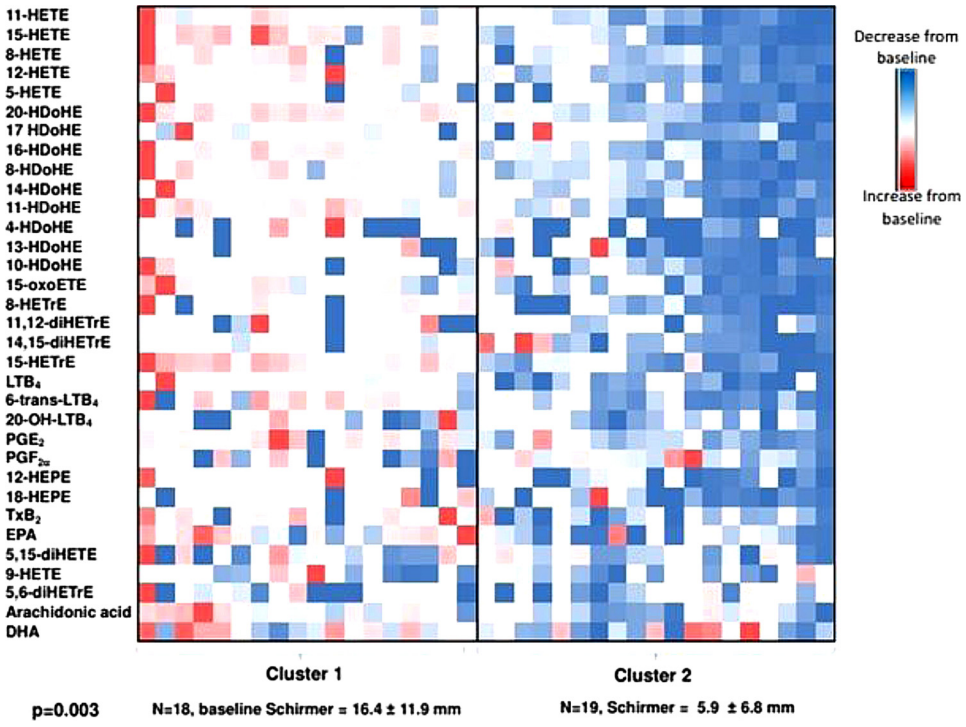

Fig. 2. The heat map shows the clustering of patients based on change of lipid mediator into 2 clusters. Blue indicates a reduction in the level of lipid mediators after treatment (a darker blue indicates a greater decrease), whereas red indicates increase in the level of lipid mediators after treatment (darker red indicates greater increase) and a white color indicates no change after treatment. Each column represents one patient and each row represents one lipid mediator that was evaluated.

plugs at baseline. The greater the number of plugs at baseline, the more the reduction of these 2 tear lipid mediators (Fig. 1 **A,B**). The change in concentration of 12-HETE was directly correlated to the baseline Schirmer test results (Fig. 1 **C**). This suggests that a low Schirmer at baseline was more likely to be associated to a reduction in the tear 12-HETE levels after treatment.

The change in the tear concentration of the 12-HEPE was significantly inversely correlated to change in the discomfort score (r = -0.50) (Fig. 1 **D**). Since 12-HEPE is a proresolution lipid an increase in levels over time may be associated with improvement (reduction) of symptoms. For some reason, the concentration of LTB_4 (r = -0.53) and 12-HETE (r = -0.53) were also significantly correlated to change in discomfort scores (Fig. 1 **E, F**).

Changes in levels of all lipid mediators tested did not correlate significantly with other clinical signs such as age, baseline symptoms, TBUT or staining severity (data not shown). Using unpaired T tests, the changes in all the lipid mediators were not significantly different between male and female participants (all $p > 0.05$).

Eighteen of the tested lipid mediators were reduced significantly in patients after 12-week treatment compared to patients at baseline (Table 2). Among which were 9 AA derived lipid mediators, 7 DHA derived lipid mediators, 2 Dihomo-γ-linolenic acid–derived lipid mediators. None of PUFAs were significantly altered. The most striking changes after treatment were observed in mono-hydroxyl class of lipid mediators including HETEs lipid mediators derived from AA, and metabolites synthesized from DHA including HDoHEs; these were

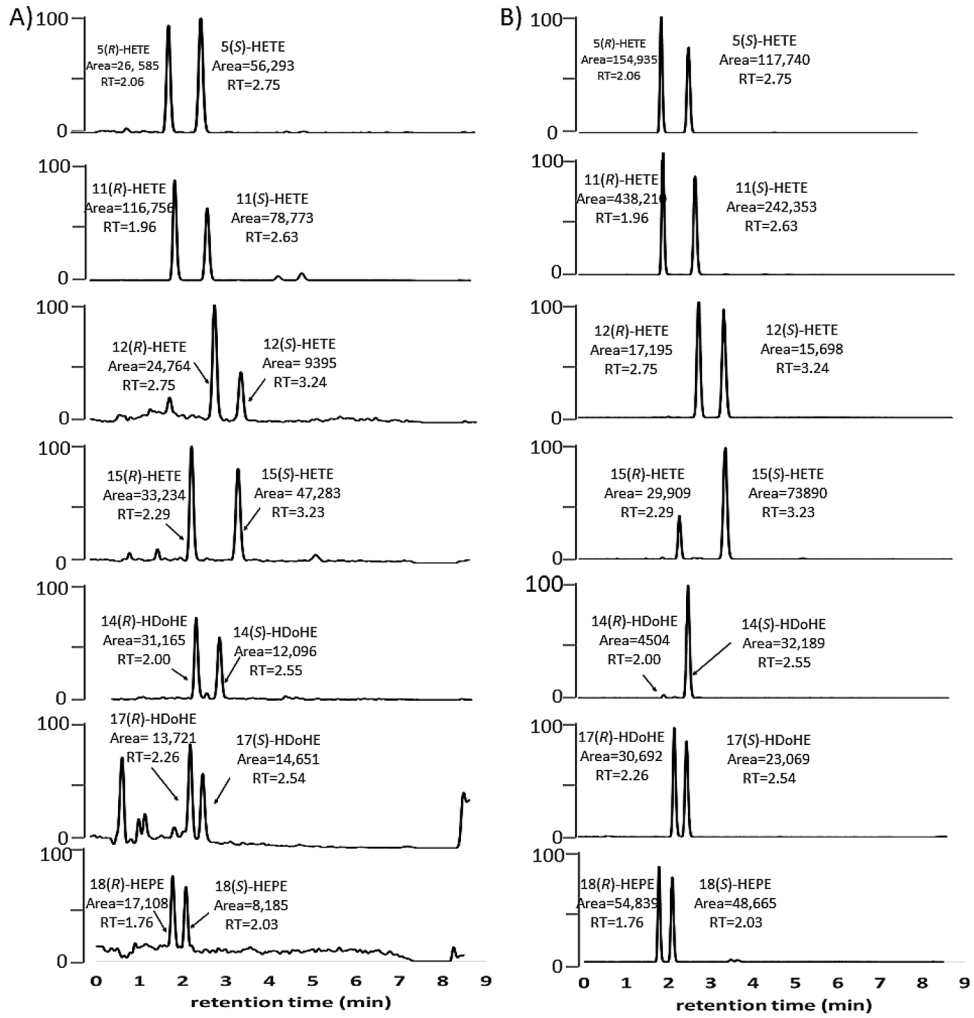

Fig. 3. Chiral LC–MS/MS chromatograms from the analysis of tear lipid mediators and standards. Shown are examples for the enantiomeric resolution of monohydroxy metabolites from **(A)** tear sample and **(B)** endogenous standards. Labels show *S, R* enantiomer. RT; retention time.

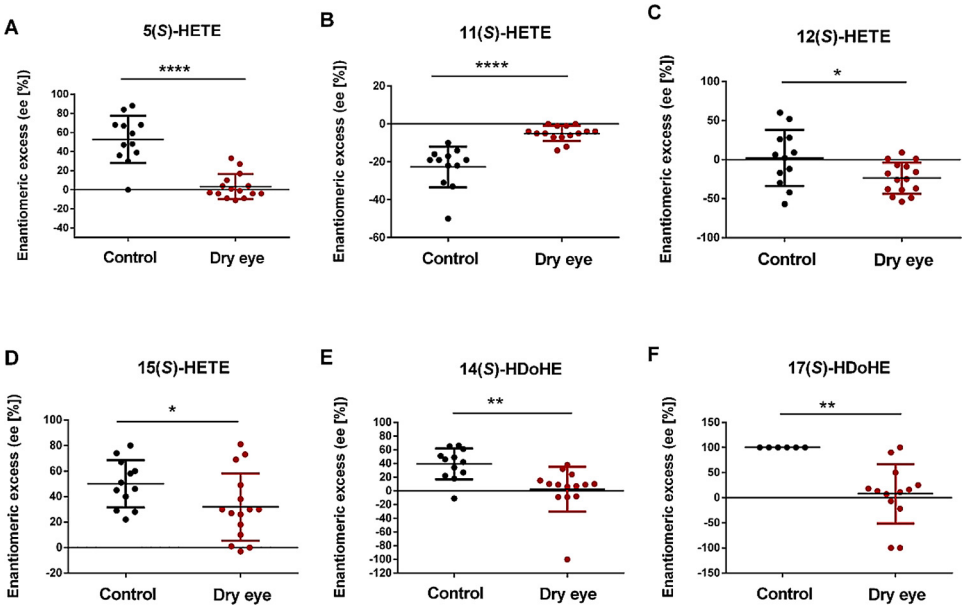

Fig. 4. The dot plots showing the different enantiomeric excess (ee %) levels in the tear between patients (before treatment) and control. A positive ee indicates excess of the (*S*)-enantiomers over (*R*)-enantiomers whereas negative ee indicates the opposite **A.** 5(*S*)-HETE **B.** 11(*S*)-HETE **C.** 12(*S*)-HETE **D.** 15(*S*)-HETE, **E.** 14(*S*)-HDoHE **F.** 17(*S*)-HDoHE. The data were presented as means ± SD. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

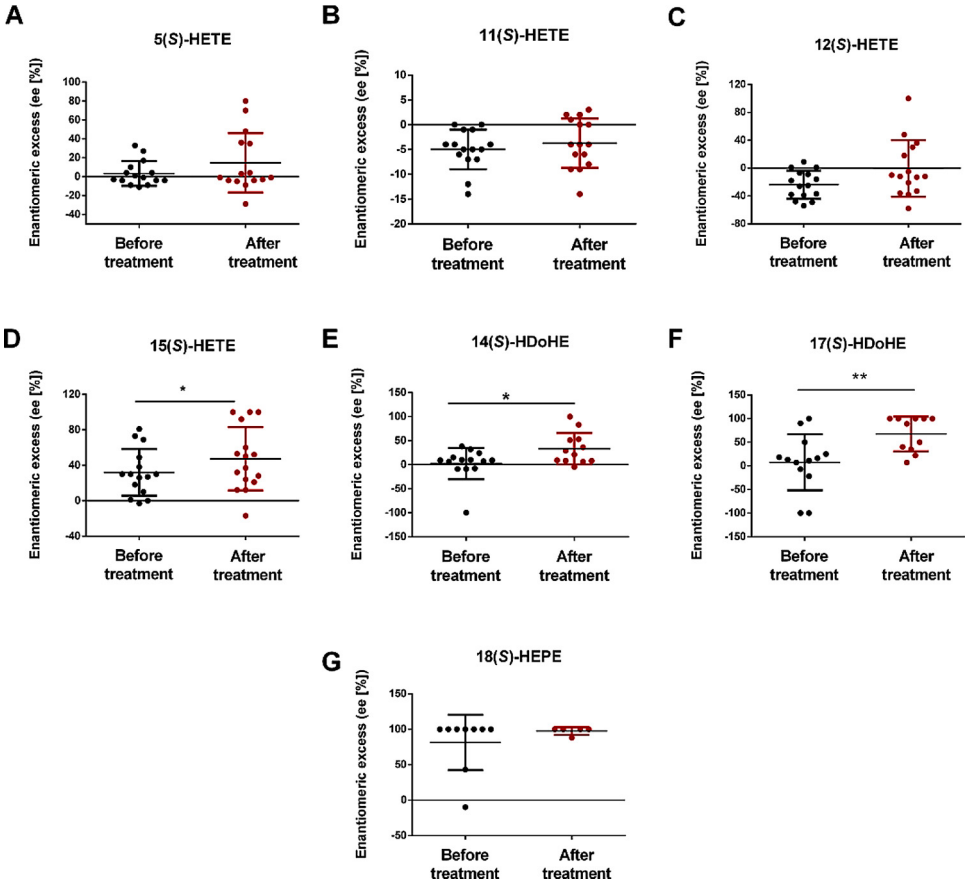

Fig. 5. Dot plots showing enantiomeric excess (ee %) in the tear between patients before treatment and after treatment. A positive ee indicates excess of the (*S*)-enantiomers over (*R*)-enantiomers whereas negative ee indicates the opposite **A.** 5(*S*)-HETE **B.** 11(*S*)-HETE **C.** 12(*S*)-HETE **D.** 15(*S*)-HETE, **E.** 14(*S*)-HDoHE **F.** 17(*S*)-HDoHE **G.** 18(*S*)-HEPE. The data were presented as means ± SD. * $p < 0.05$, ** $p < 0.01$.

significantly reduced in response to treatment (Table 2).

When the above analysis was repeated with a different method of investigation that is potentially more sensitive on sixteen participants with sufficient samples [26,27], twenty-one of the lipid mediators were found to be significantly reduced by treatment (**Table S3**). Reassuringly, lipid mediators were altered in the same direction and in similar magnitudes in both experiments after treatment.

In the present study, we identified two cluster of patients based on the amount of change in the tear lipid mediator level after treatment (blue: reduction, red: increase). Here, half the patients (cluster 2) showed a reduction in most tested lipid mediators especially in pro-inflammatory lipids/stress marker and the other half of patients (cluster 1) show no marked changes in tear lipid mediators, with one patient showing increase in most of the tested lipid mediators (Fig. 2). There was no difference in clinical parameters between the two clusters (**Table S4**), except for a significantly lower baseline Schirmer test values in the cluster that had greater reduction in lipid mediators. This significance difference in Schirmer test results between the 2 clusters remained after adjustment for age ($p = 0.003$).

3.3. Targeted chiral analysis of lipid mediators

In this study, the tear concentration of chiral lipid mediators has been assessed, including that of 5-HETE, 11-HETE, 12-HETE, 15-HETE, and 14-HDoHE, 17-HDoHE and 18-HEPE from healthy participants and MGD patients. Representative chiral chromatograms from human tear and endogenous *S*- and *R*- standards are shown in Fig. 3; which assistance to identify *S*- or *R*- enantiomer in tear. Our results show that (*S*)-HETEs, (*S*)-HDoHEs and (*S*)-18-HEPE were the major enantiomers present in healthy human tears, while 11(*R*)-HETE was more abundant than 11(*S*)-HETE (Fig. 4).

The enantiomeric excess (ee %) of *S* over *R* were calculated for detectable compounds and a positive ee indicates excess of the (*S*)-enantiomers over (*R*)-enantiomers whereas negative ee indicates the opposite. The ee values of 6 targeted lipid mediators were significantly higher in healthy human compared to dry eye patients; for example, the monohydroxy products of DHA evidenced enantioselectivity, producing 100 % 17(*S*)-HDoHE in the control, identifying the 12/15-LOX enzyme as the synthetic source of these compounds. In contrast, most monohydroxy metabolites were present as racemates in the dry eye patients (Fig. 4). There was no difference in age between control and dry eye patients ($p = 0.34$). The data therefore suggest that the ocular surface in dry eye may have been more exposed to oxidative stress, which would produce equal proportions of *R* and *S* enantiomers.

We also performed chiral analysis of lipid mediators in the tear samples obtained before and after treatment. The chiral analysis showed that the proportion of chiral enantiomers (*S* to *R* ratio) were altered in some compounds after treatment. The ee % values of 15(*S*/*R*)-HETE, 17(*S*/*R*)-HDoHE and 14(*S*/*R*)-HDoHE were increase significantly after treatment (Fig. 5). On the other hand, the proportion of enantiomers of 12(*S*/*R*)-HETE shown a marginal significance ($p = 0.08$) after treatment (Fig. 5).

4. Discussion

In this study, we found several inflammatory lipid mediators to be reduced after eyelid warming treatments. However, there were no significant differences between the levels of mediators between treatments at any of the time points. In addition, clinical parameters such as severity of MGD and Schirmer test may influence the type of lipid mediator and extent of reduction of the lipid mediator in the tear after treatment. For example, changes in tear concentrations of 10-HDoHE and 15-oxoETE were moderately correlated to number of occluded meibomian glands prior to treatment. The technique we used was able

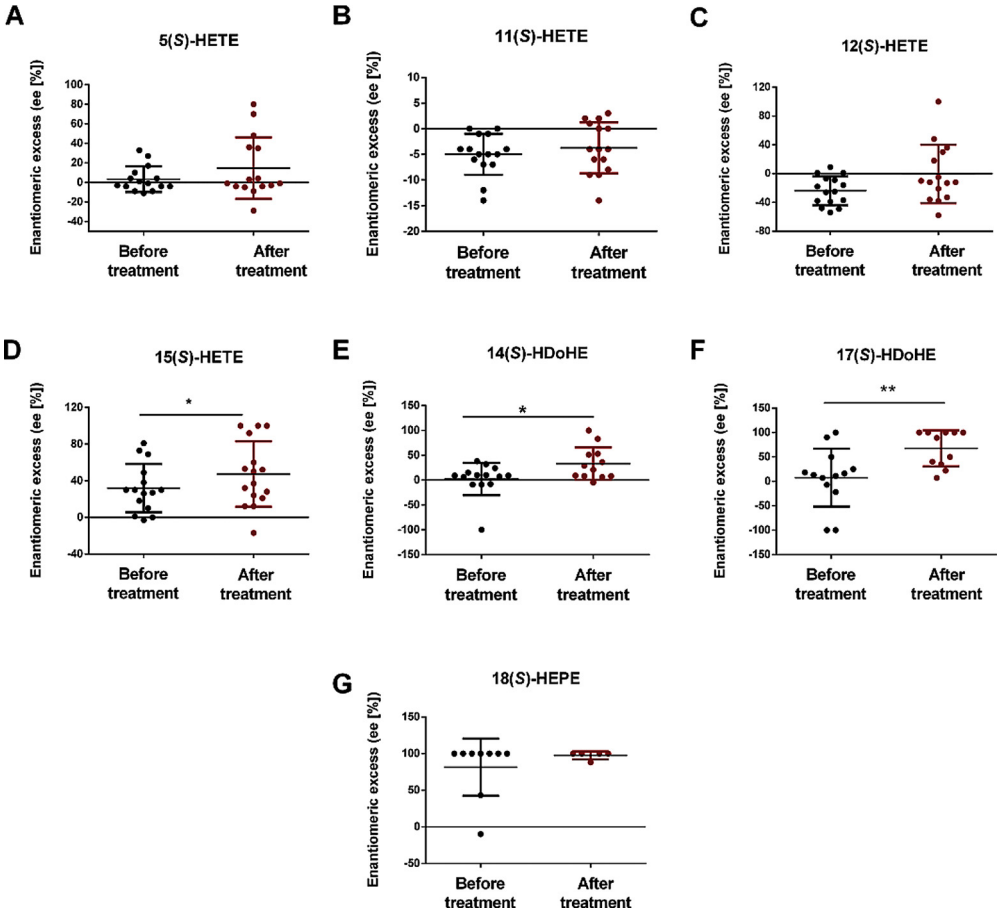

Fig. 6. Lipid mediators biosynthesis cascade. Metabolites of pro-inflammatory and pro-resolution are colored in red and blue respectively. ROS products are colored in orange. Poly unsaturated fatty acids (PUFA) and their catalyzers (enzymes) are colored in green and purple respectively. Arachidonic acid (AA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and dihomo-γ-linolenic acid (DGLA), diHETrE: Dihydroxyeicosatrienoic Acid, HDoHE: Hydroxydocosahexaenoic Acid, HEPE: Hydroxypentaenoic Acid, HETE: Hydroxyeicosatetranoic Acid, LT: Leukotriene, PG: Prostaglandin, PUFA: Polyunsaturated Fatty Acids, and Tx: Thromboxane.

to distinguish the enantiomeric excess of the isomeric compounds with high specificity and sensitivity, allowing us to segregate lipid mediators produced by enzymatic processes (chiral products) from those formed by oxidative stress (racemic products). The lipid mediators 5(*S*)-HETE, 11(*R*)-HETE, 12(*S*)-HETE, 15(*S*)-HETE, 14(*S*)-HDoHE and 17(*S*)-HDoHE were produced with significant enantiomeric excess in healthy human tear compare to dry eye patients. In contrast, most mono-hydroxy metabolites were present as racemates in the dry eye patients. However, the proportions of chiral enantiomers were significantly altered after treatment in 15(*S*)-HETE, 14(*S*)-HDoHE and 17(*S*)-HDoHE. The enzyme-catalysed formation of 5-HETE, 12-HETE, 15-HETE, 17-HDoHE, and 14-HDoHE produced *S* enantiomers, whereas 11-HETE metabolites from enzymatic catalysis produced *R* enantiomers [26,33]. An excess of these enantiomers would imply mainly enzymatic contribution compared to the racemic situation, where *S* and *R* were equally present.

So far none of reported studies have evaluated longitudinal effects of treatment. Previous cross sectional study using ELISA, higher tear PGE_2 and lower PGD_2 levels were found in dry eye patients compared to the controls. Also, the PGE_2/PGD_2 ratio was positively associated with the severity of dry eye symptoms [34]. Our recent publication has reported that higher tear 5-HETE, LTB_4, 18-HEPE, 12-HEPE and 14-HDoHE levels were associated with poorer MG expressibility [10]. A previous report has also found increased tear levels of 12-HETrE to be associated with dry eye in humans [35].

Previous studies have shown that LOX, CYP-450 Epoxygenase and non-enzymatic (ROS) mechanisms produce lipid mediators in dry eye, corneal epithelial defect and other ocular surface diseases [10,35,36]. In our current study, several metabolites derived from LOX, CYP-450 and oxidative stress were changed significantly after treatment. In dry eye and inflammatory states, there may be upregulation of enzymes like LOX, which produced increased lipid mediators [37]. When the occlusion of Meibomian glands has been relieved, the inflammation may be reduced, and so the enzymatic activity returns to normal. In the cases of reduced tear stability or evaporative dry eye, there may be reduced ocular surface protection. This results in higher levels of ROS formation on the surface of the eye, and after restoring the lipids in the tear via eyelid warming, there may be reduced ocular surface damage, resulting in reduced ROS formation. For these two reasons, there could be alteration of lipid mediators after treatment, related to enzymatic and non-enzymatic mechanisms.

In our previous report on eyelid-warming treatment for MGD, eyelid warming leads to diminished levels of lysophospholipids compared to baseline [38]. Because lysophospholipids can be metabolized to arachidonic acid and other metabolites [39,40], the reduction of lysophospholipids due to eyelid warming can lead to reduction in formation of many lipid mediators. This may explain at least in part the reduction of several lipid mediators.

In biological system, ROS initiates a non-enzymatic process which mediated formation of lipid mediators with no stereo-selectivity [33] (Fig. 6). Our results suggest that with reduced retention of the lipids, there may be less oxidative stress. Previous studies shows that detection of non-enzymatic species, such as 5(*R*)-HETE, 8(*R*)- and 8(*S*)-HETEs, 9(*R*)- and 9(*S*)-HETEs, 11(*S*)-HETE, 12(*R*)- HETE, and 15(*R*)-HETE, which could not be formed by enzymatic processes, suggest the presence of oxidative stress [41–43]. However, ocular surface stress may induce CYP450 enzymes that generate *R* enantiomers, and in addition, one LOX and COX enzymes can also generate *R* monohydroxy products.

4.1. Clinical significance

The inhibition of lipid mediators released 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 [44]. Our previous study has shown that eyelid-warming therapy reduced phospholipase activity and that may limit the precursor of lipid mediators [38]. The result of the current study shows that eyelid-warming therapy/thermopulsation can reduce pro-inflammatory and ROS-derived lipid mediators using eyelid treatments, which are safer methods of intervention than corticosteroids.

Our current and previous studies suggest that LOX derived metabolites were involved in DES [38]. Selective inhibition of LOX is possible using currently available drugs [45], which suggests that such drugs may also be used in DES. When such drugs are used for DES, tear lipid mediator levels may be used to select candidates for treatment, or to monitor effects of treatment. Our previous study shows that three lipid mediators (5-HETE, LTB_4 and 18-HEPE) were able to predict clinical obstruction of meibomian glands and may represent evaporative dry eye or MGD [10]. The lipid mediator 5-HETE may potentially be a point-of-care assay device for clinical patients, using ELISA for quantification of 5-HETE, at a low cost compared to mass spectrometry.

5. Conclusion

In the present study we have provided detailed characterization of the tear lipid mediators profiles in patients with DES/MGD. Using a targeted lipidomics approach we demonstrated that tear lipid mediators profiles were altered in patients after treatment. In the present investigation, we found that therapy of MGD reduced the tear levels of many of the lipid mediators, especially pro-inflammatory molecules and oxidative stress markers. Patients with MGD benefit from eyelid therapy as they may achieve greater reduction of inflammation. The chiral racemic mixture analysis shows the ocular surface of patients to be more affected by oxidative stress than healthy individuals, and we have shown that restoration of the enantiomeric profile can be achieved by MGD treatment.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prostaglandins.2020.106474.

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