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Intersubject and interday variability in human tear and meibum lipidomes: a pilot study

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Abstract

Purpose Our aim was to quantitate day-to-day changes in the tear and meibum lipid profile of individual subjects in a pilot study of healthy humans. **Methods** Matched tear and meibum samples were obtained from four subjects on three consecutive days. Quantitative lipid profiles of human basal tears and meibum were compared using multivariate analysis by principal components. **Results** Substantial differences in the lipid profile between subjects were observed, while lipid profiles were steady across the three consecutive days of sampling. Multivariate principal component analysis demonstrated that lysophosphatidylcholine was the largest variant lipid class between subjects in tears, while wax esters comprised the most variation between subjects in meibum secretions. **Conclusion** Interday variability is shown to be much smaller than interpatient variability, suggesting that tears and meibum subjects both have unique profiles in humans.

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Intersubject and Interday Variability in Human Tear and Meibum Lipidomes: A Pilot Study

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ABSTRACT

Purpose: Our aim was to quantitate day-to-day changes in the tear and meibum lipid profile of individual subjects in a pilot study of healthy humans.

Methods: Matched tear and meibum samples were obtained from four subjects on three consecutive days. Quantitative lipid profiles of human basal tears and meibum were compared using multivariate analysis by principal components.

Results: Substantial differences in the lipid profile between subjects were observed, while lipid profiles were steady across the three consecutive days of sampling. Multivariate principal component analysis demonstrated that lysophosphatidylcholine was the largest variant lipid class between subjects in tears, while wax ester contributed the most to variation between subjects in meibum secretions.

Conclusion: Inter-day variability is shown to be much smaller that inter-patient variability, suggesting that both tears and meibum subjects have unique profiles in individuals.

Keywords: lipids; lipidomics; multivariate analysis; tear; meibum.

Abbreviations: CE, cholesterol ester; LPC, lysophosphatidylcholine; MS, mass spectrometry; OAHFA, (O-acyl)-ω-hydroxy fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid; PS, phosphatidylserine; SM, sphingomyelin; TAG, triacylglyceride; TFLL, tear film lipid layer; WE, wax ester.

INTRODUCTION:

Dry-eye is a multifactorial disease that affects a third of the world's population and is defined as a disruption of the lacrimal functional unit. Primarily comprised of the eyelids, ocular surface, lacrimal glands and meibomian glands, the lacrimal unit delivers and maintains a thin fluid film across the cornea, which is vital in lubrication and protection of the ocular surface[1, 2]. Failure of tear film function is a critical determining factor in clinical pathologies, including dry-eye[1]. Lipids supplied principally by the meibomian glands provide the tear film lipid layer (TFLL), a complex mixture of lipids that create a thin hydrophobic film that serves to protect the tear film from evaporation. The physical properties of the tear film such as its stability and spreading are greatly influenced by the molecular lipid composition of the TFLL[3-6]. Deviations in the composition of the TFLL are likely to modulate the physical properties of the tear film and in turn effect a change in evaporation of the aqueous tear[7].

Investigation of the vanishingly small quantity of lipid in human tear film presents a unique challenge for study, and a range of analytical approaches have been applied to quantify the lipids in both tears and meibum[8, 9]. A comprehensive overview of the molecular lipid composition of human tears has recently been realized through application of contemporary mass-spectrometric analytical tools[10-12]. The advancements in instrumentation and extraction techniques have overcome challenges of small sample size and low overall lipid concentration, allowing the analysis of un-pooled samples, *i.e.* samples obtained from individual patients. These advances have opened the door to a suite of clinical studies investigating TFLL composition, correlations with dry-eye pathologies, and the potential for biomarker discovery[13, 14]. While these clinical studies have had some success in elucidating critical components of the TFLL, the day-to-day variability of the TFLL for individual subjects remains unclear. To address this question, we have applied a multivariate statistical analysis to investigate the composition of the tear film lipid layer over multiple days in the same subject. In a prior mass-spectrometric tear film lipidomics study by our team, each of four subjects was sampled three times on consecutive days[15]. That study focused on the differences in lipid profile between tears and meibum, and the profiles of individual subjects were averaged and not explored in detail. In order to investigate if subjects in fact have different lipid profiles, the current study compares the lipidomes of each human subject in both tears and meibum and explores the variation in the molecular composition between patients and sampling days.

METHODS:

Subject-matched tears and meibum secretions were collected and quantitative lipidomes were obtained. Techniques are described in detail in the original study[15], and summarized here. Tears and meibum secretions were collected from four healthy noncontact-lens wearers aged between 20 and 35 years. Left or right eye was randomized and the same for each participant throughout the study, and each subject was sampled once on each of three consecutive days. All samples were collected at approximately the same time each day by the same investigator. All patients signed an informed consent form before enrolment in the study, which was conducted in compliance with the tenets of the Declaration of Helsinki (2013).

A maximum 10 uL of tears was collected in a glass capillary tube placed at the edge of the lower eyelid, transferred to a microcentrifuge tube, centrifuged at low speed (9300 g) at 4^{o}C for 10 minutes to remove cells that may have been dislodged from the eyelid margin, then transferred to a 300 uL glass-insert vial (Chromacol, Thermo, Scoresby, VIC, Australia). Meibum was collected using either meibomian gland forceps or Korb meibomian gland evaluator, and approximately eight glands were expressed simultaneously. Meibum was collected from the whole lower eyelid moving the device from the nasal to temporal eyelid margin. Meibum was collected by gently pulling a metal spatula along the eyelid margin, then dissolved in chloroform, evaporated under nitrogen gas on a hot plate (34C), and stored in a glass vial at -80C until extraction.

Following sample extraction as described previously[15], quantification of 236 lipid molecular species was performed by chip-based tandem mass spectrometry. Lipid molecular species were normalized to total lipid and principal component analysis (PCA) was performed using MarkerView v1.2.1.1 (Sciex). Data from LipidView (Sciex) were imported into Markerview with lipid identification and quantification (represented as mol % total lipid) included. Data were scaled using Pareto scaling and no weighting was applied. PCA was performed in an unsupervised mode. Calculation of principal components was stopped when the amount of variance explained was less that 0.5% of the total variance. Ten components were used for tears and six components for meibum.

Following PCA analysis, the top two principal components were chosen to display the variability between patients. To compare lipid speciation of the abundant lipids in tears, comparisons were made between subjects of the major lipids classes as well

as the underlying species in these classes. The means (over the 3 sampling days) of the lipid classes and molecular species for each subject were compared by ANOVA using SPSS v21 (IBM).

RESULTS:

Comparisons of the means for each of the five major lipid classes and the major individual lipid species in each subject's tear samples are shown in Figure 1. The lipids reported in this study represent >99% of those reported in meibum and >97% of those reported in tears[10]. For clarity, only the ten most abundant lipid species in each class are shown in panels B, C and D. The quantity (expressed as mol %) of each of the five major lipid classes in tears differed substantially between the four individual subjects. Phospholipid (PL) was the most variable of all the lipid classes, varying from 1-30% of the total lipid across the four subjects. In all four major lipid classes, and in 27 of the 30 lipid molecular species, there was considerable variability between subjects, contrasted by only minor day-to-day changes within subjects. In consideration of the challenges of identifying key changes between subjects in a complex dataset such as this, we utilized a multivariate statistical approach to analyze our dataset for explicit lipid species that were changed between individuals.

Shown in Figure 2 are the principal component analysis (PCA) scores plots (panel A) and component loading plots (panel B) for tear samples. Principal components are defined as linear combinations of original variables that allow representation of the multivariate space into two or more dimensions. PCA allows the reduction of multivariate data into a smaller number of components representing the original variables. This technique enables the comparison of samples in multivariate datasets, revealing both the differences between samples and the variables that contribute to these differences. Principle components may be represented in simple two-dimensional plots and samples grouped their distance (termed Malahlanobis distance) between points. PCA has been applied to both IR and NMR spectrometry data to identify changes in human meibum with age and dry eye state[16-19]. In our analysis of tears, the first two principal components represented 79.4% of the variability between the 12 samples (4 subjects over 3 days), indicating a majority of the variation can be discerned from the single plot. The PCA scores plot shows tear samples clearly grouped by subject, with multiple days of sampling forming tight clusters. This demonstrates that tear lipidomes differ substantially between subjects, however an individual's tear lipidome does not vary greatly from day-to-day.

The PCA loading plot shown in Figure 2B graphically represents the contribution of each lipid species to the respective principle components. Examination of the loading plot allows identification of lipid species that contribute greatly to PCA scores, and therefore the lipid species that are substantially different between samples. For example, in Figure 2A, subject 1 (1T) is right-shifted on the plot, representing a high score for PC1. In the component loading plot shown in Figure 2B, the lipid species that contribute the most to a positive PC1 are the phospholipids, highlighted in pink. The lysophosphotidylcholine species (LPC) LPC 16:0, LPC 18:1 and LPC 18:0 (labeled on Figure 1B) are the highest contributors to PC1. Therefore, these LPC lipids explain a large proportion of the lipid profile differences between subjects with different PC1 scores.

In contrast to subject 1, subject 3 (3T) grouped to the top-left of the PCA plot, representing a low level of PC1 and a high-level of PC2. Wax ester (WE) and Cholesteryl ester (CE) species, specifically WE 18:1/25:0, WE 16:1/25:0 and CE 25:0 are identified in the loading plot as having a large contribution to the PCA scores observed for subject 3, indicating these lipid species are substantially higher in subject 3. Using the loading plot scores, 9 lipid species with maximal and minimal principle component scores (labeled in Figure 2B) were selected for further statistical analysis.

All lipid species selected from the loading plot differed between patients when analyzed by ANOVA, shown in Figure 2C. Interestingly, each cluster of lipid species identified from the PCA loading plot has similar biochemical parameters, indicating these inter-subject differences may result from differences in lipid precursor levels or lipid synthesis pathways. For example, subject 4 has high levels of 18 to 24-carbon even-chain CE species, suggesting that synthesis of very-long chain CE, i.e. those with esterified fatty acids longer than 24 carbons, may be down-regulated in subject 4. In contrast, subject 3 has high levels of lipids containing a 25:0 carbon chain, indicating this precursor may be more abundant in this subject.

PCA score plots for meibum samples (Figure 3A) also show clear grouping by subject and similar to tears, the first two principal components represented a majority (72.4%) of the variation between subjects. Component loading plots for meibum (Figure 3B) are substantially different to those from tears (Figure 2B). For example, WE containing an 18:1 fatty acid are the most substantial contributors to positive PC1 and PC2 scores in meibum, highlighted by WE 18:1/24:0, WE 18:1/25:0, and

WE 18:1/26:0. In contrast, the same wax ester species, but with a 16:1 fatty acid substitution, are highly loaded on the opposite side of the PCA scores plot. Similar to tears, the meibum lipid species with high contributions to the PCA loading plot have similar biochemical parameters. WE containing 18:1 fatty acid chains were highest in subject 1, while WE containing 16:1 fatty acids were highest in subject 2 (Figure 3C).

Interestingly, of these WE species, only WE 18:1/25:0 is a major contributor to principal components in both tears and meibum. This can be explained by considering that the major difference between tears and meibum is a substantially lower level of PL in meibum[10, 15, 20]. Without the contribution of PL to the PCA loadings and scores, the contribution of the WE (and other lipid classes) to variation between subjects becomes more pronounced.

DISCUSSION:

This study highlights the utility of PCA for investigation of lipidomic changes. Substantial challenges exist when applying univariate analysis tools to large numbers of variables, and mutivariate tools such as PCA allow the investigator to rapidly tease out subtle changes in the lipidome associated with biological factors such as age, race, gender, and disease pathology.

Lipid profiles of meibum are highly variable with 2-fold variation in CE abundance between individuals[11, 21]. This high variability of lipid profiles has also been shown in the CE abundance in human tears[22]. The current study confirms high variability of lipid profiles in tears and meibum, extends this result to all classes of lipids, and furthermore has demonstrated that this variability is indeed an inter-patient variation and not variability in collection or analysis. Data from Sullivan and Borchman have previously shown that lipid profile of meibum is affected by the donor's age and gender[18, 19, 23-26]. Sex related differences were observed only in the older age groups (mean age of 70.1 and 70.6 years for male and female, respectively). Our four subjects were three female and one male aged 25-33 years old, clearly within the young age group as defined by Sullivan, so it would be expected that age and sex differences did not contribute to the observed differences in the current study. Recent measurement of cytokines from human tear film also shows little intra-subject variability[27], further supporting the potential for biomarker discovery in tear film.

The most striking result from this multivariate analysis of human tears is the large variability of LPC between subjects. While LPC levels varied substantially on a day-

to-day basis, PCA analysis clearly shows that inter-subject variation dominates LPC variability. The physiological basis of this variation is unknown but may have a correlation with ocular disease and comfort for contact lens wearers. In contact lens studies, intolerance to lens wear is correlated with secretory phospholipase A2, an enzyme that produces LPC from PC[28]. However, LPC has protective effects from bacterial infection in vitro in a mechanism whereby LPC increases bactericidal activity of neutrophils[29], suggesting the increase in LPC may be protective on the ocular surface. The high variability of LPC may be a result of factors exogenous to the tear film, such as the removal and lysis of cells from the lid margin and subsequent enzymatic production of LPC, which suggests LPC may not be necessary for tear film function.

In contrast, in human meibum WE lipid species were the most variable across subjects. The highly hydrophobic nature of WE may result in these species being buried deeply into the hydrophobic layer, and tight biological regulation is less necessary than lipids found in the aqueous-lipid and/or lipid–air interface layers.

In summary, the data presented here suggests that tears and meibum have lipid profiles that are unique for each individual and remain consistent from day-to-day.

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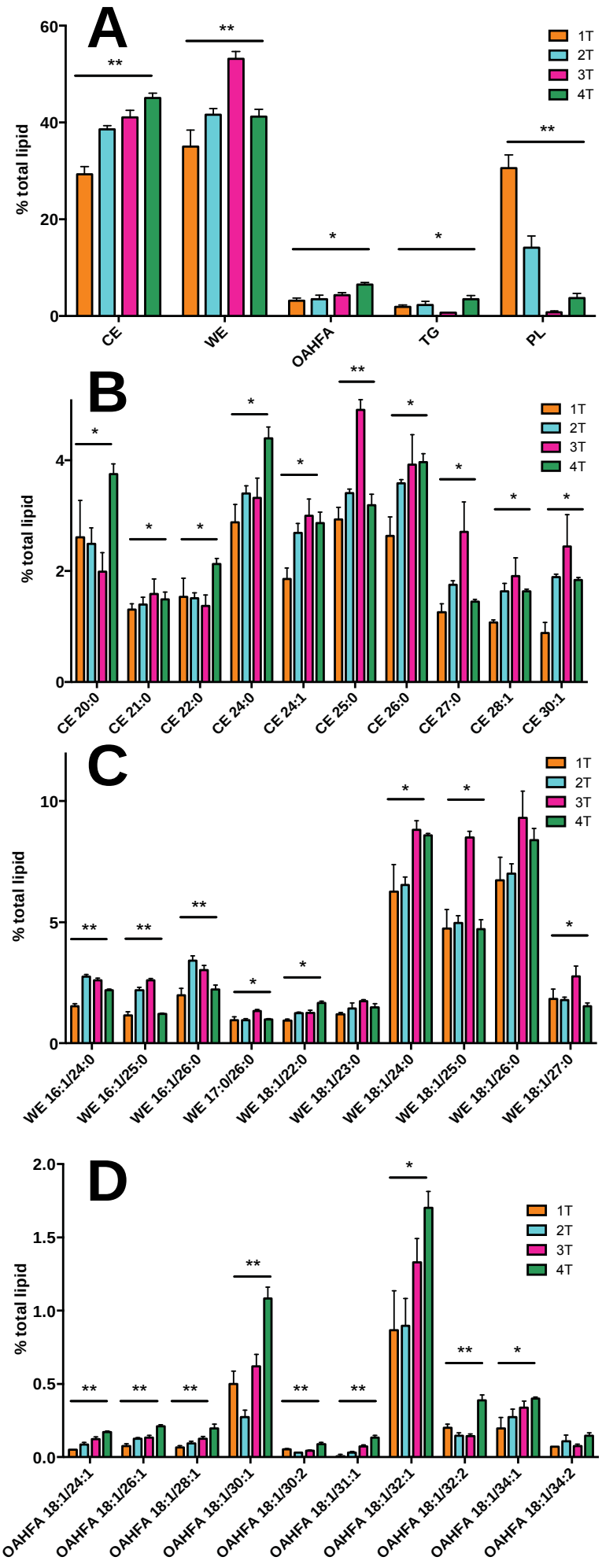

Figure 1: Tear lipid profile of four healthy human subjects. The lipid class totals shown as the mean of three sampling days are displayed in panel A. The ten most abundant lipid species for the classes CE (panel B), WE (panel C) and OAHFA (panel D) compared between subjects are shown. Lipid species are colored by subject. Shown as means ± SEM, * p<0.01, ** p<0.001. T = tear samples. CE, cholesteryl ester; WE, wax ester; OAHFA, o-acyl hydoxy fatty acid; TG, triacylglycerol; PL, phospholipid.

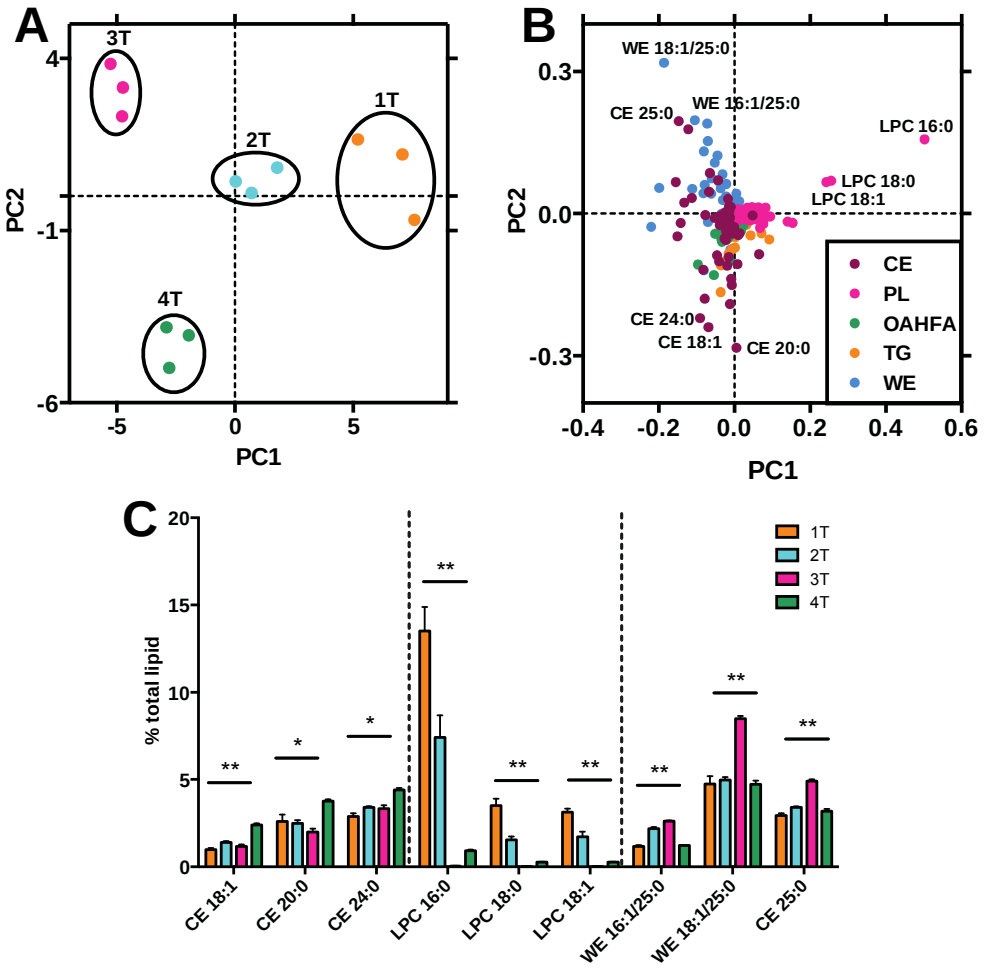

Figure 2: Principal competent analysis (PCA) of tears. Scores plot showing grouping of the 3 consecutive-day analysis of 4 subjects (A). X (PCA1) and Y axes (PCA2) represents the principal components accounting for the greatest and second greatest variability, which in total represented 79.4% of the variability. PCA loading plot indicating the contribution of individual lipid species towards the observed differences is shown in panel B. Species are colored by lipid class, and for clarity only the most substantially changed lipid species are labeled. The molar % contribution of each labeled lipid species is shown in panel (C). Data shown as means ± SEM, * p<0.01, ** p<0.001. T = tear samples

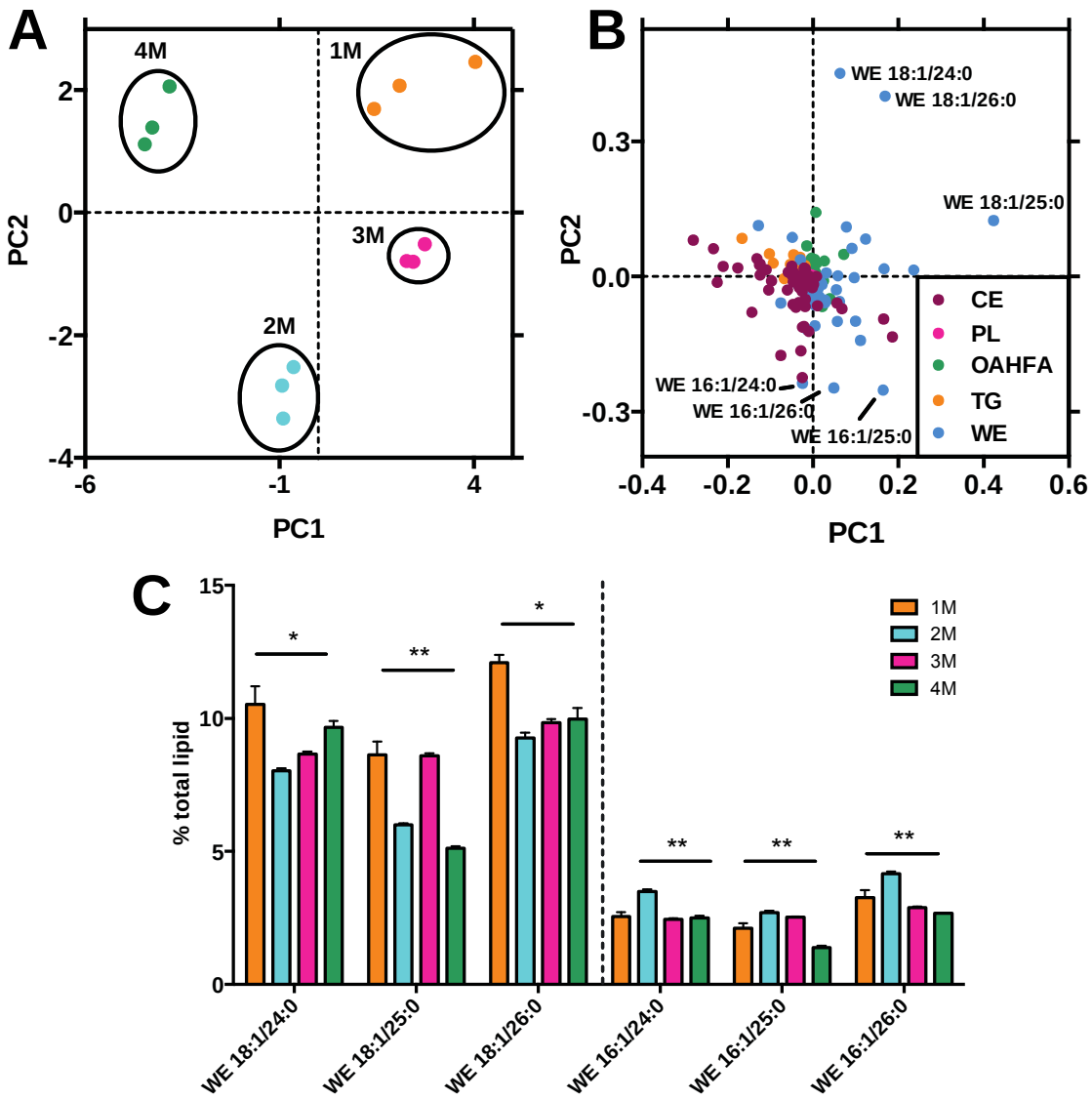

Figure 3: Principal competent analysis (PCA) of meibum. Scores plot showing grouping of the 3 consecutive-day analysis of 4 subjects (A). X (PCA1) and Y axes (PCA2) represents the principal components accounting for the greatest and second greatest variability, which in total represented 72.4% of the variability. PCA loading plot indicating the contribution of individual lipid species towards the observed differences is shown in panel B. Species are colored by lipid class, and for clarity only the most substantially changed lipid species are labeled. The molar % contribution of each labeled lipid species is shown in panel (C). Data shown as means ± SEM, * $p<0.01$, ** $p<0.001$. M = meibum samples