A Comparison of Patient Matched Meibum and Tear Lipidomes

Simon H. J. Brown,[1] Carolina M. E. Kunnen,[2–4] Eva Duchoslav,[5] Naveen K. Dolla,[6] Michael J. Kelso,[6] Eric B. Papas,[2–4] Percy Lazon de la Jara,[2–4] Mark D. P. Willcox,[3] Stephen J. Blanksby,[7] and Todd W. Mitchell[1]

[1]Illawara Health and Medical Research Institute and School of Health Sciences, University of Wollongong, Wollongong, New South Wales, Australia
[2]Vision Cooperative Research Centre, Sydney, New South Wales, Australia
[3]School of Optometry and Vision Science, University of New South Wales, Sydney, New South Wales, Australia
[4]Brien Holden Vision Institute, Sydney, New South Wales, Australia
[5]AB SCIEX, Concord, Ontario, Canada
[6]School of Chemistry, University of Wollongong, Wollongong, New South Wales, Australia
[7]Australian Research Council Centre of Excellence for Free Radical Chemistry and Biotechnology, School of Chemistry, University of Wollongong, Wollongong, New South Wales, Australia

Correspondence: Todd W. Mitchell, School of Health Sciences, University of Wollongong, Wollongong, NSW, Australia, 2522; toddm@uow.edu.au.

Submitted: July 25, 2013
Accepted: September 25, 2013

Citation: Brown SHJ, Kunnen CME, Duchoslav E, et al. A comparison of patient matched meibum and tear lipidomes. *Invest Ophthalmol Vis Sci.* 2013;54:7417–7423. DOI:10.1167/iovs.13-12916

Purpose. To quantify the molecular lipid composition of patient-matched tear and meibum samples and compare tear and meibum lipid molecular profiles.

Methods. Lipids were extracted from tears and meibum by bi-phasic methods using 10:3 *tert*-butyl methyl ether:methanol, washed with aqueous ammonium acetate, and analyzed by chip-based nanoelectrospray ionization tandem mass spectrometry. Targeted precursor ion and neutral loss scans identified individual molecular lipids and quantification was obtained by comparison to internal standards in each lipid class.

Results. Two hundred and thirty-six lipid species were identified and quantified from nine lipid classes comprised of cholesterol esters, wax esters, (O-acyl)-ω-hydroxy fatty acids, triacylglycerols, phosphatidylcholine, lysophosphatidylcholine, phosphatidylethanolamine, sphingomyelin, and phosphatidylserine. With the exception of phospholipids, lipid molecular profiles were strikingly similar between tears and meibum.

Conclusions. Comparisons between tears and meibum indicate that meibum is likely to supply the majority of lipids in the tear film lipid layer. However, the observed higher mole ratio of phospholipid in tears shows that analysis of meibum alone does not provide a complete understanding of the tear film lipid composition.

Keywords: tears, meibum, lipids, lipidome, lipidomics, OAHFA, mass spectrometry

Dry-eye disease affects up to one-third of the world's population and results in large medical and societal costs, estimated at up to $55 billion in 2007 in the USA alone.[1] Dry eye is a multifactorial disease defined as a disruption of the lacrimal functional unit, a system comprised of the eyelids, ocular surface, lacrimal glands, and meibomian glands.[2] One role of this system is to deliver and maintain the thin film of primarily aqueous tears across the cornea. This tear film serves vital roles in maintenance of the highly sensitive ocular machinery, serving to lubricate and protect the ocular surface. The physical properties of the tear film are intimately linked to the chemical composition of the tear fluid itself. Failure of the tear film to provide lubrication and protection is a critical determining factor in clinical pathologies, including dry eye.[2]

It has been proposed that the human tear film is composed of three major layers: an inner glycocalyx layer rich in glycgoproteins and mucins, an aqueous layer containing salts and proteins, and an outermost tear film lipid layer (TFLL).[3] Although the aqueous and glycocalyx layers are often described as distinctly separated, evidence indicates that secreted mucins are in fact highly soluble and suggests the glycocalyx and aqueous layers are not distinct.[4] The TFLL is composed of a complex mixture of lipids, which create a thin hydrophobic film that serves to protect the tear film from evaporation. The physical properties of the tear film, including its stability and spreading, are intimately linked to the chemical composition of the TFLL, and therefore the molecular lipid composition. The TFLL itself has an internal structure, composed of an outer region of nonpolar lipids, and an inner amphipathic region containing mainly polar lipids.[3] The inner amphipathic layer facilitates spreading of the TFLL across the bulk aqueous tear film.[5,6] Pathologies linked to deviations in the composition of the TFLL, and especially deviations in the polar lipids of the amphipathic layer, are likely to modulate the physical properties of the tear film and in turn effect a change in evaporation of the aqueous tear.[7]

The primary source of lipids in human tear film is believed to be meibum, a waxy secretion originating from the meibomian glands. Although significant progress has been made in identification and quantification of the lipids in meibum, obtaining a complete molecular composition of human tears remains an ongoing challenge.[8] Factors including

small sample size, the predominance of highly hydrophobic lipids, low overall lipid concentration, and the presence of rare and novel lipid species all contribute to the challenges of studying human tears. This is a significant limitation for clinical trials where the analysis of unpooled samples (i.e., samples from individual patients) is a necessity.

While it is accepted that meibum supplies the majority of tear film lipids, it is debatable if the composition of meibum alone reflects the complete suite of lipids found in the tear film. To address this question, we obtained patient-matched lipidomes of tears and meibum, with both collected from individual patients in a single clinic visit. To address the analytical challenges involved in quantifying tear lipids, we developed specialized extraction and data collection techniques. Lipids were extracted by biphasic methods using methyl tert-butyl ether (MTBE),[9] and the extracts analyzed using chip-based nanoelectrospray tandem mass spectrometry.[10] This study compares the patient-matched lipidomes of human tears and meibum, and explores the variation in the molecular composition of tears and meibum.

Materials and Methods

Chemicals

Methanol, chloroform, and MTBE were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia) and were high performance liquid chromatography (HPLC) or liquid chromatography-mass spectroscopy (LC-MS) grade when available. Ammonium acetate (LC-MS grade) and analytical grade butylated hydroxytoluene (BHT) were also purchased from Sigma-Aldrich. The 18:1/16:0 (O-acyl)-ω-hydroxy fatty acid (OAHFA), internal standard was synthesized in-house (see below). Phospholipid standards were from Avanti Polar Lipids (Alabaster, AL), wax ester (WE) and cholesterol ester (CE) standards were from Nu-Chek Prep (Elysian, MN) and triacyglycerol standard from CDN isotopes (Point-Claire, QC, Canada).

Preparation of OAHFA 18:1/16:0 Internal Standard

Oleoyl chloride (0.24 mL, 0.734 mM) was added dropwise via syringe to a stirring solution of 16-hydroxypalmatic acid (0.2 g, 0.734 mM) in pyridine (5.0 mL) and the mixture was stirred at room temperature for 14 to 16 hours. Reaction progress was monitored by thin layer chromatography (10% methanol in chloroform). Upon completion of the reaction the mixture was concentrated under reduced pressure and the residue obtained dissolved in chloroform (30 mL). The organic phase was washed with water (3 × 15 mL) and saturated sodium chloride (15 mL) before being dried over magnesium sulphate and concentrated under reduced pressure. Purification by column chromatography on silica gel (200–400 mesh) initially using ethyl acetate: chloroform:hexane (4:1:1) followed by 3% to 5% methanol: chloroform as eluent yielded the title compound (0.16 g; 41%) as a white waxy solid. ^{1}H nuclear magnetic resonance (NMR) (500 MHz , $CDCl_3$, ppm): δ 5.34 (m, 2H), 4.05 (t, $J = 6.5$ Hz), 2.35 (t, $J = 7.5$), 2.30 (t, $J = 7.5$ Hz), 2.08 (m, 4H), 1.61 (m, 4H), 1.25-1.28 (m, 46H), 0.87 (t, $J = 6.0$ Hz, 3H); ^{13}C NMR (500 MHz; $CDCl_3$, ppm): δ 179, 174, 129.7, 129.9, 64.0, 31.9, 29.7, 29.8, 29.6, 29.64, 29.57, 29.52, 29.42, 29.30, 29.20, 29.16, 29.13, 29.11, 29.06, 29.0, 27.3, 27.2, 27.15, 25.9, 25.0, 24.69, 24.67, 22.7, 14.1. IR (CH_2Cl_2) ν_{max} (cm^{-1}): 2924–2853, 1718, 1468, 1280; electrospray ionization (ESI)-high-resolution mass spectrometry: The theoretical mass of $C_{34}H_{64}O_4$ ($[M+H]^+$) is 537.4877. The measured value was 537.4902, an accuracy of 4.6 ppm.

Sample Collection and Preparation

Matched tears and meibum secretions were collected from four noncontact-lens wearers aged between 20 and 35 years. The choice of either left or right eye was randomized and the same for each participant throughout the study. Each patient was sampled three times on consecutive days. All samples were collected at approximately the same time 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.

A maximum 10 μL of tears was collected in a fine glass capillary tube placed at the edge of the lower eyelid and stored in a microcentrifuge tube. This tube was placed in a centrifuge at 4°C, 9300*g* for 10 minutes to remove any cellular debris and was stored in a 300-μL glass-insert HPLC vial (Thermo-Fisher Scientific, Scoresby, VIC, Australia). Meibum was collected using either meibomian gland forceps (MGF) or Korb meibomian gland evaluator (Korb MGE). The Korb MGE or MGF was placed below the eyelash line of the lower eyelid and was held in this position for 10 seconds. With this technique approximately eight glands are expressed simultaneously. Meibum was collected from the whole lower eyelid moving the device from the nasal to temporal eyelid margin.

In all cases, during meibum collection, the eyelid was pulled away from the eye so as to reduce contamination with tears. The meibum was collected by gently pulling a metal spatula along the eyelid margin, with care to minimize contact with the lid surface. The meibum sample was dissolved in 1000 μL chloroform in a glass vial, evaporated with nitrogen gas on a hot plate (34°C), and stored at –80°C until extraction.

Tear Lipid Extraction

Lipid extraction was performed using a modification of the method of Matyash et al.[9] An aliquot containing 60 μL of methanol, 200 μL MBTE, and 10 μL of internal standard mix was added to tear samples. Butylated hydroxytoluene (0.01%) was added to the mixture to prevent lipid oxidation. The extraction solvent mix was added directly to the 300-μL glass-insert HPLC vial containing the tear sample. Internal standard mix contained (per sample) 30 pmol OAHFA 18:1/16:0, 2.4 pmol phosphatidylcholine (PC) 19:0/19:0, 2.4 pmol dihydrosphingomyelin 12:0, 1.5 pmol phosphatidylserine (PS) 17:0/17:0, 1.5 pmol phosphatidylethanolamine (PE) 17:0/17:0, 10 pmol of D_5 triacylglycerol (TAG) 16:0/16:0/16:0, 20 pmol wax ester (WE) 16:0/16:0, 200 pmol of WE 18:1/18:0, and 80 pmol of cholesterol ester (CE) 14:0. The samples were then mixed on an orbital platform shaker for 1 hour, at room temperature. An addition of 50 μL of 150 mM aqueous ammonium acetate was added to induce phase separation. Tubes were vortexed and spun at 2000*g* for 5 minutes to complete phase separation. Approximately 200 μL of the upper organic layer was removed and added to a new 300 μL glass-insert HPLC vial, dried under a stream of nitrogen at 37°C, resuspended in 50 μL methanol: chloroform (2:1 vol/vol) and stored at –20°C until analysis. Samples were diluted 2-fold in methanol:chloroform (2:1 vol/vol) containing 15 mM ammonium acetate prior to mass spectrometric analysis.

Meibum Extraction

Similar to tears, meibum lipid extraction was performed using a modification of the method of Matyash et al.[9] The dry meibum sample was dissolved in 1 mL chloroform including 0.01% BHT and shaken gently for 1 hour on an orbital platform shaker. An aliquot containing 60 μL of methanol, 200 μL MTBE, 10 μL of internal standard mix and 0.01% wt/vol BHT of internal standard mix was added to a 300 μL glass-insert HPLC vial, followed by addition of 20 μL of meibum solution. Internal

TABLE. Target Lipid Class, Ion Observed, MS/MS Experiment (Precursor Ion [PI] or Neutral Loss [NL]), CID Energy, and Internal Standard Used for MS/MS Identification and Quantification of Lipids in Human Tear and Meibum

Target Lipid	Ion	MS/MS	CID Energy	Internal Standard
WE (18:1 FA)	$[M+NH_4]^+$	PI m/z 283.3	30	WE 18:1/18:0
WE (16:1 FA)	$[M+NH_4]^+$	PI m/z 255.3	30	WE 18:1/18:0
WE (17:0 FA)	$[M+NH_4]^+$	PI m/z 271.3	30	WE 16:0/18:0
WE (16:0 FA)	$[M+NH_4]^+$	PI m/z 257.3	30	WE 16:0/18:0
CE	$[M+NH_4]^+$	PI m/z 369.4	25	CE 14:0
TAG (18:1 FA)	$[M+NH_4]^+$	NL 299.3	35	D_5 TAG 48:0
TAG (16:1 FA)	$[M+NH_4]^+$	NL 271.3	35	D_5 TAG 48:0
TAG (18:0 FA)	$[M+NH_4]^+$	NL 301.3	35	D_5 TAG 48:0
TAG (16:0 FA)	$[M+NH_4]^+$	NL 273.3	35	D_5 TAG 48:0
SM	$[M+H]^+$	PI m/z 184.1	55	DHSM 12:0
PC	$[M+H]^+$	PI m/z 184.1	55	PC 38:0
LPC	$[M+H]^+$	PI m/z 184.1	55	PC 38:0
PE	$[M+H]^+$	NL 141	30	PE 34:0
PS	$[M+H]^+$	NL 185	35	PS 34:0
OAHFA (18:2 FA)	$[M-H]^-$	PI m/z 279.3	45	OAHFA 18:1/16:0
OAHFA (18:1 FA)	$[M-H]^-$	PI m/z 281.3	45	OAHFA 18:1/16:0
OAHFA (16:1 FA)	$[M-H]^-$	PI m/z 253.3	45	OAHFA 18:1/16:0
OAHFA (18:0 FA)	$[M-H]^-$	PI m/z 281.3	45	OAHFA 18:1/16:0
OAHFA (16:0 FA)	$[M-H]^-$	PI m/z 255.3	45	OAHFA 18:1/16:0

In total 236 molecular lipid species were quantified; WE, CE, TAG, SM, PC, LPC, PE, PC, and OAHFA.

standard mix contained (per sample) 30 pmol OAHFA 18:1/16:0, 2.4 pmol PC 19:0/19:0, 2.4 pmol dihydrosphingomyelin 12:0, 1.5 pmol PS 17:0/17:0, 1.5 pmol PE 17:0/17:0, 10 pmol of TAG 16:0/16:0/16:0, 20 pmol WE 16:0/16:0, 200 pmol of WE 18:1/18:0, and 80 pmol of CE 14:0. Meibum samples were mixed on an orbital shaker for 10 minutes at room temperature. An addition of 50 μL of 150 mM aqueous ammonium acetate was added to induce phase separation. Tubes were vortexed and spun at 2000*g* for 5 minutes to complete phase separation. Approximately 200 μL of the upper organic layer was removed and added to a new 300-μL glass-insert HPLC vial, dried under a stream of nitrogen at 37°C, resuspended in 50 μL methanol:chloroform (2:1 vol/vol) and stored at −20°C until analysis. Samples were diluted 2-fold in methanol:chloroform (2:1 vol/vol) containing 15 mM ammonium acetate prior to mass spectrometric analysis.

Mass Spectrometry

Mass spectra were acquired using a chip based nanoelectrospray ionization source (TriVersa Nanomate; Advion, Ithaca, NY) coupled to a hybrid linear ion trap-triple quadruple mass spectrometer (QTRAP 5500; AB SCIEX, Foster City, CA). The pipette tip in the TriVersa Nanomate aspirated 10 μL of extract from a sealed 96-well plate and delivered it into the mass spectrometer via a nano-ESI chip with an orifice diameter of 4.1 μm. The delivery gas was N_2 at a pressure of 0.4 psi and spray voltage of 1.2 kV and −1.1 kV for positive and negative ion acquisition, respectively. All samples subjected to ESI-MS were at a concentration below 100 μM, conditions at which ion-suppression effects have been shown to be minimal.[11,12] Target lipids and MS scan parameters are shown in the Table. Typical experiment conditions for positive ion mode acquisition were a declustering potential of 100 V, entrance potential of 10 V, and a scan rate of 200 m/z units.s^{-1}. Negative ion mode acquisition parameters were a declustering potential of −300 V, entrance potential −11 V, and scan rate 200 m/z units.s^{-1}. Mass spectra were averaged over 120 scans in negative mode and 104 scans in positive mode. Data were analyzed with LipidView (AB SCIEX) software version 1.1, including smoothing, identification, removal of isotope contribution from lower mass species, and correction for isotope distribution. Ionized lipids detected with a signal-to-noise ratio (s/n) over 5 were included in the analysis. Quantification was achieved in LipidView software (AB SCIEX) by comparison of the peak area of individual lipids to their class-specific internal standards after isotope correction. A post hoc correction factor was determined to allow use of PC 19:0/19:0 as an internal standard for LPC. Samples containing a 3:2 molar ratio of PC 19:0/19:0:lysophosphatidylcholine (LPC) 17:0 were quantified using the same precursor ion experiment listed in the Table. Precursor ion scan peak area per mol was determined to be 2.25-fold higher for PC than LPC; therefore, a correction factor of 2.25 was applied to the endogenous LPC peak area.

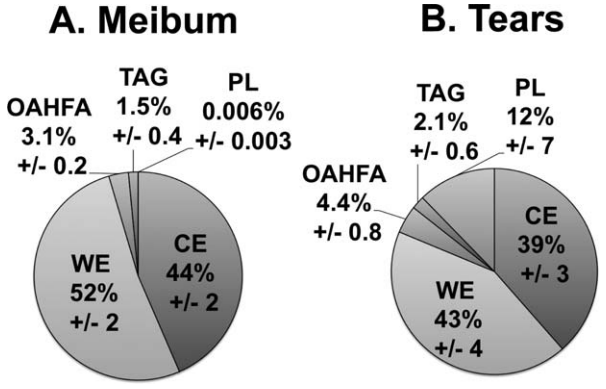

FIGURE 1. Quantitative comparison of lipid profiles between (**A**) human meibum ($N = 4$) and (**B**) human tears ($N = 4$). Values are shown as the mean of 12 measurements ± SEM ($N = 4$). Di-esters and ceramides were detected but not included in the quantitative analysis.

Statistics

Following absolute per-sample quantification, individual lipid species in each sample were normalized with respect to total lipid in each sample. Accurate masses of tear and meibum samples were not obtained during collection, and total lipid on a "per-sample" basis was found to be highly variable in tears.

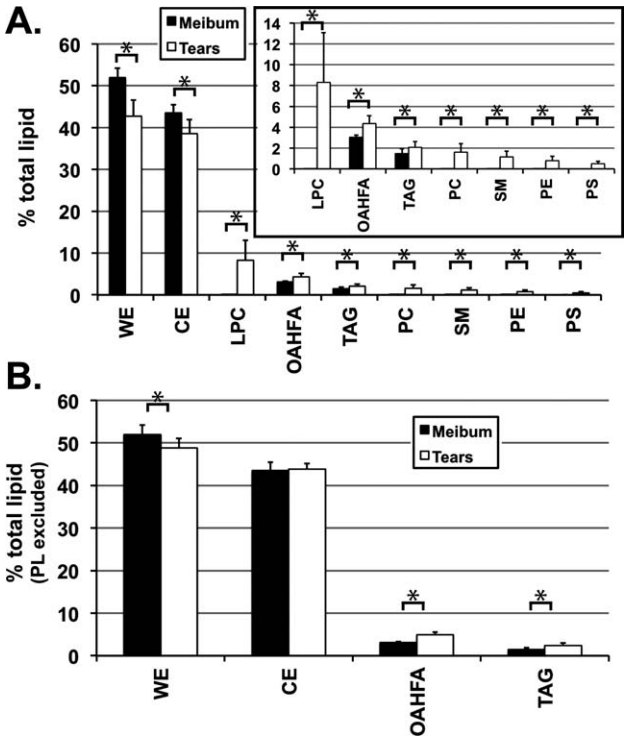

FIGURE 2. Quantitative comparison of lipid profiles in human meibum ($N = 4$) and human tears ($N = 4$) shown (**A**) including PL and (**B**) with PL excluded. Values are shown as the mean of 12 measurements ± SEM ($N = 4$). *$P < 0.05$ tears versus meibum. Di-esters and ceramides were detected but not included in the quantitative analysis.

Therefore comparison on a normalized basis was deemed the most appropriate. Results are reported as mole fraction (Figs. 1, 2A, Supplementary Tables S1–S6). To evaluate the effect that the highly variable levels of phospholipid caused on the mole fraction of other lipid species, all phospholipids (PL) were excluded and lipids were renormalized (Fig. 2B). To evaluate if speciation varied within each lipid class, lipid species were normalized with respect to the sum of all lipids within the respective class (Figs. 3, 4A). Finally, PL classes were renormalized with respect to total phospholipid (Fig. 4B). Normalization was performed on each sample. In every case, lipid profiles of tears ($N = 4$) and meibum ($N = 4$) were compared using linear mixed model. An initial analysis compared the effect of sample type (tears/meibum) for each lipid class. The factors of the model, namely, sample type and sampling day (days 1, 2, and 3) were factored as within subject repeated effects. Subjects were included as random intercepts. Following this, the effect of sample type on lipid species within each lipid class was investigated. If the interaction of sample type with lipid species was significant, the significance of sample type was determined for each lipid species. Level of significance was set at 5%. As there were only two sample types, no post hoc multiple comparisons were required. In comparisons of speciation shown in Figures 3 and 4A and discussed in results, P values were Bonferroni corrected.

RESULTS

Tear and Meibum Lipid Classes

Shown in Figures 1 and 2A are the overall lipid class profiles for tears and meibum. Sum totals for every lipid class differed between tears and meibum. Wax esters are the most abundant lipid class, representing 52 ± 2% (meibum) and 43 ± 4% (tears) ($P = 0.001$, meibum versus tears). Cholesterol esters were present at a slightly lower mole fraction at 44 ± 2% (meibum) and 39 ± 3% (tears) ($P < 0.001$, meibum versus tears). Other major classes were substantially lower in abundance, with OAHFA at 3.1 ± 0.2% (meibum) and 4.4 ± 0.8% (tears) ($P = 0.002$, meibum versus tears); and TAG at 1.5 ± 0.4% (meibum) and 2.1 ± 0.6% (tears) ($P = 0.009$, meibum versus tears). Phospholipids are the lipid class with the lowest abundance in meibum, representing only 0.006 ± 0.003%. In stark contrast, PL was highly abundant in tears at 12 ± 7%.

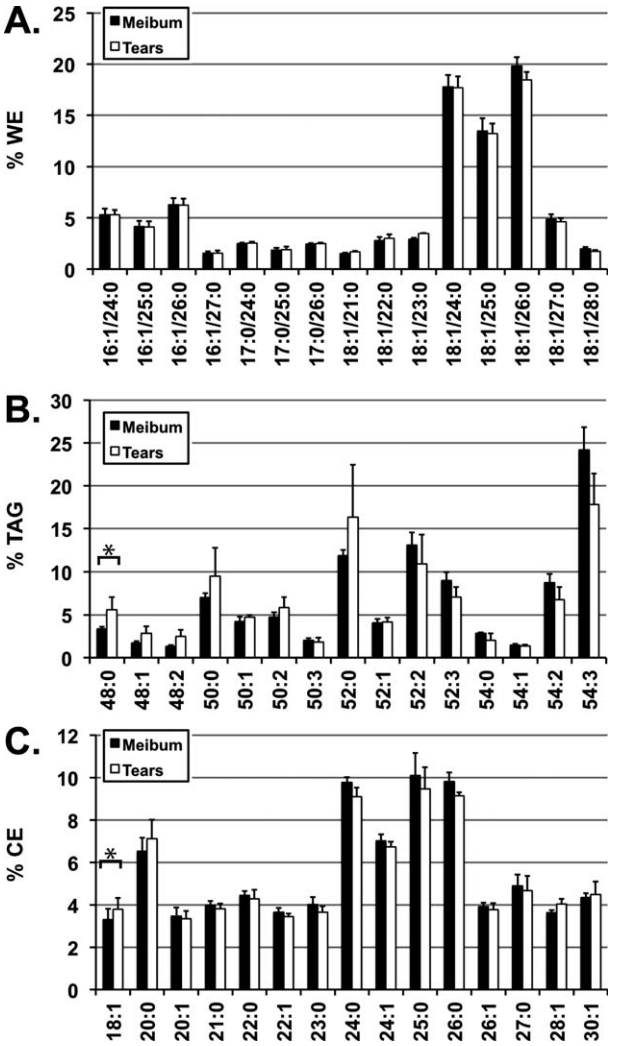

FIGURE 3. Molecular lipid speciation of major classes of nonpolar lipids identified in human tears and meibum including: (**A**) WE, (**B**) CE, and (**C**) TAG. The most abundant 15 species are shown, normalized to the sum total of lipids in respective class. Values are shown as the mean of 12 measurements ± SEM ($N = 4$). *$P < 0.05$ tears versus meibum.

While all lipid classes differed between meibum and tears, this variation was primarily due to a large increase in PL in tears; therefore, a second comparison between meibum and tear samples was made. Phospholipids was excluded from the sum total, and lipid class profiles were generated (Fig. 2B). With PL excluded, the mole fractions of CE was no different between meibum and tears, CE representing 44 ± 2% (meibum) and 44 ± 1% (tears). Wax esters represented 52 ± 2% (meibum) and 49 ±2% (tears), a 6% decrease in tears compared with meibum ($P = 0.008$). Other classes were higher in tears, with OAHFA 61% higher (3.1% vs. 4.9%, $P < 0.001$) and TAG 61% higher (1.5% vs. 2.4%, $P = 0.001$).

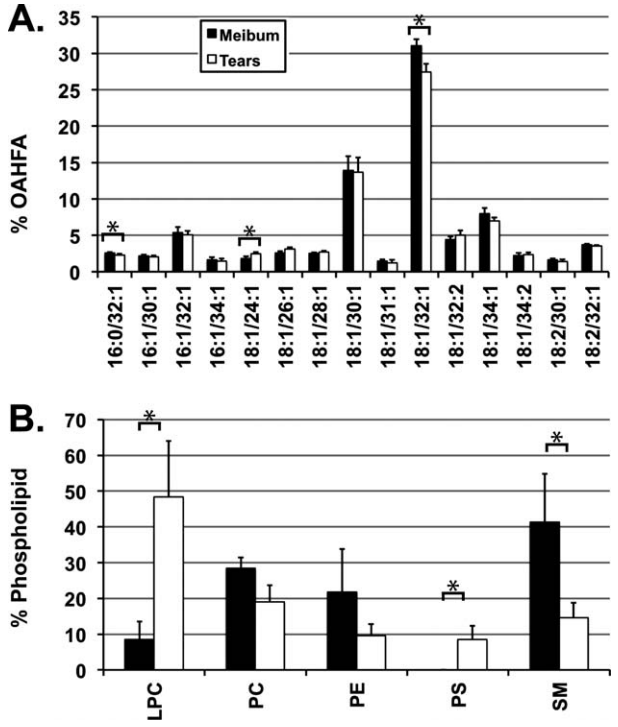

FIGURE 4. (**A**) Molecular lipid speciation of OAHFA identified in human tears and meibum. The most abundant 15 species are shown, normalized to the sum total of OAHFA. (**B**) PL class profiles of PL compared between human tears and meibum, normalized to the sum total of PL. Values are shown as the mean of 12 measurements ± SEM ($N = 4$). *$P < 0.05$ tears versus meibum.

Speciation of Nonpolar Lipid Classes

Molecular speciation of the three major nonpolar lipid classes (CE, WE, and TAG) was compared between tears and meibum. The fifteen most abundant molecular species (representing over 80% of each class total) are shown in Figure 3. Minor species are not shown but all species identified are listed in Supplementary Tables S2 (CE), S5 (TAG), and S6 (WE). Precursor ion (CE and WE) and neutral loss scanning (TAG) allowed identification of fatty acid chain lengths, fatty alcohol chain lengths (WE), and lipid unsaturation. Identification of chain branching, double bond position, and double bond stereochemistry was not pursued in the study.

Wax esters are dominated by species containing 18:1 fatty acids (FA), with such species averaging 65.2% of the total WE observed. Lower abundance WE included species containing 16:1 (20.7%), 17:0 (8.8%), and 16:0 (5.3%) FA. Fatty alcohols (FAl) observed were fully saturated, dominated by species containing 24, 25, or 26 carbon acyl chains (27.0%, 20.4%, and 29.4%, respectively), in total representing 76.8% of total WE. A similar pattern of FAl chains was observed for each esterified FA. No differences were observed in the speciation of abundant WEs between tears and meibum.

Similar to WE, the most abundant CE species contain 24, 25, or 26 carbon acyl chains (17.0%, 10.1%, and 13.7%, respectively), in total representing 40.9% of total CE Only fully saturated odd-chain species were observed, while even-chain species were a mixture of fully saturated, mono-unsaturated and di-unsaturated. The only observed difference in the speciation of abundant CEs between tear and meibum was a 15% increase in the level of CE 18:1 in tears compared with meibum (3.8% vs. 3.1%; $P = 0.014$).

Triacylglycerol species were identified and quantified using neutral-loss scans for each FA of the TAG molecular species. Preliminary data indicated that only scans for 16:0, 16:1, 18:0, and 18:1 yielded signals of sufficient signal-to-noise ratio to quantify accurately, therefore, only molecular species containing these FAs are reported. The only observed difference in the speciation of abundant TAGs between tear and meibum was a 65% increase in the level of TAG 48:0 in tears compared with meibum (5.6% vs. 3.4%; $P = 0.027$).

Speciation of Polar Lipid Classes

Molecular speciation of OAHFA was compared between tears and meibum. Precursor ion scanning allowed identification of FA chain lengths and the degree of unsaturation, as well as ω-hydroxy FA lengths and the degree of unsaturation. The fifteen most abundant molecular species (representing over 80% of the total OAHFA) are shown in Figure 4A. Low abundance OAHFA species are not shown in Figure 4A, however, all OAHFA species identified are listed in Supplementary Table S3. (O-acyl)-ω-hydroxy fatty acid is dominated by species containing 18:1 FAs, with such lipids averaging 69.5% of the total OAHFA observed. Less abundant OAHFA included species containing 16:1 (11.5%), 18:2 (9.2%), 16:0 (6.6%), and 18:0 (3.1%) FAs. The ω-hydroxy fatty acid chains range from 24 to 34 carbons long and are primarily monounsaturated, however di-unsaturated species were observed at lower abundance. No OAHFAs with fully saturated ω-hydroxy FAs were detected. Three molecular species dominated the OAHFA lipid profile: 18:1/30:1, 18:1/32:1, and 18:1/34:1 comprised 50.5% of the total OAHFA's observed. Observed differences in the speciation of abundant OAHFAs between tear and meibum included an increase in 18:1/24:1 by 36% (2.5% vs. 1.8%; $P < 0.001$), and a decrease of 16:0/32:1 and 18:1/32:1 in tears by 10% and 12%, respectively (2.3% vs. 2.6%; $P = 0.049$, 27.5% vs. 31.1%; $P = 0.005$).

Class totals of PL were compared between tears and meibum. Precursor ion and neutral loss scanning allowed identification of PL lipid class, total FA carbon numbers and unsaturation for each PL species, shown in Supplementary Table S4. Due to low abundances of PL, the molecular identification (e.g., the degree of unsaturation, assignment of FAs) of PL was not attempted in the current study. Distribution of PL classes was compared between tears and meibum, and is shown in Figure 4B. PLs were found to be highly variable, with the coefficient of variation (across all 12 samples from 4 individuals) for the sum of PL of over 100% in tears and meibum. Lysophosphatidylcholine represented nearly half of the PL in tears at 48%, yet only represented 8% phospholipid in meibum ($P < 0.001$). Conversely, sphingomyelin (SM) represented 15% of the PL in tears and represented 41% phospholipid in meibum ($P = 0.013$). Phosphatidylserine represented 8.5% of the PL in tears and was not detected in meibum. Phosphatidylcholine and PE class distribution did not differ between tears and meibum.

DISCUSSION

This study identifies CE and US as the dominant lipid classes in meibum, representing 52% and 44% of the mole fraction, respectively. (O-acyl)-ω-hydroxy fatty acid was the most abundant polar lipid at 3.1%, while TAG represented only 1.5% and PL were 0.006%. These findings agree with a recent meibum study by Lam et al.,[13] where CE and WE were highly abundant, TAG and OAHFA represented less than 5% of the lipidome, and PL were the least abundant at less than 1%. However, while Lam et al.[13] reported a similar total quantity of CE and WE, they found CE (66%) contributed over twice the fraction compared to WE (25%). However, the authors

commented that their use of a single fully saturated WE internal standard may have led to errors in quantification. In the current study, we observed saturated and unsaturated WE produce the characteristic protonated FA ion at different abundances. Therefore, we used a separate WE internal standard for saturated and unsaturated WE quantification. Lam et al.[13] concluded that PL are a minor component of the meibum lipidome, but reported substantially higher levels than found in our current study. Some factors that may contribute to this difference are discussed later.

The WE species detected at the highest abundance included a FA chain of 18:1 and FAl chain lengths of 24:0, 25:0, and 26:0, in agreement with analysis of meibum WE species by ESI–tandem mass spectroscopy (MS/MS)[13,14] and gas–liquid chromatography MS/MS.[15] This is also in agreement with the recent ESI-MS study by Chen et al.,[16] who report the highest abundance WE species of WE 42:1, WE 43:1, and WE 44:1. Similar to WE FA chains, the CE species detected at the highest abundance contained esterified FA chains of 24:0, 25:0, and 26:0. This is in agreement with the recent study by Chen,[16] as well as earlier studies of Chen,[14] Lam,[13] and Nicolaides.[17] Butovich et al.[18] characterized CE speciation in meibum by HPLC atmospheric pressure chemical ionization MS, and their study reported CE 24:0, CE 25:0, and CE 26:0 as highly abundant, but also reported CE 26:1 and 28:1 as highly abundant. However, due to chromatographic co-elution (CE 24:0 with CE 26:1, and CE 26:0 with CE 28:1) the individual contribution of these species could not be determined. In the current study, a series of higher mass peaks were observed in the precursor ion 369.4 scan. This series had major peaks at m/z 1117, 1145, and 1173 and are likely to represent the ammonium adducts of the ω Type I cholesterol di-esters[19–21] CE 18:1/30:1, CE 18:1/32:1, and CE 18:1/34:1. However, due to low abundance (<5% of total CE ion abundance) and a lack of internal standards, their quantification was not performed in the current study. The lipidome of tears was remarkably similar to that of meibum, with the exception of PL. Molecular speciation of all lipid classes was similar between tears and meibum, again with the exception of PL.

Ceramide, free cholesterol, squalene, di-esters, and free FA have been reported in tears[22,23] and/or meibum.[13,21,24,25] In the current study, low levels of ceramide were detected in precursor ion scans of tears, however, the signal-to-noise ratio was below the limit of detection. Quantification of di-esters was not attempted due to the lack of appropriate internal standards. Free cholesterol was not measured in the current study as it is not well ionized by ESI. Detection of free fatty acids and squalene was not attempted as these lipid classes are not amenable to the MS/MS approach used.

A key result in the current study is the substantially higher mole fraction of PL in tears compared with meibum. While relative quantities of WE, CE, OAHFA, and TAG remain consistent, the mole fraction of PL is increased by four orders of magnitude in tears. Phospholipids have been detected in tears in a number of studies[22,23,26,27] and the species detected are relatively consistent across reports. In contrast, detection of PL in meibum has been controversial, with some groups identifying a range of PL,[13,26] and others failing to detect their presence or reporting detection at concentrations below a reasonable level of quantification.[28,14,29] We suggest the discrepancy may be a result of a few of factors, primarily differences in sampling techniques and analytical methods. Firstly, meibum samples are likely to have some degree of contamination from tears, and both tears and meibum may contain contamination from cells of the lid margin. Additionally, considering the high level of PL found in tears, meibum samples would require only a minute amount of tear contamination to allow detection of PL.

Secondly, the analytical techniques used by different groups have different sensitivities for PL. Recent studies that detected PL in meibum used ESI coupled with MS/MS (precursor ion and/or neutral loss), an approach that is well suited to ionization of polar PL and has proven highly effective in detecting low-abundance species in mixtures. Notably, Lam et al.[13] and Saville et al.[26] both detected PL in meibum. Saville detected total PC and SM concentrations of 0.0018% (wt/wt) while Lam detected total PC and SM concentrations of 0.222% (% mole fraction). These results are comparable with the current study at 0.0037% mole fraction. Butovich reported an upper limit of total PC and SM concentrations of 0.015%. This finding was based on the detection of a phosphocholine fragment below the limit of quantification.[28] Chen et al.[14] did not detect PL in meibum using ESI-MS, however, they did not use MS/MS techniques. In contrast, Dean et al.[27] used precursor ion experiments to detect a range of PC species in tears, and in fact demonstrated a very similar PC lipid profile to the current study.

A number of possible factors may explain our data reporting a higher mole ratio of PL in tears compared with meibum: (1) meibum is not the sole source of PL in tears of healthy subjects. Cells from the lid margin, lacrimal gland, as well as cells from the meibomian gland may supply PL to the tear film, (2) a physical mechanism (e.g., preferential partitioning) transfers PL selectively into the lipid and/or aqueous layer of the tears. The polar nature of PL (and also OAHFA) may cause rapid transfer from the eyelid meibum into the lipid layer of the tears, increasing the relative proportion in the tear samples as compared with meibum. (O-acyl)-ω-hydroxy fatty acid is found at a nearly 50% higher mole fraction in tears and may represent a similar effect. These polar lipids may also partially partition into the aqueous layer of the tear film, increasing their relative proportion in tears compared with meibum, and (3) a mechanism of biological sequestration concentrates PL in tears. PL have been identified bound to lipocalin in the tear film,[27] suggesting an active biological sequestration mechanism for PL lipids. Depending on relative turnover rates of meibum and the tear film, this sequestration may elevate the relative PL levels in tears.

A second notable result is that LPC is the most abundant PL in tears. In healthy human tissue, LPC represents approximately 1% to 5% of the total PC. In tears however, LPC represented nearly 50% of the total PL, and were more than twice as abundant as PC. This is in agreement with Dean et al.[27] and Rantamaki et al.,[23] who both report LPC to be a dominant PC subclass, while Saville et al.[26] did not search for PL in the mass range of LPC Interestingly, this high level of LPC raises the possibility that LPC is being produced in the tear film itself, likely by degradation of PC. The most common biosynthetic pathway for LPC is the removal of the *sn*-2 acyl chain of PC by phospholipase A_2 (PLA_2), resulting in free FA and LPC. High concentrations of secretory PLA_2 have been reported in human tears,[30] suggesting that the breakdown of PL to LPC could occur in the tear film. In addition, phospholipase C, which catalyses the removal of the head group of PC, has been detected at high levels in human tears, and its product, diacylglycerol has been reported in tears.[31] These studies indicate tear fluid has two independent pathways for enzymatic removal of PC, suggesting that PC may in fact be detrimental to the physical properties of tear fluid, possibly at the interface of the lipid and aqueous layers.

Conclusions

Identification of the complete lipidomes of human meibum and tears has been an ongoing analytical challenge for over 40

years. With the advances in biomarker detection, personalized medicine and targeted treatments, the drive to analyze complete lipidomes of individual patient samples has grown substantially. The analytical techniques described in this study have been shown capable of identification and quantification of what is believed to be the major classes of tear and meibum lipids. Our study represents the most comprehensive quantitative lipidome of individual subjects of tears yet published.

By comparing tears and meibum in patient-matched samples from healthy human subjects, the current study has shown that both class and molecular lipid profiles are nearly identical between tears and meibum. The one notable exception being PL, having a mole ratio four of orders of magnitude greater in tears than in meibum. It is therefore important to note that by analyzing meibum alone one does not gain a complete understanding of the lipids present on the ocular surface. Whether phospholipids are an integral part of the tear film, or simply a byproduct of cell debris in the eye is still to be determined.

Acknowledgments

The authors thank Varghese Thomas for technical assistance with statistical analysis, Jennifer Saville (UOW) for helpful discussion, and Mark Allen at Advion Biosciences, Inc. for assistance with the nanomate hardware.

Supported by grants from the Australian Research Council (ARC) and the Brien Holden Vision Institute (New South Wales, Australia) through the Linkage Project scheme (LP0989883). A Future Fellowship from the ARC (FT110100249; TWM) and supported by the ARC Centre of Excellence for Free Radical Chemistry and Biotechnology (CE0561607; SHJB).

Disclosure: **S.H.J. Brown**, None; **C.M.E. Kunnen**, None; **E. Duchoslav**, None; **N.K. Dolla**, None; **M.J. Kelso**, None; **E.B. Papas**, None; **P. Lazon de la Jara**, None; **M.D.P. Willcox**, None; **S.J. Blanksby**, None; **T.W. Mitchell**, None

References

1. Yu J, Asche CV, Fairchild CJ. The economic burden of dry eye disease in the United States: a decision tree analysis. *Cornea*. 2011;30:379–387.
2. The definition and classification of dry eye disease: report of the definition and classification Subcommittee of the International Dry Eye WorkShop (2007). *Ocul Surf*. 2007;5:75–92.
3. Green-Church KB, Butovich I, Willcox M, et al. The international workshop on meibomian gland dysfunction: report of the subcommittee on tear film lipids and lipid-protein interactions in health and disease. *Invest Ophthalmol Vis Sci*. 2011;52:1979–1993.
4. Gipson IK. Distribution of mucins at the ocular surface. *Exp Eye Res*. 2004;78:379–388.
5. Shine WE, McCulley JP. Polar lipids in human meibomian gland secretions. *Curr Eye Res*. 2003;26:89–94.
6. Nicolaides N, Ruth EC. Unusual fatty acids in the lipids of steer and human meibomian gland excreta. *Curr Eye Res*. 1982;2:93–98.
7. Kulovesi P, Telenius J, Koivuniemi A, Brezesinski G, Vattulainen I, Holopainen JM. The impact of lipid composition on the stability of the tear fluid lipid layer. *Soft Matter*. 2012;8:5826–5834.
8. Pucker AD, Nichols JJ. Analysis of meibum and tear lipids. *Ocul Surf*. 2012;10:230–250.
9. Matyash V, Liebisch G, Kurzchalia TV, Shevchenko A, Schwudke D. Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. *J Lipid Res*. 2008;49:1137–1146.
10. Schultz GA, Corso TN, Prosser SJ, Zhang S. A fully integrated monolithic microchip electrospray device for mass spectrometry. *Anal Chem*. 2000;72:4058–4063.
11. Deeley JM, Mitchell TW, Wei X, et al. Human lens lipids differ markedly from those of commonly used experimental animals. *Biochim Biophys Acta*. 2008;1781:288–298.
12. Han X, Yang K, Gross RW. Multi-dimensional mass spectrometry-based shotgun lipidomics and novel strategies for lipidomic analyses. *Mass Spectrom Rev*. 2012;31:134–178.
13. Lam SM, Tong L, Yong SS, et al. Meibum lipid composition in Asians with dry eye disease. *PLoS One*. 2011;6:e24339.
14. Chen J, Green-Church KB, Nichols KK. Shotgun lipidomic analysis of human meibomian gland secretions with electrospray ionization tandem mass spectrometry. *Invest Ophthalmol Vis Sci*. 2010;51:6220–6231.
15. Butovich IA, Arciniega JC, Lu H, Molai M. Evaluation and quantitation of intact wax esters of human meibum by gas-liquid chromatography-ion trap mass spectrometry. *Invest Ophthalmol Vis Sci*. 2012;53:3766–3781.
16. Chen J, Green KB, Nichols KK. Quantitative profiling of major neutral lipid classes in human meibum by direct infusion electrospray ionization mass spectrometry. *Invest Ophthalmol Vis Sci*. 2013;54:5730–5753.
17. Nicolaides N, Kaitaranta JK, Rawdah TN, Macy JI, Boswell FM III, Smith RE. Meibomian gland studies: comparison of steer and human lipids. *Invest Ophthalmol Vis Sci*. 1981;20:522–536.
18. Butovich IA. Cholesteryl esters as a depot for very long chain fatty acids in human meibum. *J Lipid Res*. 2009;50:501–513.
19. Butovich IA, Wojtowicz JC, Molai M. Human tear film and meibum. Very long chain wax esters and (O-acyl)-omega-hydroxy fatty acids of meibum. *J Lipid Res*. 2009;50:2471–2485.
20. Butovich IA, Borowiak AM, Eule JC. Comparative HPLC-MS analysis of canine and human meibomian lipidomes: many similarities, a few differences. *Sci Rep*. 2011;1:24.
21. Nicolaides N, Santos EC. The di- and triesters of the lipids of steer and human meibomian glands. *Lipids*. 1985;20:454–467.
22. Saville JT, Zhao ZJ, Willcox MDP, Blanksby SJ, Mitchell TW. Detection and quantification of tear phospholipids and cholesterol in contact lens deposits: the effect of contact lens material and lens care solution. *Invest Ophthalmol Vis Sci*. 2010;51:2843–2851.
23. Rantamaki AH, Seppanen-Laakso T, Oresic M, Jauhiainen M, Holopainen JM. Human tear fluid lipidome: from composition to function. *PLoS One*. 2011;6:e19553.
24. Nichols KK, Ham BM, Nichols JJ, Ziegler C, Green-Church KB. Identification of fatty acids and fatty acid amides in human meibomian gland secretions. *Invest Ophthalmol Vis Sci*. 2007;48:34–39.
25. Borchman D, Foulks GN, Yappert MC, Milliner SE. Differences in human meibum lipid composition with meibomian gland dysfunction using NMR and principal component analysis. *Invest Ophthalmol Vis Sci*. 2012;53:337–347.
26. Saville JT, Zhao ZJ, Willcox MDP, Ariyavidana MA, Blanksby SJ, Mitchell TW. Identification of phospholipids in human meibum by nano-electrospray ionisation tandem mass spectrometry. *Exp Eye Res*. 2011;92:238–240.
27. Dean AW, Glasgow BJ. Mass spectrometric identification of phospholipids in human tears and tear lipocalin. *Invest Ophthalmol Vis Sci*. 2012;53:1773–1782.
28. Butovich IA, Uchiyama E, McCulley JP. Lipids of human meibum: mass-spectrometric analysis and structural elucidation. *J Lipid Res*. 2007;48:2220–2235.
29. Butovich IA, Uchiyama E, Di Pascuale MA, McCulley JP. Liquid chromatography-mass spectrometric analysis of lipids present in human meibomian gland secretions. *Lipids*. 2007;42:765–776.
30. Saari KM, Aho V, Paavilainen V, Nevalainen TJ. Group II PLA(2) content of tears in normal subjects. *Invest Ophthalmol Vis Sci*. 2001;42:318–320.
31. Campbell D, Griffiths G, Tighe BJ. Tear analysis and lens-tear interactions: part II. Ocular lipids-nature and fate of meibomian gland phospholipids. *Cornea*. 2011;30:325–332.

Investigative Ophthalmology & Visual Science