Extensive characterization of human tear fluid collected using different techniques unravels the presence of novel lipid amphiphiles

Authors: Sin Man Lam\textsuperscript{1,2}, Louis Tong\textsuperscript{3,4,5,6}, Xinrui Duan\textsuperscript{7}, Andrea Petznick\textsuperscript{3}, Markus R. Wenk\textsuperscript{2,8*}, Guanghou Shui\textsuperscript{1*}

Affiliations:

\textsuperscript{1}State Key Laboratory of Molecular Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China

\textsuperscript{2}Department of Biological Sciences, National University of Singapore, Singapore, Singapore.

\textsuperscript{3}Singapore Eye Research Institute, Singapore, Singapore.

\textsuperscript{4}Singapore National Eye Centre, Singapore, Singapore.

\textsuperscript{5}Duke-NUS Graduate Medical School, Singapore, Singapore.

\textsuperscript{6}Department of Ophthalmology, Yong Loo Lin School of Medicine, Singapore, Singapore

\textsuperscript{7}Life Sciences Institute, National University of Singapore, Singapore, Singapore.

\textsuperscript{8}Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore

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*To whom correspondence should be addressed:

**Guanghou Shui**

State Key Laboratory of Molecular Developmental Biology  
S2-416, Institute of Genetics and Developmental Biology  
Chinese Academy of Sciences  
No. 1 West Beichen Road  
Chaoyang District  
Beijing, 100101, China  
Email: ghshui@genetics.ac.cn  
Tel: +86 6480 7781  
Fax: +86 6480 7781

**Markus R. Wenk**

National University of Singapore  
Yong Loo Lin School of Medicine  
Centre for Life Sciences  
28 Medical Drive, CELS #04-26  
Singapore 117607  
E-mail: markus_wenk@nuhs.edu.sg  
Tel: +65 6516 3624  
Fax: +65 6777 3271
ABSTRACT

The tear film covers the anterior eye and the precise balance of its various constituting components is critical for maintaining ocular health. The composition of the tear film amphiphilic lipid sublayer, in particular, has largely remained a matter of contention due to the limiting concentrations of these lipid amphiphiles in tears that render their detection and accurate quantitation tedious. Using systematic and sensitive lipidomic approaches, we validated different tear collection techniques and report the most comprehensive human tear lipidome to date; comprising more than 600 lipid species from 17 major lipid classes. Our study confers novel insights to the compositional details of existent tear film model, in particular the disputable amphiphilic lipid sublayer constituents, by demonstrating the presence of cholesteryl sulfate, O-acyl-ω-hydroxy fatty acids, and various sphingolipids and phospholipids in tears. The discovery and quantitation of the relative abundances of various tear lipid amphiphiles reported herein are expected to have profound impact on the current understanding of the existent human tear film model.
Supplementary Keywords: Lipidomics; mass spectrometry; dry eye syndrome; meibum; tear lipidome; cholesteryl sulfates; O-acyl-ω-hydroxy fatty acids

Abbreviations: AqD: aqueous-deficiency; APCI, atmospheric pressure chemical ionization; Cer, ceramides; CE, cholesteryl esters; CS, cholesteryl sulfate; DAG, diacylglycerides; DES, dry eye syndrome; Cho, free cholesterols; GM3, NeuAcα2-3Galβ1-4Glcβ-Cer; GC, gas chromatography; GluCer, glucosylceramides; S1P, sphingosine-1-phosphates; LBPA, lyso-bisphosphatidic acids; LPS, lyso-PS; MGD, meibomian gland dysfunction; OSDI, modified ocular surface disease index; MRM, multiple reaction monitoring; NP, normal phase; OAHFA, O-acyl-ω-hydroxy-fatty acids; PA, phosphatidic acids; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PG, phosphatidyglycerols; PI, phosphatidylinositol; PS, phosphatidylserines; PLTP, phospholipid transfer protein; RP, reverse phase; Schir I, Schirmer’s type I length; SM, sphingomyelins; TBuT, tear breakup time; TAG, triacylglycerides; WE, wax esters.
INTRODUCTION

The tear fluid covers the anterior surface of the cornea and serves critical functions in maintaining the homeostasis of the ocular surface. Tears hydrate and lubricate the mucous membranes constituting the ocular surface, supply nourishment to the avascular corneal epithelium and provide a smooth optical surface essential for visual acuity. The drainage of tears via the lacrimal puncta flushes contaminants and irritants out of the eye, thereby functioning as a first line of defense for the anterior eye against invading pathogens (1,2). The typical volume of tears in normal eyes ranges from 3.4 to 10.7 μl per eye (3). Despite its small volume, tear represents a biological fluid of immense complexities with a wide array of proteins/peptides, electrolytes, lipids and small molecule metabolites contributed by distinct sources (1). The precise balance of these various metabolites is crucial in ensuring proper physiological function and maintaining biophysical integrity of the precorneal tear film. Perturbations in this delicate equilibrium may be manifested in various ocular conditions such as the dry eye syndrome (DES) and blepharitis (1,4,5).

Recent decades have witnessed tremendous progress in the systematic profiling of proteins as well as small molecule metabolites present in tears (1,6-9). Furthermore, with technological advancements in mass spectrometry and nuclear magnetic resonance spectroscopy, the lipid composition of the human meibomian gland secretions, the predominant source of lipids for the precorneal tear film, has been elucidated with great compositional and structural details (10-19). A comprehensive lipidomic analysis of the tear fluid per se, however, has lingered behind. The appreciably lower lipid concentration in human tears, which is largely aqueous compared to the meibum, as well as the limiting amount of unstimulated tears that could be obtained from individual subjects, present a new level of challenge for the field of analytical lipidomics. In spite of these analytical challenges, a comprehensive elucidation of the tear lipidome is imperative to unravel the biophysical properties of the tear film. As a proximal fluid to the anterior eye, the tear
is a dynamic reflection of metabolites present at the ocular surface and therefore represents a rich source for the discovery of ocular disease-related biomarkers. A systematic comparison of tear collection techniques will therefore facilitate the use of human tears for biomarker discovery pertaining to both ocular and systemic diseases, which would be of immense importance to the field of ocular research and beyond.

A preliminary analysis of human tears using mass spectrometry had concluded that meibum represents the prominent source of lipids for the tear film (13). The presence of phosphatidylcholines (PC), sphingomyelins (SM), wax esters (WE) and free cholesterol (Cho) in tears was demonstrated using infrared spectroscopy (20). Saville et al subsequently provided the relative abundances of individual species of PC and SM in human tears (21). The polar lipid composition was further expanded by two other groups, who reported a predominance of lysophosphatidylcholines (lyso-PC) (2,22). In addition to PC and SM, Rantamäki et al also reported the presence of triacylglycerides (TAG), ceramides (Cer) and phosphatidylethanolamines (PE) in tears. As most of these studies only focused on selected lipid groups in tears, a comprehensive lipidome with sufficient details to encompass the sheer complexities of the various lipid classes in tears is therefore still lacking. Apart from the analytical challenges posed by the limited abundance but yet great complexities of lipid species in tears, the biochemical analysis of tears has also been plagued by the problem of specimen collection. Thus, a systematic comparison of tear lipidomes obtained using different collection methods will therefore be of immense practical value for the ocular community in facilitating the standardization of collection procedures. This will also aid future clinical designs to use human tears as a proximal fluid for biomarker discovery.

Herein, we report HPLC/MS-based approaches for the comprehensive qualitative and comparative characterization of human tear and meibum lipidomes from individual subjects. Our analyses revealed that human tears comprise more than 600 individual lipid species from 17 distinct lipid classes in volumes less than 10 μL per sample. We also report, for the first time, the
presence of a novel lipid amphiphile, cholesteryl sulfate (CS), in the human tears and meibum, which would have considerable impact on existent tear film model. In addition, systematic comparisons of the tear lipidomes collected with different sampling techniques were evaluated.

MATERIALS AND METHODS

Study group

For preliminary analysis and method validation, 45 patients and 15 volunteers were recruited to contribute pooled or individual samples of tears and meibum. The patients were diagnosed with dry eye syndrome at the Singapore National Eye Center. Detailed demographic information for the study group can be found in the supplemental section (Supplementary Tables S1-S2). Written informed consent was obtained from all participating subjects and the procedure for the project was specifically approved by the SingHealth Centralised Institutional Review Board (CIRB Reference No: 2008/611/A). We adhered to the tenets of the declaration of Helsinki for all human research conducted in this study. The detailed clinical procedures had been reported elsewhere (10).

Sample collection

Capillary tears were collected using 5μL glass microcapillary tubes (Blaubrand® intraEND, Wertheim, Germany). The collection time was limited to a maximum of 5 min with collection volumes between 2 to 10 μL. The capillary tube rested in the lateral tear meniscus and care was exercised to minimize contact with the bulbar conjunctiva or the lid margin. Reflex tearing was observed in some subjects (Supplementary Table S2). Flush tears were collected by instilling 20μL of non-preserved, unit dose saline (sodium chloride injection 0.9%, B Braun, Germany) into the inferior palpebral fold using an Eppendorf pipette. Care was taken not to contact the eye with the pipette tip. Participants were then instructed to gently close and rotate...
their eyes. Tears were then immediately collected by the capillary tubes as aforementioned. Tears were expelled from the capillary tubes into glass vials. Capillary tubes were washed two times with chloroform:methanol (2:1) to remove residual lipids and the washings were also pooled together with the tear samples and stored at -80°C until further analysis. Schirmer’s tears were collected from both eyes of the subjects without anesthesia (Bausch & Lomb® Sno strips, New York, USA) and the strips were stored at -80°C in glass vials until further analysis. Meibum samples were collected as previously described (10).

### Lipid extraction

**Capillary tears.** Lipids were extracted at 4°C for 1h with 200μL ice-cold of chloroform:methanol (1:1) at 1200 rpm in a thermomixer. Samples were then centrifuged at 3000 rpm for 5min at 4°C and 200 μL of clear supernatant was transferred to fresh glass vials. Second extraction was carried out by repeating the procedures and the extracted organic fractions were pooled and dried using speed-valco (Thermo Savant, Milford, USA). Dried lipid extracts were stored at -80°C until mass spectrometric analysis.

**Schirmer’s tears.** The first three 5-mm segments of the strips were cut into fine pieces (of approximately 2mm) using micro-scissors pre-washed with methanol between samples. Lipids were extracted overnight at 4°C with 900μL of ice-cold chloroform:methanol (1:2) at 1200 rpm in a thermomixer. 540 μL of deionised water and 300 μL of chloroform were added to separate phases. Samples were vortexed and incubated on ice for 3min. Samples were then centrifuged at 3000 rpm at 4°C for 5min and the lower organic phase was extracted. The aqueous phase was re-extracted with 500 μL of chloroform and 50 μL of 0.1M hydrochloric acid. The extracted organic fractions were pooled and dried using speed-valco (Thermo Savant, Milford, USA). Dried lipid extracts were stored at -80°C until mass spectrometric analysis.

**Meibum.** Meibum samples were extracted as previously described (10).
Mass spectrometric analysis

All lipid species were quantitated using LC-MRM in a combined workflow, but high-resolution MS was used for characterization and confirmation of lipid identities, or for illustrative purposes. Concentrations of individual samples were pre-adjusted for mass spectrometric analyses by taking out 5 µL of each sample for estimation of total CE and total WE content such that the final adjusted concentrations for mass spectrometric analyses lie within the linear dynamic range of the various lipid classes analysed.

*Normal-phase LC/MS.* Polar lipids were analyzed using an Agilent 1200 HPLC system coupled with an Applied Biosystem Triple Quadrupole/Ion Trap mass spectrometers 3200Qtrap as described previously (23). Separation of individual lipid classes of polar lipids by normal phase HPLC was carried out using a Phenomenex Luna 3µ-silica column (i.d. 150x2.0 mm) with the following conditions: mobile phase A (chloroform:methanol:ammonium hydroxide, 89.5:10:0.5), B (chloroform:methanol:ammonium hydroxide:water, 55:39:0.5:5.5). Multiple reaction monitoring (MRM) transitions were set up for comparative analysis of various polar lipids. Individual lipid species were quantified by referencing to spiked internal standards. PC-14:0/14:0, PC34:1-d31, LPC-C20, PE-14:0/14:0, PE34:1-d31, LPE-C17, PS-14:0/14:0, LPS-C17, PA-17:0/17:0, LPA-C14, PG-14:0/14:0, C14-LBPA, C8-GluCer, C17-Cer, C17-S1P and C12-SM were obtained from Avanti Polar Lipids (Alabaster, AL, USA) and LIPIDMAPS. Dioctanoyl phosphatidylinositol (PI, 16:0-PI) was obtained from Echelon Biosciences, Inc. (Salt Lake City, UT, USA) and used together with PI34:1-d31 (LIPIDMAPS) for phosphatidylinositol and lysophosphatidylinositol quantitation. Qualitative deuterated lipid standards from LIPIDMAPS were pre-corrected using quantitative lipid standards prior to their use for quantitation. GM3 species were quantified using C17-GM3 as an internal standard. OAHFA species were quantitated using OAHFA 18:1/16:0, which was synthesized as previously described (17).
Reverse-phase LC/MS. Phospholipids, sphingolipids, CS, glycerol lipids (diacylglycerides, DAG; triacylglycerides, TAG), WE, cholesteryl esters (CE) were analyzed using a modified version of reverse phase HPLC/ESI/MS/MS described previously (24). Briefly, separation of lipids aforementioned was carried out on a Phenomenex Kinetex 2.6µ-C18 column (i.d. 4.6x100mm) using an isocratic mobile phase chloroform:methanol:0.1M ammonium acetate (100:100:4) at a flow rate of 150 µl/min for 22 min. CS and individual CE species were quantified using d7-CS (CDN isotopes) and d6-C18 CE (CDN isotopes) as internal standards, respectively. MRM analysis of CS was validated using d7-CS as internal standard (Supplementary Figure S1). Using neutral loss-based MS/MS techniques, the levels of TAG were calculated as relative contents to the spiked d5-TAG 48:0 internal standard (CDN isotopes), while DAG species were quantified using 4ME 16:0 Diether DG as an internal standard (Avanti Polar Lipids, Alabaster, AL, USA). Levels of WE were quantified using three standards, palmitoyl palmitate (C16:0C16:0) for wax esters with saturated fatty acyl heads, while wax esters containing unsaturated fatty acyl heads were quantitated using C18:1C26:0 and C18:1C28:0, which were synthesized as in-house using oleic acid-1,2,3,7,8,9,10-13C7 following the procedure described previously (25). The detailed mass spectrometric procedures for analysis of wax esters had been reported elsewhere (26).

High-resolution MS. Single stage mass spectrometry profiles of lipid extracts were also carried out using an Accela HPLC system coupled with an LTQ Orbitrap XL hybrid Fourier Transform Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA) using both reverse phase HPLC/MS and normal phase HPLC/MS approach as aforementioned. In the case of reverse-phase LCMS, 5 µL of each extract was injected into a Phenomenex Kinetex 2.6µ-C18 column (i.d. 4.6x100 mm) with the mobile phase as aforementioned at a flow rate of 120 µL/min for a total duration of 30 min. In normal phase LCMS, separation of individual lipid classes of polar lipids by normal phase HPLC was carried out using a Phenomenex Luna 3µ-silica column (i.d. 150x2.0 mm) with the following conditions: mobile phase A
(chloroform:methanol:ammonium hydroxide, 89.5:10:0.5), mobile phase B (chloroform:methanol:ammonium hydroxide:water, 55:39:0.5:5.5). Mass spectrometry profiles were recorded under both positive and negative modes in separate runs (resolution 60 000), and mass accuracy of less than 2 ppm were obtained throughout the analytical runs.

Atmospheric Pressure Chemical Ionization (APCI). Free Cho and CE were further analyzed using HPLC/APCI/MS/MS as previously described with corresponding d6-Cho and d6-C18 CE (CDN isotopes) as internal standards (27). As we had previously shown that HPLC/APCI/MS allows for a wide dynamic linearity range in measuring the levels of CE in plasma samples due to less ion suppression compared to ESI mode (27), the absolute amount of total CE obtained in APCI/MS/MS was used to correct results obtained in ESI/MS/MS analysis of individual CE species.

Statistical Analysis

The absolute levels of lipids in tears obtained using different collection techniques were compared using one-way ANOVA with post hoc TukeyHSD test. For all analyses, **p<0.001; ** p<0.01; *p<0.05; #0.05<p<0.10.

RESULTS

Validation of Schirmer’s strip method of tear collection

In order to ensure that tears collected using Schirmer’s strips represent an accurate reflection of the dynamic lipid microenvironment at the ocular surface we considered a few key issues: (1) What is the lipid background in the paper strip per se? (2) To what extent does the strip act as a chromatographic system? (3) Does the portion of tears captured by the strip represent a true reflection of the concentrations of lipid metabolites in the tear reservoir?
We found that lipids were concentrated in the first two segments of the strip, with the -5-0 mm segment, which is in direct contact with the conjunctiva during the collection process, having the highest abundance of all lipid classes (Supplementary Fig. S2). The blank Schirmer’s strips gave rise to considerable background noise in the mass spectrometric analysis of the individual lipid classes, but did not produce appreciable peak shapes in the mass spectra (data not shown). This indicates that the strip materials did not contain the endogenous tear lipids and the Schirmer’s strips are suitable for collecting tears intended for lipid analysis. In order to maximize the accuracy of our analytical results, we had chosen to extract and analyse lipids only from the first three segments (i.e. -5-10mm, Schirmer’s length=10mm) of the strips regardless of the actual length of the entire wetted portion, as it is apparent from our preliminary analyses that lipids, especially the nonpolar lipids such as CE and WE, do not travel along the strips as efficiently as the aqueous portion of the tears. This approach could minimize the level of background noise presented by the strip materials without significantly compromising the extraction efficiency of the endogenous tear lipids, as the first three strip segments contained at least 80% of all lipids along the entire wetted area (Supplementary Table S3). Furthermore, lipid extraction from a fixed length of the strip could standardize the background noise in individual samples for unbiased comparison of endogenous lipid levels between samples. Also, the region of Schirmer’s strips furthest away from the notch of the papers might unavoidably contact with facial skin during the collection process, especially for subjects with longer Schirmer’s lengths, and therefore by considering only the first three 5-mm segments of the strips could minimize contamination with skin lipids.

We next investigated if the Schirmer’s strips captured lipids in a manner that reflects the actual concentrations of lipids from the respective classes in the tear reservoir using a lipid cocktail consisting of different lipid standards with concentrations that approximate the relative abundances of various lipid classes in the human tears. The lipid cocktail was first dried under pure nitrogen gas and the dried samples were then reconstituted in phosphate buffered saline and
diluted to create different concentrations of “artificial tear lipids”. Schirmer’s strips were placed in glass vials containing varying concentrations of the artificial tear lipids for five minutes and lipid extraction and mass spectrometric analyses were subsequently carried out on the first three 5-mm segments of the strips. An approximately linear relationship was observed between the raw intensities of individual lipid standards and their relative concentrations in the reconstituted lipid mixture (Supplementary Fig. S3), implying that Schirmer’s strip-collected portions were reflective of the actual concentration of lipids in the original aqueous lipid mixture.

Collectively, the data obtained demonstrated that the first three 5-mm segments of the wetted strip area (regardless of total wetted length) were sufficient to provide a tear lipidome that was reflective of the actual lipid compositions in the tear reservoir with negligible background interferences (Fig. 1; Supplementary Fig. S1-3; Supplementary Tables S3). The Schirmer’s strip method of tear collection is thus suitable for use in lipid analysis.

**Comparison of tear lipidomes obtained using different collection techniques**

As it had been documented that conflicting reports on the clinical biochemistry of tears could be attributed to differences in specimen collection (e.g. Schirmer’s strips or capillary tubes) (28), we next investigated the quantitative and qualitative differences in tear lipids collected using different techniques. Thus far, only preliminary statements had been made on the generally similar lipid profiles between tears collected using Schirmer’s strips and capillary tubes, but no detailed comparison was conducted (13). We systematically compared the different clinical techniques for tear collection based on the quantity and composition of lipids captured (Fig. 1-2). The Schirmer’s method of tear collection generally captures the highest absolute amounts of lipids (Fig. 2). CE and WE were found to be the predominant nonpolar lipid classes and PC was the major polar lipid fraction in all three collection procedures. Higher levels of all lipid classes, except NeuAcα2-3Galβ1-4Glcβ-Cer (GM3), in tears collected using Schirmer’s strips could be attributed to overall greater amount of tears collected with this method. The enhanced detection
of specific lipid classes under an overall limiting absolute concentration of total lipids in capillary-collected tears could be attributed to the different limits of detection using MRM for the various lipid classes investigated (data not shown). We found no appreciable differences in lipid composition of tears collected using capillary without saline flush from that with saline flush (apart from the lower absolute amounts of lipids in the latter), which is in good agreement with a previous study that reported an essentially similar tear proteome using the two methods (29).

On the basis of the similar tear lipid profiles collected using the three methods, we adopted the Schirmer’s method for subsequent tear analysis in our clinical cohort primarily due to the overall greater absolute amounts of tear lipids that this method could capture. Furthermore, the Schirmer’s method is a routine clinical procedure in dry eye clinics and could be performed with relative ease. Aside from providing tear samples for analysis, the Schirmer’s method of tear collection also confers additional clinical information pertaining to the ocular health status of subjects compared to the capillary method (i.e. lower Schirmer’s I implies impaired tear secretion).

**Analytical workflow facilitated characterization of polar lipid species based on retention times, accurate masses and specific MRM transitions**

In the current study, all individual lipid species in human tears were measured in a simple analytical workflow, using either normal phase (NP)- or reverse phase (RP)-HPLC/MS (Fig. 3-4 See Supplementary Fig. S4). Pooled tear samples from DES patients were first analyzed to determine representative baseline values (Supplementary Table S4). The lipid composition of tear fluid derived from the pooled patient sample had also been validated in individual Schirmer’s samples from a cohort of 28 DES patients with a reasonable range in individual DES clinical indicators, including the tear breakup time (TBuT), Schirmer’s type I length (Schir I), modified ocular surface disease index (OSDI) and Baylor score for corneal staining (Baylor) (See Supplementary Fig. S5 and Table S5). The lipidomic composition of tear fluid displayed a
striking overall consistency even for samples with very low wetted lengths (i.e. 0-3mm), demonstrating the sensitivity of our analytical methods and the feasibility of our protocol to be translated for use in dry eye clinics for large-scale analysis of tear samples from severe DES patients with significantly impaired lacrimal function.

Our analyses revealed the astonishing complexities of the human tear fluid lipidome, consisting of the nonpolar lipid classes of CE, WE, TAG, DAG, and free Cho; the glycerophospholipid classes such as PC, PE, phosphatidylinositol (PI), phosphatidylglycerols (PG), phosphatidylserines (PS), phosphatidic acids (PA) and lyso-bisphosphatidic acids (LBPA); the sphingolipid classes including SM, Cer, glucosylceramides (GluCer), GM3 and sphingosine-1-phosphate (S1P); as well as CS and O-acyl-ω-hydroxy fatty acids (OAHFA), a unique class of amphiphilic compounds first reported in meibum by Butovich (17). The tear (and meibum) lipidomes presented in this study are a significant expansion from previous report by our group (10) and others (2,21,22). First, CS, LBPA, S1P and GM3 have neither been previously reported in tear nor meibum (Table 1). Second, we expanded the list of phospholipid classes found in tears to include PI, PG and PA from previously reported PC, PE and PS (Table 1) (2,22,30). In addition, we report the relative abundances of individual OAHFA species in the tear fluid using the MRM method we had previously developed based on meibum samples (10).

Of particular relevance to tear film structural integrity, a diverse range of amphiphilic lipid compounds was detected. CS (Fig. 4A) and total OAHFA (Fig. 4B) were found to constitute approximately 0.14% and 2.52% of the total tear lipidomes, respectively (Table 1). Besides, we detected the unambiguous presence of LBPA in the human tears, with LBPA 36:2 as the most abundant species (Fig. 4G). LBPA represents an isobaric class of lipids to PG, for which the predominant species in tears was found to be PG34:2. Individual species of LBPA and its corresponding isobaric PG species were distinguishable via their different retention times using HPLC separation. In addition, various classes of phospholipids and their lyso-forms were also detected. In accordance with previous reports (2,22), lysophospholipids formed a major proportion
of the phospholipid pool (Fig. 3B; See Supplementary Table S4). In agreement with Rantamäki et al, we detected appreciable quantities of PE (Supplementary Fig. S3C) and lyso-PE (Fig. 4D) in human tears collected using the Schirmer’s strips in both pooled and individual samples. An appreciable amount of lyso-PS (LPS) species was found in human tears that consisted predominantly of three distinct species, namely LPS16:0, LPS18:0 and LPS18:1 (Fig. 4E), which were different from the unusual species (LPS19:3; LPS20:2) earlier reported by Rantamäki et al (2).

**Quantitative corrections of major neutral lipid classes in tears and meibum**

Consistent with previous reports (13,20), CE and WE were unambiguously detected in human tears as the predominant nonpolar lipid classes in our study. A notable analytical advancement in our current study is the quantitative corrections of the proportions of WE and CE in the human tears and meibum (See Materials and Methods). Using our improved approach incorporating in-house synthesized $^{13}$C-labelled WE as internal standards (26), we found that WE constitute approximately 43% of the total lipids in the human meibum (Fig. 3B; See Supplementary Table S4), which is in excellent agreement with a recent value (40±10% (wt/wt)) determined by gas-chromatography (GC)-MS (19). Furthermore, we had detected and characterized the comprehensive profile of DAG species in human tears (Supplementary Fig. S6), which were in appreciably higher levels than that in human meibum (Supplementary Table S4). This class of compounds corresponded to an earlier preliminary observation stating that the human tears contained a new range of compounds with higher polarity than the nonpolar components of meibum that co-eluted with authentic DAG (13). On the other hand, Ham et al had also previously reported the presence of a few DAG species, such as DAG16:0/16:0 and DAG 16:0/18:0, in the human tears (31).

**DISCUSSION**
The foregoing results evaluated the suitability of the Schirmer's strip for collecting tears designated for lipid analyses; and systematically compared the different tear collection methods currently available in eye clinics in terms of both quantity and quality of lipids captured. A comprehensive lipidome of the human tear fluid was presented; and the relative abundances of various novel lipid amphiphiles in tears including CS and OAHFA were reported. The compositional details of the human tear lipidome reported herein have major implications on ocular surface biochemistry as well as tear film biophysics.

**Physiological significance of the tear lysophospholipidome**

In considering the possible physiological functions of the novel lipids detected in the human tears, it is important to reckon the unique biochemistry at the ocular surface and the critical importance of ocular homeostasis in maintaining precise vision. Valuable insights can be drawn from the tear fluid proteome, of which bactericidal proteins such as lysozyme, lactoferrin and lipocalin form the bulk constituents (1). In line with this, lyso-PC had been reported to increase the bactericidal activity of neutrophils (36), while lyso-PE were shown to possess antifungal and antibacterial activity in housefly (37). Therefore, the physiological significance of the tear lysophospholipidome seems to coincide with that of the tear proteome, which comprises a predominance of metabolites with microbicidal properties.

**Implications of lipid compositional details on existent tear film model**

The longstanding perception that phospholipids serve as an amphiphilic layer of the tear film lipid layer has been recently challenged due to the ambiguous occurrence of phospholipids in human meibum, the predominant source of lipids for tears (10,32). Our current analysis of the tear lipidome revealed that phospholipids comprise a substantial amount (approximately 6%) of the total tear lipids. The significant enrichment of phospholipids and sphingolipids in the human tear fluid compared to the meibum therefore has important implications for our understanding of tear
film physiology. On the other hand, the detection of considerable amount of CS in the tear fluid (approximately 0.14%) has introduced a novel, hitherto unknown amphiphile to the tear film (Fig. 5). CS had been shown to interact with phospholipids in a manner akin to cholesterol, inducing hydrocarbon ordering in lipid bilayers and stabilizing model membranes (33). Moreover, under physiological conditions, the sulfate moiety of CS is ionized, converting CS from a relatively hydrophobic molecule into a highly amphiphilic compound comprising a highly charged headgroup (34). While the physiological significance of CS in tears is presently obscure, CS might be a suitable candidate for the amphiphilic sublayer of the tear film based on its biophysical property as a stabilizing agent of biological membranes (34). Furthermore, CS (p<0.001) and OAHFA (p<0.05) represent the only lipid classes that were positively correlated with TBuT, which is a proximal measure of tear film stability after blinking (35).

CONCLUSION

Our in-depth analysis of the human tear lipidome has provided new insights pertaining to ocular surface biochemistry and tear film biophysics, especially with regard to composition of the amphiphilic lipid sublayer of the tear film. We have shown that the tear fluid is appreciably enriched in phospholipids and sphingolipids relative to the meibum, and demonstrated that a surplus of phospholipids and sphingolipids is available in tears to constitute the amphiphilic lipid sublayer. We have also put forth CS as a novel candidate for this interfacial lipid sublayer. The detailed evaluations of various sampling techniques could help standardize collection protocols, thereby facilitating future biomarker studies on tears. Furthermore, we had demonstrated the translational feasibility of using Schirmer’s strip for tear collection designated for lipid analyses, which confers high reproducibility in terms of lipid profiles across samples with a wide range of wetted lengths; and is thus applicable even to patients with severe DES.

The ocular tissues and fluids are potential reporters of integrated metabolic stress over prolonged periods, otherwise obscured by complex homeostatic mechanisms on a systemic scale.
The tear fluid, being the most accessible of all ocular fluids, therefore also represents a novel source of biomarkers for systemic diseases marked by long prodromal periods, extending the translational significance of the comprehensive human tear lipidome reported herein from the ocular field to other realms of clinical medicine.

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References


FIGURE LEGENDS

Figure 1. Schematic diagram illustrating the procedures for validating and comparing different tear collection techniques. Schirmer’s tears collected from four volunteers on two separate occasions were used to validate the gradient of lipid metabolites along the wetted length of the strips. Briefly, the wetted portion of each strip was cut into 5-mm segments and lipid extractions were carried out on individual segments. The segment before the notch of the Schirmer’s strip, which was not taken into account during the measurement of the Schirmer’s length, was denoted the -5-0mm segment. The representative lipidome of Schirmer’s strip-collected tears from normal volunteers was compared with that of capillary-collected tears with and without saline flush.

Figure 2. Comparison of the absolute amounts of lipids captured using the Schirmer’s strips (n=10) and capillary tubes with (n=5) or without (n=5) saline flush. Mean values were plotted. Error bars indicate standard errors of the means. ST: Schirmer’s tears; CT: capillary tears; CT saline: capillary tears with saline flush.

Figure 3. Establishment of a comprehensive lipidomic platform for qualitative and comparative elucidation of human tear and meibum lipidomes. (A) Schematic summary of analytical workflow employed. Human tear and meibum lipidomes were elucidated using HPLC/MS in different ionization modes (ESI +/-; APCI+). Different lipid classes were analyzed and quantitated chiefly using HPLC-MRM in the reverse phase (RP) and normal phase (NP). (B) Pie-charts illustrate the lipidomes of pooled sample of Schirmer’s strip-collected tears (n=8) and meibum (n=7) from DES patients.
**Figure 4. High resolution mass spectra for selected classes of polar lipids in tears.** Accurate masses of major species from lipid classes of (A) CS, (B) OAHFA, (C) LPC, (D) LPE, (E) LPS, (F) LPA and (G) LBPA were presented. Mass spectra were obtained using the LTQ Orbitrap XL with normal phase LC-separation.

**Figure 5. Human tear film model.** The tear film lipid layer comprises the superficial sublayer consisting predominantly of nonpolar lipids and an inner, amphiphilic sublayer, which facilitates the interaction between the polar and nonpolar components of tears. The composition of the amphiphilic lipid sublayer has remained a matter of contention (32,40). Our global lipidomic analysis had introduced CS as a novel candidate for the amphiphilic sublayer. Other possible amphiphilic lipid candidates were also listed. Percent abundances of respective lipid classes were presented in parentheses. Figure is not drawn to scale.
Table 1. An overview of major lipid classes in human tears. Values were presented as molar percentages of total lipids measured. Comparisons were made only with selected recent studies on human tear lipidomes derived using mass spectrometry. While the presence of PA in tears had been earlier suggested based on TLC results (22,41), no prior mass spectrometric analysis of tears had confirmed the presence of PA in tears. In contrast to earlier works, we found appreciable amounts of serine-containing phospholipids in tears. This apparent disparity might possibly be due to the usage of only ESI positive mode in the analysis of tears by Rantamäki et al, as PS species ionize preferentially in the negative mode. On the other hand, while Dean et al had employed neutral loss scan (NLS) in the negative mode for analysis of PS, they did not detect appreciable amount of PS in tears, which was possibly due to insensitive methodology (Limit Of Detection, 12ng/µL). The lack of column separation in the method used by Dean et al, coupled with the complex pool of phospholipids inherent in tear samples might have also significantly suppressed the ionization of PS species. Contrary to Saville et al and Dean et al who reported PE to be lower than the limit of detection in their analyses, we noted the unambiguous presence of PE in our tear samples.

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a: Undetectable using MS but presence indicated via TLC.

b: Detected using TLC; estimated to be approximately twice of TAG in abundance.

c: Amount of total SM approximately equal to total PC detected in tears.

d: Values presented were sum of diacyl- and lyso-phospholipids.

e: Below the limit of detection in their study.

+ Detected but relative abundances were not reported; - Not detected.
Quantification of lipid content in individual segments

Comparison between collection techniques (Quantity & Quality)

First three 5-mm segments
- Contained approx. 90% of major lipid classes regardless of wetted length
- Discarded to reduce background noise

Collection using Schirmer's strip
- For 5 minutes without anesthesia
- Schirmer's length (e.g. 13.5 mm)
- Wetted portion cut into 5mm segments; Un-wetted portion discarded
- Lipids extracted separately from individual segments
- Mass spectrometric analysis

Collection using glass microcapillary tubes
- With 20μL saline flush
- Without saline flush
- Lipid extraction
- Mass spectrometric analysis

- Capillary tears with saline flush (CT saline)
- Capillary tears (CT)

Schirmer's length (e.g. 13.5 mm)
- 0-5mm
- 5-10mm
- 10-15mm

Wetted portion cut into 5mm segments; Un-wetted portion discarded

For 5 minutes without anesthesia

- 5-0 mm
- 0-5mm
- 5-10mm
- 10-15mm

Lipids extracted separately from individual segments

Mass spectrometric analysis

Quantification of lipid content in individual segments
FIGURE 3

A

Tear or Meibum

NP-LCMS

PS PC PA PE PG PI LBPA SM GM3 S1P Cer GluCer OAHPA

ESI - + - - - - + + + - + + + + + + - + +

RP-LCMS

CE WE TAG DAG CS Cho PE

APCI

B

Tear Lipidome

Meibum Lipidome

CE

WE

PS PC PA PE PI SM CER LPA LPE LPC LPS LPI

TAG DAG FREE CHO CS OAHPA SPL&PL (~8%)

PS PC PA PE PI SM CER LPA LPE LPC LPS LPI

TAG DAG FREE CHO CS OAHPA SPL&PL (~0.6%)

SPL&PL (~8%)

(~0.6%)
FIGURE 4

A  
Relative Abundance

B  
Relative Abundance

C  
Relative Abundance

D  
Relative Abundance

E  
Relative Abundance

F  
Relative Abundance

G  
Relative Abundance
FIGURE 5

- Evaporation

40-90nm

- Air
  - Nonpolar Lipid Sublayer
    - CE (44.8), WE (35.2), TAG (2.8), DAG (0.3), Free Cho (5.9)
  - Amphiphilic Lipid Sublayer
    - OAHFA (2.5), CS (0.1), lysoPL (2.4), PL (4.1), SPL (1.8)
  - Aqueous-Mucin Gel Layer
    - water, salts, proteins, carbohydrates etc
  - Glycocalyx Layer
  - Corneal Epithelial Cells

Up to 4000nm

Side View of an Eye

Rotate 90° anticlockwise

Molecular Structure of Cholesteryl Sulfate