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Delineating a Novel Metabolic High Triglycerides-Low Waxes Syndrome That Affects Lipid Homeostasis in Meibomian and Sebaceous Glands

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Abstract

Meibomian glands that are embedded in tarsal plates of human eyelids, and sebaceous glands found in the skin, including that of eyelids, are two related types of holocrine glands that produce lipid-rich secretions called meibum and sebum. Pervasive ocular disorders, such as Meibomian gland dysfunction and dry eye, have been linked to changes in the lipid composition of meibum. However, in most described cases the changes were either small, or random, or insufficiently characterized on the molecular level. Here, we present results of comprehensive lipidomic analyses of meibum, tears and sebum of a patient whose secretions were highly abnormal (abnormal meibum, tears and sebum, or AMTS, patient). The lipidomes were characterized on the level of individual lipid species using ultra-high performance liquid chromatography and high resolution mass spectrometry. The major differences between the AMTS patient and normal age- and gender-matched subjects included, among others, severely diminished pools of normal meibomian lipids such as wax esters and cholesteryl esters in meibum and tears, a 2x increase in total cholesteryl esters to wax esters ratio, their skewed molecular profiles, a ~3x increase in free cholesterol to cholesteryl esters ratio, and, most importantly, a 20x to 30x increase in the triglycerides fraction over the norm. Sebum of the AMTS patient was also highly abnormal lacking major wax esters. Notably, the routine blood lipid panel test of the AMTS patient showed no

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Compliance with ethical standards: All procedures used for collecting human meibum samples were approved by the Institutional Review Boards of UTSW and KPUM and performed in accordance with the principles of the Declaration of Helsinki. Written informed consents were obtained from all study volunteers.

Data and materials availability: All data pertinent to this paper presented and discussed in the text.

Supplementary Data can be found at: Table S1

Declaration of competing interests: The authors have no conflicts of interest to disclose.

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abnormalities. The data imply that the AMTS patient had a severe, previously unreported, metabolic disorder that affected meibogenesis in Meibomian glands and sebogenesis in sebaceous glands. This is, to the best of our knowledge, a first observation of the condition that we have termed High Triglycerides/Low Waxes (HTLW) syndrome.

Keywords

Metabolic profiling; Meibum, human; Sebum, human; High triglycerides/low waxes syndrome; Lipidomics

1. Introduction

Meibomian gland (MG) impairments are associated with several ocular pathologies, most common of which are MG dysfunction (MGD) [1], Dry Eye (DE) syndrome [1,2], chalazia [3,4] and meibomitis-related keratoconjunctivitis [5–7]. Holocrine MGs produce secretions (also called *meibum* [8]) that are composed mostly of lipids, with some non-lipid inclusions of various nature [9]. The process of biosynthesis of meibum in differentiated meibocytes located in the acini of MGs [termed *meibogenesis* [10–12]] has not been characterized in full details yet. A widely accepted view is that meibum forms the outermost lipid layer of a multilayered tear film, whose physiological functions are being studied and discussed in detail [13–17]. The lipid profiles of normal and abnormal meibum and tears have been compared in previous studies [18–24]. However, in most, if not all, described cases of abnormal meibum, reported changes were either small [18,21,23–25], or characterized in a way that precluded identification of individual lipid species or lipid classes and was more descriptive than structural and quantitative [25–30]. Here, for the first time, we describe results of a comprehensive lipidomic analysis of meibum, tears, and sebum of a patient whose secretions were severely depleted of regular Meibomian and sebaceous lipids (MLs and SLs, respectively), and, instead, were highly enriched with those lipids that are typically not present in the norm (“abnormal meibum, tears and sebum”, or AMTS, patient). As standard lipid profiling techniques and tests that are used in clinics are not very specific and cannot discriminate between closely related lipid species, *e.g.* series of homologous compounds or structural isomers, we utilized reverse phase ultra high performance liquid chromatography/high resolution mass spectrometry (RP-UPLC/MS) as the main analytical tool for lipid profiling. This method is considered to be a current golden standard in lipidomics and can be used as a valuable diagnostic tool, as we demonstrate in this manuscript.

2. Materials and Methods

2.1. Ethics statement.

The study was conducted at the Kyoto Prefectural University of Medicine (Japan; KPUM) and the University of Texas Southwestern Medical Center in Dallas (TX, USA; UTSW). All procedures used for collecting human meibum samples were approved by the Institutional Review Boards of UTSW and KPUM and performed in accordance with the principles of the Declaration of Helsinki.

2.2. Clinical assessment of study subjects.

Each normal, non-DE/non-MGD volunteer (n=8; males; mean age 67.5 ± 5.5 years) answered a questionnaire to determine eligibility for the study, signed informed consent forms and then underwent routine ocular examinations such as slit lamp evaluation of the ocular surface, meibography, cornea staining with fluorescein and/or Lissamine green, measurement of the tear film breakup time (TBUT), and measurement of their tear secretion rate using Schirmer 1 test. A 63-year old male AMTS patient was recruited during a routine ocular evaluation at KPUM.

2.3. Sample collection.

Samples of meibum, tears, sebum and blood of the AMTS patient and normal age-matched male controls (eight in total) were collected at KPUM and UTSW. Samples collected at KPUM were shipped to Dallas on cold packs chilled to below -20°C using an expedited next day delivery service. Upon arrival to UTSW, the temperature of the samples was still below -20°C .

At both locations, surgical microscopes were used for collecting meibum samples. At KPUM, meibum samples were obtained from the subjects' eyelids by the use of a Daviel cataract spoon after gently squeezing the eyelid margin with a Yoshitomi MG compressor (T.M.I. Co. Ltd., Saitama, Japan). At UTSW, meibum was expressed using Hardten Eyelid Compression Forceps (Katena Products, Parsippany, NJ, USA), and collected with a platinum microspatula. At both locations, strict attention was paid to avoid contamination by the tear fluid and other deposits from the eyelids around the MG orifices. Meibum specimens were then dissolved in ~1mL of HPLC-grade solvent mixture (chloroform : methanol, 2:1, v/v) and stored in 2-mL sample glass vials with Teflon®-lined lids at -20°C .

Tears samples were collected using Schirmer strips and processed as described before [31]. Sebum samples were collected using Sebutape® (from CuDerm, Dallas, TX, USA) as suggested by the manufacturer. Lipids were extracted with $3 \times 1\text{mL}$ of *iso*-propanol : *n*-hexane mixture (1:1, vol/vol), pooled, evaporated to dryness under stream of nitrogen, and then redissolved in 1 mL of *iso*-propanol. Blood samples of the AMTS patient and normal controls were drawn and routine lipid profiling was conducted in a clinical laboratory. Also, lipid profiles of their plasma and serum samples were assessed in-house using RP-UPLC/MS approach similarly to other types of lipid samples.

2.4. Analytical procedures.

Chemical analyses of samples were conducted at UTSW using procedures that were described earlier [10,11,32]. Lipids were analyzed as intact species using RP-UPLC/MS. The analyses were conducted on a Synapt G2-Si high resolution quadrupole time-of-flight (qTOF) mass spectrometer, with either an IonSaber-II atmospheric pressure chemical ionization (APCI) ion source or a LowFlow electrospray ionization (ESI) ion source, with Zspray LC interface (all instruments from Waters Corp., Milford, MA). Chromatographic separations were conducted using an M-Class UPLC system and an Acquity UPLC C18 BEH column (1mm $\times$ 100mm, 1.7 μm) (also from Waters Corp). The elution of the analytes was conducted using a binary *iso*-propanol : acetonitrile gradient [10,32]. Both solvents had

5% of 10 mM aqueous ammonium formate in them. All solvents, obtained from different manufacturers, were of LC-MS or HPLC grade.

Note that the chemical nature of meibomian lipids had been established in our previous studies using MS/MS and MSⁿ approaches. Thus, it was deemed unnecessary to repeat those targeted experiments in the current study. Instead, the identities of the compounds were verified by their high resolution mass spectra with better than 6 mDa accuracy, which allowed for a reliable determination of their elemental (CHNO) composition. Also, two additional parameters – RP-UPLC retention times and fragmentation patterns of the analytes in the automatic MS^E mode – were used to verify the chemical nature of the lipids.

Nonpolar and neutral lipids, such free cholesterol (Chl), squalene (Sql), wax esters (WEs), triacyl glycerols (TAGs), cholesteryl esters (CEs), and cholesteryl esters of (*O*)-acylated ω -hydroxy fatty acids (Chl-OAHFAs), were detected and analyzed in positive ion mode primarily as (M + H)⁺, (M + Na)⁺ or (M + NH₄)⁺ adducts. In addition to these ions, most of lipid species offered alternative ways of their detection and identification, which provided additional tools for verification of their structures. For example, Chl, CEs, and Chl-OAHFAs were also observed using their common analytical ion with a unique *m/z* value of 369.35, which offered a convenient way of their quantitation [33,34]. TAGs, in addition to (M + H)⁺, (M + Na)⁺ and (M + NH₄)⁺ ions, were also observed as spontaneously formed (M – FA + H)⁺ adducts. For example, a major TAG in normal meibum is triolein with an elemental formula of C₅₇H₁₀₄O₆. Triolein has three C_{18:1} oleic acid residues, totaling 54 carbons and 3 double bonds, and a glyceryl moiety with 3 carbons (C_{57:3}). Triolein was detected as a (M + H)⁺ adduct with *m/z* value of 885.7905, a (M + Na)⁺ adduct with *m/z* 907.7872, a (M + NH₄)⁺ adduct with *m/z* 902.8171, and a DAG (diolein) fragment (M – FA + H)⁺ with *m/z* value of 603.5321 (in MS and MS^E modes).

The same underivatized samples were also analyzed using gas chromatography/mass spectrometry as described earlier [35]. The method proved to be most useful for analyzing fully saturated WEs which are poorly detectable in the conditions of RP-UPLC/MS.

3. Results

3.1. General evaluation of the AMTS patient's health.

The 63-year-old male AMTS patient was referred to the Kyoto Prefectural University of Medicine (KPUM) affiliated hospital for treatment of MGD in 2016, complaining of a “foreign body” sensation in his eyes, and has been regularly seen for four years. For the purpose of this study, the patient was examined thrice during a one-year period. The following clinical observations were made. In both eyes best corrected visual acuity was 20/20 and intraocular pressure was within normal limits. On slit lamp examination, as shown in Figure 1, protrusions of abnormal, white, lard-like meibum at the MG orifices of the lower eyelid margins as well as pluggings on the upper eyelid margins were observed. Chalazia was ruled out because the AMTS condition did not resemble it clinically: A chalazion is defined as a chronic inflammatory granuloma caused primarily by the retention of meibomian glands, but no granuloma formations were observed anywhere in all the eyelids of the AMTS patient. Also, the AMTS condition persisted for several years, affected all

eyelids and multiple glands/orifices simultaneously, and caused no pain, which all are symptoms that are atypical of chalazia.

Although difficult to quantify, noticed was an abnormally large amount of meibum in the patient's MG. Oil-like droplets were seen on the tear film with fluorescein dye in right eye and mild superficial punctate keratopathy on the nasal upper cornea of the left eye was observed. Unexpectedly, the tear film break-up time (TBUT) measured during a slit lamp examination with fluorescein was found to be normal at 11 seconds in the right eye and 8 sec – in the left eye (average of three measurements), and so was the tear production rate (Schirmer 1 test produced 33 mm in the right eye and 35 mm in the left eye). Meibography showed globular appearance of MGs without ductal shadow. Results of the routine blood lipid test of the AMTS patient were normal: Triglycerides, 54 mg/dL; total cholesterol, 161 mg/dL; high density lipoproteins, 48 mg/dL; low density lipoproteins, 102 mg/dL.

3.2. Untargeted lipidomic analyses of meibum of the study subjects.

As the first step, meibum samples that had been collected from the AMTS patient and control subjects were “fingerprinted” using total ion chromatograms (TIC) obtained in RP-UPLC/MS experiments. As an illustration, normal meibum samples are shown in Figure 2A as overlaid uncorrected raw TIC, while abnormal ones are displayed in Figure 2B. The chromatograms were essentially indistinguishable from each other within their respective groups implying their high *intra*-group similarities. On the other hand, their *inter*-group differences were staggering: a large portion of lipids in the abnormal samples eluted considerably faster than normal MLs, indicating a significant shift in their lipid profiles.

Since human meibum is composed of hundreds of various lipid species, the first step in comparing samples of normal and abnormal types was untargeted (i.e. “unsupervised”) analysis of the MS datasets using the Principal Component Analysis (PCA) approach. Initially, raw RP-UPLC/MS datasets that had been obtained for eight individual samples of normal meibum from eight control subjects were evaluated and presented as a PCA biplot (Figure 2C), which demonstrated tight clustering of the samples (blue dots) with no outliers – atypical observations would have been outside the encircled area. The analytes (grey dots that represent individual lipids with particular *m/z* values and retention times) did not show any visible clustering or outliers, either, which indicated high inter-donor similarities in this group of samples. Importantly, when one normal (blue dots) and one abnormal (red dots) samples were analyzed as technical replicates four times each, the datasets produced two tight, clearly separated clusters of both samples and analytes (blue and red ovals in Figure 2D). This experiment provided realistic estimates of reproducibility of the implemented experimental approaches and defined possible boundaries for the intra-group, inter-donor variability of the lipid composition of the samples. Notably, when meibum samples from normal subjects were compared with samples from the AMTS patient, equally tight clustering of samples and variables was observed (Figure 2E). Thus, a conclusion was made that both normal and abnormal samples demonstrated rather high similarities within their respective study groups, but they were vastly different when the two groups were compared with each other.

3.3. Targeted analysis of meibum specimens.

Then, targeted (or “supervised”) lipidomic experiments were conducted. As discussed above, TIC of normal and abnormal meibum were distinctively different from each other (Figure 3, Panels A and B). Observation spectra of samples from both groups were obtained by extracting MS signals from their TIC (Figure 3, Panels C and D). Next, CHNO compositions of individual MLs were derived from their m/z values using the Elemental Composition (v.4.0) built-in routine of the MassLynx software. Finally, structural assignments were made based on the CHNO composition of the analytes as well as their RP-UPLC retention times and MS^E fragmentation patterns.

The following considerations must be taken into account when discussing the data: 1) lipids that are described below belong to several major groups of lipids in meibum, tears, and sebum, and include TAGs, WEs, Scl, Chl, CEs, OAHFAs, and Chl-OAHFAs; 2) many of those lipids were present as complex mixtures of often isobaric compounds composed of different combinations of fatty acids (FAs) and fatty alcohols; 3) lipids were separated using RP-UPLC before the MS analyses, which helped with their identification due to their unique chromatographic retention times; 4) the chromatographic step eliminated the problem of misidentification of analytes caused by the commonly observed in MS experiments spontaneous in-source fragmentation of complex lipids [36]; 5) to optimize detection of various classes of lipids, two major approaches – APCI MS and ESI MS – had to be used, as different types of analytes respond differently to different analytical conditions; 6) most of the WEs were detected as $(M + H)^+$ adducts; 7) TAGs were observed mostly as $(M + H)^+$ and $(M - FA + H)^+$ species; 8) Chl was observed as $(M - H_2O + H)^+$ ions with a unique theoretical m/z value of 369.3521; 9) CEs and Chl-OAHFAs were detected as $(M + H)^+$, $(M + Na)^+$ and $(M + NH_4)^+$ adducts; also, they were visible due to formation of the same analytical ion m/z 369.35 as Chl, but because of the esterification with FAs, all CE and Chl-OAHFA had much longer chromatographic retention times than free Chl.

The lipid profiles of normal meibum samples (Figure 3C) replicated those that were characterized earlier ([10] and references cited therein). However, mass spectra of lipids in abnormal meibum samples (Figure 3D) were strikingly, and consistently, different from the norm: a large number of major analytes in the “abnormal” group were TAGs, while normal MLs, such as WEs, CEs, and Chl-OAHFAs, were considerably suppressed. Characteristically, analytes that belong to the class of TAGs [TAGs themselves, and products of their spontaneous in-source fragmentation $(TAG - FA + H)^+$] were tightly clustered on the left side of the biplot graph and correlated negatively with an equally tightly clustered “normal” lipids on the right side of the graph (Figures 2D and 2E).

The chemical nature of TAGs was elucidated using accurate m/z values of their $(M + H)^+$ adducts and $(M - FA + H)^+$ fragments (Figure 3C), and it was determined that most, if not all, TAGs were based on C₁₂-C₂₀ FAs with 0 to 3 double bonds per FA residue. The dominant FAs were those with 0 to 2 double bonds per FA residue, resulting in 0 to 5 double bonds per TAG molecule. The most prominent TAG species are listed in Supplemental Table S1 and shown, as spectra and heat maps, in Figure 4, Panels A1, A2, B1 and B2. Highly unsaturated TAGs (with >5 double bonds per molecule) were relatively minor members of the family regardless of the donor. A series of homologous saturated TAGs, such as

$C_{47}H_{90}O_6$, $C_{48}H_{92}O_6$, $C_{49}H_{94}O_6$, $C_{50}H_{96}O_6$, and $C_{51}H_{98}O_6$, and others was also observed, but quantitation of these species was not performed at this time due to their weak ionizability in chosen experimental conditions, and will be pursued later.

Notably, the profile of TAGs in abnormal samples was clearly different from the corresponding profile of normal meibum featuring a six-fold drop in $C_{57:3}$ TAG (mainly, triolein and various positional isomers of stearyl-oleoyl-linoleoyl glycerol), and more than a ten-fold increase in shorter chain TAGs, primarily $C_{46:n}$ to $C_{54:n}$ with 0 to 3 double bonds per molecule. The tipping point was a group of TAGs $C_{55:1}$, $C_{55:2}$, and $C_{55:3}$, which did not differ much between the samples.

In order to evaluate the role of blood lipids in meibogenesis, the blood lipid profiles of the AMTS patient and normal controls had been compared and the following observations were made. Firstly, no intra-group differences between serum and plasma lipids (such as TAGs and CEs) were noted: their lipid profiles were essentially identical (data not shown). Thus, only the data on serum lipids are discussed below. Secondly, the lipidomic analyses revealed no major differences between serum TAGs of the AMTS subject and those of normal donors (Figure 4; Panels C1, C2, D1, and D2). Two omnipresent species of CEs – a $C_{18:1}$ -CE and a $C_{18:2}$ -CE, which accounted for more than 90% of all CEs in serum, – were also equally present in abnormal and normal serum samples. No very long chain (VLC) and extremely long chain (ELC) FA-containing TAGs and CEs were detected in any of the samples.

Importantly, neither normal, nor abnormal meibum TAGs seemingly bore any resemblance with serum TAGs: the latter were dominated by a group of tri-unsaturated $C_{55}H_{100}O_6$ TAGs, followed by their di- and tetra-unsaturated counterparts. The second largest group of TAGs in serum was formed of $C_{57:3}$, $C_{57:2}$, and $C_{57:4}$ compounds. In general, serum TAGs were a rather simple mixture that was based almost exclusively on even-chain FAs with almost nonexistent odd-chain variants: Note the virtual absence of odd-chain FA-containing C_{52} , C_{54} , C_{56} , C_{58} and C_{60} TAGs in the mass spectra of serum samples. On the contrary, the very same odd-chain TAGs were among major lipids of abnormal meibum, and, to a lesser extent, of the normal one.

The relative abundances of major analytes within their respective lipid classes in meibum were calculated using extracted ion chromatograms of the compounds of interest. Data for Chl/CE ratio (detected and quantified using the Chl and CE common analytical ion m/z 369.35) are shown in Figures 5A and 5B, from which one can conclude that there was a considerable shift in the CE profiles toward shorter compounds with C_{14} - C_{18} fatty acids in the “abnormal” group of samples. Other lipids of interest were compared using the same approach. A summary graph of the changes in major TAGs, CEs, and WEs (Figure 6, Panels A-D) illustrate this landslide shift in lipid homeostasis in the MGs of the AMTS patient.

3.4. Targeted analysis of tear lipids.

As MGs are major sources of lipids for tears [35], tear samples of normal and abnormal subjects were compared next (Figure 7, Panels A1, A2, B1 and B2). Lipids of abnormal tears followed the patterns established for abnormal meibum: 1) abnormal tears had higher Chl/CE ratio than the normal tears; 2) shorter CEs were increased; and 3) tear levels of

TAGs were highly elevated, while WEs were severely diminished. However, the balance of individual TAGs in tears thoroughly replicated that of meibum (compare Figure 7, Panel B2, Figure 4, Panel A1, and Figure 3, panel C). These observations reinforce our previous conclusion that MLs is a dominant feature of the tear film lipidome, and imply that the source of abnormal TAGs in tears of the AMTS patient is indeed abnormal meibum.

3.5. Targeted analysis of sebaceous lipids.

Finally, sebum of the AMTS patient was compared with sebum of two normal age- and gender-matched controls (Figure 7, Panels C1, C2, D1 and D2). In all cases, sebum samples were collected from the nasal and the forehead areas. No noticeable differences between sebum from the two locations were detected – the lipidomes were qualitatively and quantitatively almost identical (data not shown). Then, sebum specimens from the AMTS patient and normal volunteers were compared. Superficially, the sebaceous lipid profile of the AMTS patient was qualitatively and quantitatively very close to that of normal controls (Figure 7, Panels C1 and C2): They all were composed primarily of identical TAGs, Chl, and Scl which were expressed in similar ratios. Characteristically, both normal and abnormal sebum had similar profiles of TAGs. However, upon close examination, a clear defect in the AMTS patient's sebaceous lipids was discovered: The WEs/TAGs ratio in abnormal sebum was much lower than in the norm. An example of this change for the TAG fragments with molecular formulas $C_{40}H_{70}O_4$, $C_{40}H_{72}O_4$, and $C_{40}H_{74}O_4$, and WEs with molecular formulas $C_{42}H_{78}O_2$, $C_{42}H_{80}O_2$ and $C_{42}H_{82}O_2$ is shown in Figure 7, Panels D1 and D2. Note that the $(M - FA + H)^+$ fragments of TAGs and $(M + H)^+$ adducts of WEs have almost identical m/z values, which can be resolved only using high resolution MS (Figure 7, Panel D2) or in targeted fragmentation studies. A range of other WEs followed the trend suggesting a shift in sebogenesis that mirrors that of meibogenesis.

Based on these data we established that: 1) the majority of newly observed meibomian lipids in abnormal AMTS samples belong to the class of TAGs, which increased their TAG fraction by a factor of 20x to 30x, at the expense of all other MLs; 2) the meibomian WE/(Chl + CE) ratio decreased by a factor of 2; 3) the meibomian Chl/CE ratio increased by a factor of 3; 4) the profiles of meibomian WEs and CEs demonstrated a shift toward shorter chain lipids; 5) the tear lipid profiles closely resembled those of meibum for each type of donors; 6) the blood lipid panel testing showed no abnormalities; and 7) sebum of the AMTS patient was also affected as its long chain WEs were considerably suppressed. Importantly, no improvement in the patient's meibum was observed a year after the first round of samples had been collected: Meibum remained highly abnormal which indicated that the patient's condition was not a short-term aberration, but a rather persistent abnormality.

4. Discussion

In this first report on a patient with apparently normal blood lipid levels and profiles, but abnormal meibum, sebum, and tears, we present results of lipidomic analyses that clearly demonstrate massive changes in their compositions compared to the norm. The changes affected, to various extents, several major classes of lipids including Chl, CEs, WEs, and TAGs. Note that meibum, sebum and tears are composed of hundreds of different, but often

homologous and/or isomeric lipids, whose comprehensive evaluation is a formidable task which goes beyond the scope of this manuscript. Therefore, only the major, firmly established facts that characterize the abnormal lipid metabolism in MGs and SGs of the AMTS patient will be discussed below.

The first, and most obvious, difference was the excessive accumulation of TAGs in abnormal meibum. In normal meibum, TAGs are present as a tiny fraction (less than 0.1%) of the overall lipid pool [Figure 3C and reference [10]]. However, the total TAG fraction in the samples of meibum from the AMTS patient was increased by a factor of 20x to 30x, which transformed TAGs into one of the major lipid species in the abnormal MG secretions.

The second important difference was the altered balance of various TAGs in abnormal meibum, such as a 6-fold decrease in the fraction of C_{57:3} TAG [mainly, triolein – the most abundant TAG in *normal* meibum (Figures 3 and 4)], and an increase in shorter chain C₅₁-C₅₅ TAGs (Figures 3C and 3D).

Thirdly, the TAG profile of abnormal meibum was very different from that of normal MLs which are known to be based on VLC and ELC FAs and alcohols of the C₂₄-C₃₆ family [8,15,33,37]. Meibomian TAGs, unlike WEs, CEs, OAHFAs and Chl-OAHFAs, did not have any VLC- and ELC FA moieties in their structures: The longest TAG that was reliably detected in the samples was C₆₃H₁₁₆O₆, i.e. had 20 carbons per FA chain, on average. Indicatively, no VLC- and ELC FA moieties were detected among the DAG fragments of TAGs either, with the upper range of FA being C₂₀.

Profound differences between serum and meibum TAGs, such as changes in the overall lengths of lipids, the altered balance of odd- and even-chain FA residues in their structures, and a much broader spectrum of TAG species in abnormal meibum, can be explained only if assume that these TAGs are biosynthesized by MGs in situ and/or de novo by metabolizing the pool of serum TAGs and CEs. Our previously published data on the high expression levels of genes that are related to biosynthesis of FAs and glycerolipids in human MGs (such as *DGAT2*, *ELOVL6*, *FASN*, *GPAM* and others [10]) support this conclusion. Also reasonable is to conclude that meibomian TAGs are early precursors of regular MLs as no VLC and ELC TAGs have been detected in any types of samples. The excessive accumulation of TAGs in abnormal meibum clearly indicates a disconnect between classical lipogenesis (e.g. biosynthesis of TAGs of normal lengths), and meibogenesis (i.e. biosynthesis of VLC and ELC meibomian-type lipids [8], [11,12,37]).

Similarly to TAGs, changes in the CE and WE profiles also indicated a decrease in longer chain members of these families and an increase in their shorter homologs (Figures 5 and 6). The tipping points for the changes were CEs with C₁₇-C₂₂ FA residues and a C₄₁ WE with *m/z* 605.6233, formed mostly of C_{18:1} oleic acid and a C_{23:0} alcohol. As serum and plasma TAGs are virtually depleted of FAs longer than C₂₀, VLC/ELC FAs for meibomian-type CEs and WEs with all likelihood are biosynthesized in MGs in situ. Therefore, the shift in their profiles may involve genes and enzymes of MGs that are responsible for catabolism of dietary lipids, transesterification of FAs, and/or their elongation [10,12,38], among other possible reasons.

The changes in meibum composition can be explained using a generalized diagram of normal and abnormal meibogenesis shown in Figure 8. It is reasonable to assume that the processes start with absorption of serum-type TAGs and CEs from the blood stream followed by their metabolic transformation into meibomian-type TAGs in a series of reactions (labeled as Core 1 in the Figure). In normal conditions, these meibomian-type TAGs and CEs of normal C₁₆ and C₁₈ length may serve as precursors for biosynthesis of VLC and ELC WEs, CEs, Chl-OAHFAs and other meibomian lipids in Core 2 reactions (i.e. meibogenesis per se), but once this pathway is permanently affected, one can anticipate rapid and sustained accumulation of their precursors – shorter-chain TAGs and CEs – in the secretion, and a large drop in VLC and ELC meibomian lipids.

To the best of our knowledge, a change in lipid homeostasis in MGs of this magnitude is unparalleled in the published literature, except, possibly, pathologies like chalazia [3,4], which was shown to lead to a similarly abnormal MG lipidome. However, the differences between chalazia and the newly described syndrome are significant: Advanced chalazia is characterized by an almost complete loss of normal meibomian lipids, such as CE, and a severe shift in their molecular profiles toward shorter chain compounds, while the AMTS patient's meibum still had a large pool of normal meibomian lipids in its composition even a year after the initial analyses.

These shifts in the lipid profile of meibum had a deep impact on the lipid composition of tears as well: the tear lipids were highly abnormal being enriched with Chl, shorter-chain CEs, and TAGs (Figure 7, Panels A1-B2). Paradoxically, this deficiency had no negative impact on the patient's TBUT (8–11s), indicating a rather high stability of the tear film. However, the “foreign body” sensation reported by the patient may be related to large lipid aggregates that were observed in the tear film as “lipid droplets”. Formation of the latter with all likelihood is related to the abnormal composition and/or quantity of meibum, and its altered rheological and thermotropic properties: Thick, lard-like consistency of the abnormal secretion under physiological conditions (Figure 1, Panels A1 and A2) warrants changes in its aggregation state on the ocular surface of the patient. The lower fluidity of meibum may also result in its lower, and inconsistent, expressibility, causing its accumulation and stagnation within MGs and plugging of their orifices, which indicatively were observed upon ophthalmic evaluation of the patient.

Lastly, the lipid composition of the abnormal sebum needs to be discussed. SGs and MGs are considered to be related despite the fact that the lipid secretions they produce – normal sebum and normal meibum – are very different (compare Figure 3, Panel C and Figure 7, Panels C1). However, certain commonalities between two secretions, such as presence of VLC and ELC WEs, made it interesting to compare sebum and meibum samples of the AMTS patient and normal controls.

As there is no easy method for quantification of sebum production by SGs in vivo (the amount of sebum that is expressed onto the skin surface depends on many factors, such as ambient and body temperatures, humidity, diet, age, etc.), the best way to characterize sebogenesis was to measure the lipid *ratios* in the secretion. These experiments revealed that the WE/TAG ratio in sebum of the AMTS patient was lower than that in normal controls

(Figure 7, Panels D1 and D2), meaning that lipid homeostasis in SGs of the patient was also atypical and affected in a similar way to meibogenesis in MGs.

Interestingly, the TAG profile of abnormal meibum was a close replica of that of abnormal sebum (compare Fig. 3, Panel D and Figure 7, Panel C2), which further substantiated our hypothesis of the major failure in meibogenesis and redirection of the pathway toward accumulation of shorter-chain precursors of normal VLC and ELC lipids of meibum, such as TAGs.

In summary, abnormal meibum was found to be highly enriched with Chl and TAGs, and depleted of WEs and CEs, starting to resemble human sebum. Within their respective classes, short and medium chain lipids were increased, while VLC and ELC lipids were decreased, compared to the normal secretions. The pattern did not change during a one-year period. A well-documented shift of this magnitude in lipid homeostasis in MGs, to the best of our knowledge, has not been described and reported before, except for chalazia [3,4]. The extent of changes in the lipid profiles of the otherwise extremely invariant MG secretions [10,32], and the fact that sebum of the AMTS patient was also affected, point toward a genetic mutation that affects multiple tissues and/or organs. Experiments are in progress to characterize the condition in more details and elucidate its molecular causes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AMTS	abnormal meibum, tears and sebum patient
APCI	atmospheric pressure chemical ionization
CE	cholesteryl ester(s)
Chl	free cholesterol
Chl-OAHFA	cholesteryl ester(s) of (<i>O</i>)-acylated ω -hydroxy fatty acid(s)
DE	Dry eye
EIC	extracted ion chromatogram(s)
ELC	extremely long chain
FA	fatty acid(s)

FAI	fatty alcohol(s)
MG	Meibomian gland(s)
MGD	Meibomian gland dysfunction
ML	meibomian lipids
OAHA	(<i>O</i>)-acylated ω -hydroxy fatty acid(s)
PCA	principal component analysis
PIM	positive ion mode
RP UPLC/MS	reverse phase ultra high performance liquid chromatography/mass spectrometry
SG	sebaceous gland(s)
TAG	triacylglycerol(s)
TBUT	tear film breakup time
TIC	total ion chromatogram(s)
TP	tarsal plate(s)
VLC	very long chain
WE	wax ester(s)

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Figure 1.

Abnormal ocular surface manifestation of the eyes of the AMTS patient.

Right (**A1-C1**) and left (**A2-C2**) eyes are shown. Protrusions of white thick meibum at the MG orifices of the lower eyelid margins (arrows) (**A1, A2**). Fluorescein staining image shows oil-droplets in the tear film (**B1**) and SPK (**B2**). Meibography images show globular appearance of MGs without ductal shadow (**C1, C2**).

Figure 2.

Untargeted lipidomic analyses of normal and abnormal meibum samples.

(A) Representative TIC of samples collected from 5 normal subjects. (B) TIC of 4 samples from the AMTS patient. (C) PCA revealed high similarities between 8 normal meibum samples. A scores-loadings biplot is shown: blue dots are individual samples; grey dots – individual lipid analytes. (D) A scores-loadings biplot of one normal and one abnormal meibum samples analyzed 4 times each. The analyses produced two extremely tight clusters of, correspondingly, normal (blue oval) and abnormal (red oval) samples and analytes. (E) Untargeted PCA revealed large differences between 8 normal (blue dots and oval) and 4

abnormal (red dots and oval) samples. To differentiate between normal Asian and Caucasian samples, the latter are labeled with “c”.

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Figure 3.

Representative APCI PIM chromatograms and mass spectra of normal and abnormal meibum sample.

(A) Total ion chromatogram of a normal meibum sample. (B) Total ion chromatogram of an abnormal ion sample. (C) Mass spectrum of normal meibum. Compounds were detected primarily as $(M + H)^+$ and $(M - H_2O + H)^+$ adducts. (D) Mass spectrum of abnormal meibum. For clarity, portions of the spectra between m/z 750 and m/z 1,200 in Panels C and D were magnified by x100 and x18, correspondingly.

Figure 4.

Targeted RP-UPLC/MS evaluation of meibum and serum triacylglycerols of the AMTS patient and normal controls.

Observation mass spectra of the TAG fractions of abnormal meibum (**A1**), normal meibum (**B1**), abnormal serum (**C1**) and normal serum (**D1**). Heat maps of the TAG fractions of abnormal meibum (**A2**), normal meibum (**B2**), abnormal serum (**C2**) and normal serum (**D2**).

Figure 5.

Targeted RP-UPLC/MS analyses of free cholesterol and cholesteryl esters in normal and abnormal meibum.

Analysis of Chl and CEs in normal meibum using normalized chromatograms of analytical ion m/z 369.35. Each chromatographic peak is labeled with its retention time (upper number, min) and peak area (lower number, arbitrary units) (**A**). Analyses of abnormal meibum demonstrated a relative increase in Chl and shorter-chain CEs compared to normal samples (**B**).

Figure 6.

Targeted lipidomic analysis of normal and abnormal meibum: Comparison of lipid profiles of normal (black bars) and abnormal (white bars) meibum samples.

Normalized distribution of molecular species of TAGs (**A**), Chl and CEs (**B**), and WEs (**C**) in normal and abnormal samples is shown. The overall lipid profiles of normal (n=8) and abnormal (n=4) samples are shown in Panel (**D**). Labels: Chl – free cholesterol; CE – cholesteryl esters; SWE – saturated wax esters; MUWE – mono-unsaturated wax esters; DUWE – di-unsaturated wax esters; TAG - triacylglycerols.

Figure 7.

Comparative RP-UPLC/MS lipidomic analysis of normal and abnormal tears and sebum. Normal tears (**A1**) had a much lower content of Chl than abnormal tears (**A2**). Also, the CE profile of abnormal tears was altered, demonstrating an increase in shorter chain compounds with shorter elution times. The lipid profiles of normal (**B1**) and abnormal (**B2**) tears were strikingly different: note a massive accumulation of TAGs and a decline in regular meibomian WEs in the abnormal sample. Samples of normal (**C1**) and abnormal (**C2**) sebum were superficially similar. However, normal sebum had higher levels of regular meibomian WEs, such as those with m/z values of 615.6058, 617.6194, and 619.6361, than the abnormal specimens (panels **D1** and **D2**, respectively). Note that MS peak with m/z 663.45 was a previously described contaminant – oxidized Irgafos 168 [31], which is found in many organic solvents and plastics, and is difficult to control.

Figure 8.

Lipid homeostasis in Meibomian glands.

Serum-type lipids are shown in yellow, abnormal meibomian lipids and/or intermediate products/by-products of meibogenesis are represented in white, while normal meibomian-type lipids are shown in orange. Newly biosynthesized meibomian lipids form lipid droplets within differentiated meibocytes, which then release meibum via the holocrine mechanism. More information on meibogenesis can be found in our earlier publications [10–12,32].