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Time-dependent degenerative transformations in the lipidome of chalazia

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Abstract

The aim of this prospective study was to conduct histopathologic and lipidomic analyses of chalazia, in order to evaluate time-dependent changes in the lesion.

Samples of surgically excised chalazia were collected over a period of 12 months from 10 patients (mean age 41 years; range, 23–58) with clinically diagnosed chalazia, who underwent scheduled surgery. The ages of chalazia varied from 2 to 28 weeks. To confirm the clinical diagnoses, the morphology of collected tissue samples was evaluated histologically after hematoxylin and eosin staining. The lipids from individual chalazia were analyzed by high-performance liquid chromatography-mass spectrometry and compared with authentic lipid standards and with the lipids of meibum collected from normal controls.

We observed gradual, lesion age-dependent transformation of the lipidome of chalazia from an almost normal meibum-like composition to a very different kind of lipidome. A rapid initial increase in the free cholesterol content was followed by a gradual replacement of extremely long chain meibomian-type lipids with a mixture of shorter-chain cholesteryl esters of the C₁₄-C₁₈ family, triacylglycerols, ceramides, phospholipids and sphingomyelins. In addition, a rapid disappearance of wax esters and cholesteryl esters of (1-O)-acyl-omega-hydroxy fatty acids from the lipidome of aging chalazia was observed.

Our results are indicative of dramatic, time-dependent changes in the lesion that may involve cholesterol as a trigger and/or a marker of subsequent degeneration of the meibomian lipidome.

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We hypothesize that early inhibition of these transformations may be useful in reversing the course of the disease.

Keywords

chalazion; lipidomics; cholesterol; inflammation; meibomian gland; HPLC; mass spectrometry; statins

1. Introduction

Eyelid lesions are amongst the most frequently encountered pathologies that general practicing ophthalmologists face in all patients age groups (Ho et al., 2013; Paul et al., 2011). Chalazion is one of the most common benign eyelid lesions (Al-Faky, 2012; Deprez and Uffer, 2009) frequently associated with blepharitis and acne rosacea (Lempert et al., 1979; Nemet et al., 2011). The term chalazion comes from the Greek word *chalaza* (hailstone) (The Online Etymology Dictionary. Available at: <http://www.etymonline.com/index.php?term=chalazion>). It is a chronic lipogranulomatous inflammatory response secondary to endogenous degeneration of the lipid secretions of either meibomian or Zeis glands (Eagle, 2011). Chalazia may develop spontaneously as a result of 1) accumulation of the lipid-enriched secretions in the ducts of the glands and their subsequent extravasation into the neighboring tarsal plate, or 2) it may evolve from an internal hordeolum (Lederman and Miller, 1999). The histology of chalazion is characterized by the presence of macrophages, epithelioid histiocytes, plasma cells, eosinophils, lymphocytes, polymorphonuclear leukocytes (PMN) and multinucleated giant cells that typically surround empty spaces corresponding to lipid vacuoles (Eagle, 2011). Although chalazion may be a self-limited disease, its management often represents a challenge for both the ophthalmologist and the patient, due to its recurrent incidence and/or resistance to treatment (Benitez-Del-Castillo, 2012).

To the best of our knowledge, there is only one published study which describes the lipid composition of chalazion in which Nicolaides et al. evaluated five samples of chalazia by a combination of thin layer chromatography (TLC) and gas chromatography (GC) (Nicolaides et al., 1988). TLC and GC methods were popular (if not the only) choices in earlier studies of the lipid composition of meibum. We previously have discussed the strengths and disadvantages of these two analytical procedures (Butovich, 2009c; Wojtowicz et al., 2009). Despite their limitations, very important information on the overall lipid composition of chalazion was obtained in a seeding study by Nicolaides et al (Nicolaides et al., 1988). However, considering the rapid technological developments in bioanalytical chemistry in general, and lipidomics in particular, we felt that a re-visit of the topic was long overdue. The use of modern high-pressure liquid chromatography/mass spectrometry (HPLC/MS) methodology currently available for lipidologists was to expand our knowledge of the lipid composition of the chalazion. Also of interest was elucidation of the time course of the biochemical changes that might be occurring during the development of chalazia – information that had not been available before, but could be useful in developing novel therapeutic approaches for chalazion.

Therefore, the goal of this study was to conduct a histological and lipidomic evaluation of chalazion in order to unravel the underlying mechanisms of its development.

2. Materials and Methods

2.1. Selection and evaluation of study participants

This prospective study was approved by the University of Texas Southwestern Medical Center Institutional Review Board and was conducted according to the Declaration of Helsinki. HIPPA regulations were followed. Signed informed consent forms were obtained from participating volunteers with a clinical diagnosis of chalazion who underwent surgical removal at the Oculoplastic Clinic at the Department of Ophthalmology. The following parameters were evaluated and recorded: demographics (age and gender) and clinical features (duration of lesion, primary or recurrent chalazia, anatomical location of the lesions, and other clinical diagnoses associated the disease). Samples of chalazia were collected over a period of 12 months from 10 patients (mean age of 41 years; range, 23–58) with clinically diagnosed chalazia, who were scheduled to have surgery. The tissue samples were collected by surgical excision and immediately sent for both histological and lipid analyses. Three healthy, non-dry eye patients were recruited as donors of normal meibum. A larger pool of normal meibum donors was deemed to be unnecessary for the current study because 1) normal meibum has been extensively characterized in our previous publications [(Butovich, 2013), and references cited therein]), and 2) no differences between previously characterized samples of meibum and those three samples collected for the current study were detected.

2.2. Reagents and equipment

HPLC-grade acetic acid, chloroform, *n*-hexane, methanol, *i*-propanol, and water were of HPLC or spectroscopy grade, and were purchased from Aldrich (Milwaukee, WI) and/or from Burdick and Jackson (Muskegon, MI) through VWR (West Chester, PA). Lipid standards were products of Nu-Chek Prep, Inc. (Elysian, MN), Avanti Polar Lipids, Inc. (Alabaster, AL) and Sigma Chemical Co. (St. Louis, MO). A Lichrosphere Diol HPLC column (3.2 × 125 mm, particle size 5µm) was obtained from Phenomenex (Torrance, CA). A reversed-phase Hypersil Gold HPLC column (2.1 × 150 mm) was from ThermoElectron Corp. (San Jose, CA). An Alliance 2695 HPLC Separations Module (from Waters Corp., Milford, MA) was connected to a LCQ Fleet ion trap mass spectrometer (ThermoElectron Corp.) equipped, depending on the application, with either an electrospray ionization (ESI) or an atmospheric pressure chemical ionization (APCI) ion source (from the same manufacturer). The instrument was operated under the Xcalibur software (Thermo Electron Corp.). Sigma Plot software version 11 was from Systat Software, Inc. (Richmond, CA). Additional details are provided in the corresponding sections of the manuscript, and in the Legends to figures.

2.3. Collection and histopathological evaluation of study samples

Ten samples of chalazia were surgically excised from patients in the Oculoplastic Clinic by trained surgeons. Histopathologic examination of samples was performed to confirm the accuracy of the clinical diagnosis as chalazion. To prepare samples for histopathologic evaluation, two different approaches were used.

The first approach included incubation of fragments of the five largest freshly excised chalazia in buffered Carson's formalin solution (Carson et al., 1973) for at least 24 hours (the remaining portions of the same chalazion samples were saved for lipid analyses; see below). Then, the fixed tissue samples were dehydrated by successive passages through 50, 70, 95 and 100% ethanol before paraffin embedding. Serial tissue sections of 5 μm thickness were mounted on SuperFrost Plus Microscope slides (Fisher Scientific, Pittsburgh, PA), re-hydrated through decreasing ethanol concentrations and then stained with hematoxylin and eosin (Statlab Medical Products, McKinney, TX) (Osborn and Brandfass, 2006). Their photomicrographs were taken using an Axio Observer D1 microscope (Carl Zeiss Microscopy, LLC; Thornwood, NY) equipped with an AxioCam ICC1 camera (Carl Zeiss). Images were analyzed with image-processing software AxioVision Rel.4.8.2 (Carl Zeiss).

The second approach involved prior extraction of lipids from all the study tissue samples with chloroform (see below), and subsequently the very same tissue samples were treated with the Carson's solution as described above. The later approach was employed to better correlate the results of histological and biochemical evaluations of the samples, and to maximize the yield of lipid extracts. As our study samples were excised from very heterogeneous ocular lesions during surgery, no attempts to quantitate cells of different types were undertaken: the cell count would vary from slide to slide, would represent only the slides shown and might produce misleading results.

Control meibum samples from three healthy, non-dry-eye volunteers were expressed using a soft-expression technique from the lower eyelids of healthy volunteers with no ocular pathologies by applying light pressure using a cotton swab and harvesting expressed secretion with a platinum spatula (Butovich, 2008).

2.4. Extraction of lipids

Surgically excised tissue samples were transferred into small glass vials with approximately 2 mL of chloroform, vortexed, and then stored at $-20\text{ }^{\circ}\text{C}$ in the dark for more than 24 hours with occasional vortexing. In order to preserve the samples for subsequent histopathologic evaluation, no homogenization of the tissue was performed. Preliminary range-finding HPLC-MS experiments demonstrated the presence of all expected meibomian and chalazion lipids after a 24-hr incubation of the tissue samples in chloroform. The fast extraction was primarily due to the very small physical size of the tissue samples (a few mm^3 or a few mg, or less). Moreover, no noticeable changes in the lipid profiles were observed during their incubation in the indicated conditions for at least six months. The latter confirmed the extractability and stability of the lipids in the tested conditions.

2.5. Mass spectrometric analyses

Lipids were analyzed using HPLC-MS as described in our earlier publications (Butovich, 2009a, b, 2010; Butovich et al., 2007; Butovich et al., 2009; McMahon et al., 2007). For a discussion on various analytical approaches pertinent to the lipidomic analysis of meibum and related samples see our earlier reviews on the topic (Butovich, 2009c, 2011, 2013). Briefly, the overall presence of free cholesterol (Chl; defined as all forms of non-esterified cholesterol) and cholesteryl esters (CE) was evaluated in normal phase HPLC-APCI MS on

a Diol silica gel column in positive ion mode (Butovich, 2009b; Butovich et al., 2007). A more detailed analysis of molecular species of the CE family was performed using reversed-phase HPLC-APCI MS on a Hypersil Gold C18 column (Butovich, 2010). CE were detected using their common analytical fragment with a mass-to-charge (m/z) ratio of 369. Ceramides (Cer) were evaluated as previously described (McMahon et al., 2007). Wax esters (WE) and triacylglycerols (TAG) were monitored similarly to Chl and CE. Phospholipids (PL) and sphingomyelins (SM) were evaluated using reversed-phase HPLC on the Hypersil Gold column and ESI MS detection of the analytes in both negative and positive ion mode in a water-methanol gradient as suggested in an Application Note 20527 (ThermoScientific. Available at: <http://www.thermoscientific.com/accucore>), or as described in our previous publications (Butovich et al., 2007; Butovich, 2009b). Detection of eicosanoids had been attempted, but was found to be impractical because of the small size of the samples and the extremely low levels of the compounds in tissues, which were below limit of detection and quantitation in our samples.

The molecular masses, retention times (RT), and fragmentation patterns of the lipids of chalazia were compared side-by-side with available authentic lipid standards of the same types, and with known meibomian lipids that had been characterized in our previous studies. Results of the HPLC-MS experiments were analyzed and plotted using Xcalibur and SigmaPlot software. Note that the MS spectra and chromatograms shown in Figures were normalized (0–100%, where 100% was the height of the tallest peak in the given figure) using the default settings of the Xcalibur software. This is a routine procedure which is used to compare study samples with high inter-sample variations in the analytes. The correlations between different lipid species and the ages of chalazia were routinely assessed using SigmaPlot's built-in functions.

3. Results

Ten patients (eight women and two men) with chalazia were enrolled. For the reader's convenience, detailed information on the patients is presented in Table 1. The subjects were between the ages of 23 and 58 yrs, with a mean age of 41. The patients had chalazia for 2 to 28 weeks (12 weeks on average). The left eye was involved in seven patients and the right one in three. Six patients developed chalazia in their lower eyelids (LL) location, while the remaining four – in the upper eyelid (UL). Five cases of chalazia were primary, one was recurrent, and the remaining four had a new onset chalazion after a previous episode within a mean interval of six years. The most frequent clinical diagnoses associated with chalazia were blepharitis (six patients) followed by meibomian gland dysfunction (two patients), acne rosacea and allergic conjunctivitis (detected in one patient each). One patient (#9) was taking simvastatin to treat an unrelated condition.

Histological staining of a typical chalazion section is shown in Figure 1, Panel A. One can observe a lipogranuloma that surrounds an empty cavity (formerly lipid vacuoles, with the lipid content removed during the treatment of the tissue section with xylene). A detailed microscopic examination of the samples revealed the presence of mixed inflammatory cells, predominantly epithelioid histiocytes, lymphocytes, PMN, and multinucleated giant cells (Figure 1, Panels B–E). The heterogeneous nature of the small, irregularly shaped, surgically

excised lesion samples made it impractical to obtain accurate cell counts for all observed immune cell types. In addition, the types of cells observed would also vary depending on the direction of the dissection, and the slice of the tissue examined.

There were no histological signs of cell atypia in any of the samples, which confirmed the initial clinical diagnoses. Among the ten samples evaluated in this study, there was a complete concordance between the clinical and histologic diagnoses for eight patients/samples, while the remaining two were reported as unspecified inflammatory responses due to the absence of lipogranuloma and giant cells. Importantly, no gross differences in the morphology of the samples that underwent the prior lipid extraction with chloroform, compared to the samples that did not, were detected.

All ten collected samples of chalazia, and three control samples of normal meibum, were processed for lipid analyses. Note that the absolute quantitation of the lipids of chalazia was not feasible for two reasons: 1) unavailability of proper chemical standards for many lipids, and 2) inherent inhomogeneity of the tissue samples. The latter was due to the nature of the collected samples, which were harvested during scheduled surgeries, and whose size, shape, etc. could not be controlled or planned. Therefore, we evaluated the relative ratios of lipid species in our samples using the abundances of the corresponding lipid signals that were recorded in mass-spectrometric experiments.

Initially, we performed an overall evaluation of the samples using normal phase HPLC-APCI MS in positive ion mode as described earlier (Butovich et al., 2007). These experiments allowed us to make direct comparisons of the current study samples with a large body of samples analyzed in our previous projects. For the reader's convenience, results are summarized in Table 2 and in Supplementary Table 1.

Observation spectra of the lipids of normal meibum (Figure 2, Panel A) were found to be no different from those described earlier (Butovich, 2013). A typical distribution of very and extremely long chain WE, a large presence of Chl, CE, cholesteryl esters of (O-acyl)-hydroxy fatty acids (Chl-OAHFA), and an almost complete lack of TAG (except for minor amounts of unsaturated C₁₈-TAG, see below), Cer, PC, and SM were also observed.

In contrast, lipids from a 28-week old chalazion (sample #8) were very different from those found in normal meibum (Fig. 2, Panel B; Fig. 3; Supplemental Figures 1–3, and Supplemental Table 1). In concordance with the observations of Nicolaides et al., lipids of chalazia were markedly enriched with Chl, shorter chain CE (such as C₁₄, C₁₆, and C₁₈-CE) (Fig. 3), phosphatidyl cholines (PC) and SM (Suppl. Fig. 1), Cer (Suppl. Fig. 2), and TAG (Suppl. Fig. 3). At the same time, WE and very and extremely long chain CE of the C₁₉- to C₃₂-CE family were almost completely absent.

Next, an important observation was made with regard to the changing lipid profiles in developing chalazia. Unlike meibum, which has relatively small inter- and intra-donor variability (Brown et al., 2013; Butovich, 2010; Butovich et al., 2007; Lam et al., 2011), the lipid composition of chalazia was very dynamic. Notably, we found that the lipid profiles of chalazia changed gradually with the age of the lesion.

First, the overall molar ratio of Chl to total CE in the samples as determined in normal phase HPLC-MS experiments (Fig. 4, Panels A–C), was found to positively correlate with the *age* of the lesions (Fig. 4, Panel D). With the exception of sample #1, the study samples produced a quasi-linear dependency of the Chl/CE ratio on the age of the lesion ($r = 0.94$). Sample #1 demonstrated an almost 4-x higher ratio of Chl to CE than expected, and was designated as an extreme outlier.

Second, in-depth lipidomic analyses demonstrated that the shifts in the lipid profiles included a time-dependent decline in the presence of very and extremely long chain CE and WE, and a concomitant increase in the molar fractions of Chl and shorter-chain CE. Simultaneously, variable amounts of TAG, Cer, PC and SM were detected in all samples of chalazia (Suppl. Figs. 1–3).

As an illustration, progressive changes in the profiles of cholesteryl-containing compounds (Chl, CE, and Chl-OAHFA) are shown in Fig. 5. The first major peak with a RT of about 4 min is that of Chl, while the other peaks are produced by various CE and Chl-OAHFA (Butovich, 2010; Butovich et al., 2009). The peaks of Chl-OAHFA are those with RT of 27–35 min. Note a rapid increase in the ratio of relatively short-chain C_{14} – C_{18} -CE (RT between 9 and 14 min) to their long-chain counterparts (C_{19} – C_{32} -CE; RT between 15 and 27 min), whose molar fractions were calculated by integrating their HPLC peaks (Fig. 6A). Importantly, a rapid, lesion age-dependent increase in the levels of Chl in chalazia preceded other changes in their lipidomes: during the initial two to three weeks, Chl increased more than ten-fold over the basal level, while all short- and long-chain CE remained virtually unaltered (Fig. 6B). It was only after those few initial weeks of the disease that the profiles of CE started to change, and the presence of other normal meibomian lipids started to decline

The lesion age-related changes in other lipids, such as Cer, PC/SM, and TAG, were more difficult to evaluate. Unlike extremely long chain meibomian CE and WE (which are signature lipids of meibomian gland secretions), Cer, PC/SM, and TAG are not usually present in normal meibum in appreciable quantities (Butovich, 2013). At the same time, these lipids are ubiquitous constituents of any tissue (healthy or not), and, therefore, were expected to be abundantly present in the tested human samples regardless of the age of the lesion. In fact, Cer, PC/SM, and TAG were detected in all tested chalazion samples. The most common species of these lipids are listed in Supplemental Table 1. The very nature of the tested samples – surgically excised fragments of eyelids with varying degrees of inclusion of healthy tissue, - led to uncontrollable changes in their lipid profiles. Thus, any changes in the levels of Cer, PC/SM, and TAG measured in our experiments would not be representative of actual changes in meibomian lipid metabolism, and were excluded from further discussion in this paper.

4. Discussion

Chalazion is one of the most common benign eyelid lesions (Al-Faky, 2012; Deprez and Uffer, 2009). However clinical misdiagnosis can occur. Among the samples in our study, good agreement between clinical and histopathological diagnoses was noted, although

discrepancies between the two have previously been reported in 6.4% out of 1060 cases (Ozdal et al., 2004), and in 6 % out of 48 cases in another study (Al-Faky, 2012). Histopathological evaluation of the surgical tissue samples showed they had characteristic cell populations composed primarily of macrophages, neutrophils, giant cells, epithelial cells and showed typical lipogranulomatous reactions (Dhaliwal et al., 2004) (Fig. 1). Observation of all chalazia did not reveal obvious changes with age in the presence of the different types of immune cells. If there was any difference it may have been an apparent underrepresentation of macrophages in both the very early and older chalazia.

Since the initial work of Nicolaides (Nicolaides et al., 1988), rapid progress has been made in developing and validating experimental approaches for lipidomic analysis of complex samples. The chalazion lipidome, similarly to the meibomian one, is an extremely complex mixture of lipids. Its complexity forced us to concentrate mostly on evaluation of changes between major lipid classes instead of changes on the level of individual lipids.

Our experiments revealed that the lipid composition of chalazia differed from sample to sample, with some samples being biochemically closer to meibum than others. Two distinctive features of all tested chalazion samples were a) the increased Chl/CE ratio (compared with normal meibum), and b) a gradual, time-dependent decline in the presence of normal meibomian lipids (Figs. 2–5). Importantly, we observed a strong positive correlation between the Chl/CE ratio and the age of the lesion (Fig. 6). There could be a number of reasons why these changes occurred, some of which are discussed below.

First, the initial abrupt increase in Chl levels might send a chemotactic signal to the inflammatory cells to infiltrate the gland (here, we do not distinguish between free, plasma-soluble Chl, and non-esterified Chl incorporated into LDL). Infiltration of the inflammatory cells could cause a physical blockage of the meibomian gland because of, for example, swelling of the tissues or thickening of meibum, which would impede expression of meibum through the meibomian gland ducts onto the ocular surface. In return, this excessive lipid material might infiltrate surrounding tissues and escalate the inflammatory response in the glands and the tarsal plate even further. The invasion of inflammatory cells could also enrich the lesion with lipids of the inflammatory cell origin.

Second, the sheer number of inflammatory cells in the pathological tissue that require nutrients to function could cause starvation of meibocytes and other normal cells present in meibomian glands. This could decrease the output of meibum, and shift the overall lipid profile of the lesion closer to the lipid composition of inflammatory cells. A catabolic consumption of normal meibomian lipids by inflammatory cells is also a possibility.

Third, large amounts of exogenous (to meibomian gland) lipids that are brought by inflammatory cells could inhibit, or re-direct, physiological lipid metabolism in meibomian glands because of: a) reversibility of many (if not all) enzymatically-catalyzed reactions (Cornish-Bowden, 2012); and b) the possibility of a negative feedback regulation. The former can be a factor when the final products of a reversible enzymatic reaction are abundantly present, thus reversing its course because of the law of mass action, while the latter might be implemented through various cell signaling mechanisms.

Let's consider these possibilities in more detail.

In 1988, Nicolaides et al. suggested that the large amounts of Chl found in chalazia could come from phagocytic cell membranes (Nicolaides et al., 1988). The latter would be consistent with a simple enrichment of meibomian lipids with lipids derived from inflammatory cells, i.e. the *first* mechanism described above. Indeed, a typical granulomatous response, such as chalazion, contains a large population of inflammatory cells, predominantly macrophages (Ito et al., 2013). We also observed a pronounced infiltration of macrophages in the chalazia. Therefore, it is possible that the lipid profiles of the aging lesion might be changing because of the profound infiltration of the meibomian glands by inflammatory cells. Supportive of this idea is data from Massai et al. (Massai et al., 2007) who presented experimental evidence of meibomian gland adenomeres being invaded by fused macrophages (i.e. multinucleated giant cells). Also pertinent to this mechanism are the lipidomic responses of murine macrophages to an inflammatory stimulus as described by Dennis et al. (Dennis et al., 2010). The authors reported that over a 24-h period of stimulation, intracellular Chl levels doubled in the cell, while the presence of C₁₄- to C₂₂-CE simultaneously increased two- to three-fold over the same period of time.

This mechanism, however, does not seem to be the only cause of the changes in the lipidome of chalazia. Indeed, despite fast infiltration of inflammatory cells, we did not detect any simultaneous and proportional increase in Chl and relatively short-chain C₁₄ to C₁₈-CE in young chalazia, which would be indicative of a purely inflammatory cell origin of those lipids. Instead, the levels of C₁₄ to C₁₈-CE in the tissues remained relatively unchanged for the initial two to three weeks, while the levels of free Chl increased more than ten-fold (Fig. 5 and Fig. 6). Then, a period of more profound changes in the distribution of the individual molecular species of CE followed, with normal meibomian CE and Chl-OAHFA of the C₂₂-C₃₂ family being gradually replaced with much shorter C₁₄ to C₁₈ CE indicative of the pro-inflammatory cells. Thus, while likely being a contributing factor, the first mechanism (lipid enrichment) taken alone cannot explain the seemingly complete disappearance of normal meibomian lipids, such as C₂₀-C₃₂-CE, Chl-OAHFA, and WE, in older chalazia.

The *second* mechanism (starvation of meibocytes) can be hypothesized because of the intensive lipid metabolism in stimulated inflammatory cells that will inevitably compete with meibocytes for sources of energy required for making extremely long chain meibomian lipids. Indeed, it has been demonstrated that inflammatory cells quickly increase the number of lipid bodies [or lipid droplets, LD (Martin and Parton, 2005; Walther and Farese, 2012; Melo et al., 2011; and references cited therein)] in their cytoplasm in response to stimulation. The growth of LD would require a fast increase in the turnover of ATP and NADPH in the cells for the de novo biosynthesis of the inflammatory cell-specific lipids.

Also plausible is absorption (or endocytosis) of meibomian lipids by inflammatory cells followed by their catabolism (through initial hydrolysis by esterases and lipases, followed by β -oxidation of meibomian fatty acids), to support the formation of new LD, now possessing characteristic features of the inflammatory cells. Since the most common lipid constituents of LD are various shorter-chain CE and TAG (Martin and Parton, 2005; Walther and Farese,

2012) and free cholesterol, all of which are abundantly present in developed chalazia, a possible involvement of this mechanism seems to be supported by our experimental observations.

Finally, the high Chl/CE ratio could be a result of shifts in lipid metabolism in meibocytes, e.g. a decreased activity of acyl-CoA cholesterol acyltransferase which catalyzes formation of CE from Chl and free fatty acids, or increased cholesterologenesis via the 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) pathway. These factors (alone or in combination) may impact the overall lipid composition of chalazion through the *third* mechanism – negative feedback regulation of lipid-metabolizing enzymes by lipids derived from inflammatory cells, – mentioned above. Importantly, activation of the HMG-CoA pathway could also be envisioned as the first step in mechanism 1.

With all likelihood, the changes in the lipidome of chalazia are related to (or caused by) a combination of the above discussed, and possibly other, processes. However, a central role for Chl and Chl-containing compounds in the development of chalazia seems to be confirmed, and may be hypothesized as a plausible therapeutic target in treating chalazia.

In existing therapies, estimated success rates vary from 72–79% for incision and curettage treatment (Ben Simon et al., 2011; Goawalla and Lee, 2007; Jackson and Beun, 2000) to 81–84% for triamcinolone acetonide injection (Ben Simon et al., 2011; Goawalla and Lee, 2007), to 29–80% for conservative treatment (Goawalla and Lee, 2007; Jackson and Beun, 2000; Perry and Serniuk, 1980).

Based on the rapid increase in Chl, followed by the gradual replacement of the meibomian lipidome with a macrophage-like one (Dennis et al., 2010), a combined approach to the treatment of chalazia might include a localized (topical or intralesional) use of a combination of anti-inflammatory and cholesterol-lowering treatments. Indeed, recent studies have shown that hypocholesterolemic drugs such as statins are able to modulate chronic inflammatory diseases (Bracht et al., 2012). Statins usually act by inhibiting HMG-CoA reductase – a key enzyme that catalyzes a rate-limiting step in Chl biosynthesis. However, in earlier studies of human monocytic cells, inhibition of HMG-CoA reductase by pravastatin reduced chemotaxis in the human monocytic cell line U937 (Kreuzer et al., 1991) and led to inhibition of monocyte chemoattractant protein-1, inhibition of metalloproteinase-9, and to a range of other effects that suggested potential anti-inflammatory activity of the drug (Grip et al., 2000). Moreover, statins have demonstrated a lipid-independent anti-inflammatory mechanism of action (Bonetti et al., 2003; Bracht et al., 2012; Golia et al., 2014). Therefore, we hypothesize that simultaneous inhibition of Chl biosynthesis and inflammation in chalazia by a localized treatment with statins could have potential applications as an alternative, or a complementary addition, to the currently existing treatments of chalazia.

Considering the very complex chemical nature of meibum and the chalazion, and complex effects of cholesterol and cholesterol-lowering treatments, further investigative efforts in this area are justified.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

APCI	atmospheric pressure chemical ionization
CE	cholesteryl esters
Cer	ceramides
Chl	total non-esterified cholesterol of any sort – soluble, protein-bound, or incorporated in HDL, LDL, and cell membrane
Chl-OAHFA	cholesteryl esters of (O-acyl)- ω -hydroxy fatty acids
GC	gas chromatography
HPLC/MS	high-pressure liquid chromatography/mass spectrometry
LD	lipid droplets
PC	phosphatidylcholines
PL	phospholipids
RT	retention time
SM	sphingomyelins
TAG	triacylglycerols
TLC	thin layer chromatography
WE	wax esters

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Appendix. Supplementary data

Supplementary data related to this article can be found at <http://>

Highlights

Time-dependent changes in the chalazion lipidome were observed.

They start with a fast accumulation of free cholesterol in the tissue.

Then, normal meibomian lipids are replaced with the lipids similar to those of macrophages.

Lowering cholesterol is proposed to counteract these degenerative transformations.

Figure 1. Photomicrographs of a chalazion (sample #7) and enlarged views of inflammatory cells identified in the sample

Panel A. A large lipogranuloma (marked with a black arrow) encircles a previously lipid-filled vacuole (marked with an asterisk). The section was stained with hematoxylin and eosin.

Panel B. Multinucleated giant cell.

Panel C. Lymphocyte.

Panel D. Epithelioid histiocyte.

Panel E. Polymorphonuclear lymphocyte.

Panel A – magnification 40×; Panel B – 400×; Panels C through E – 490×. All panels: specific cells are marked with white arrows.

Figure 2. Observation mass spectra of normal human meibum (Panel A) and lipids of chalazion #8 (Panel B)

Experimental conditions: mass spectra were recorded in positive ion mode APCI experiments.

The following analytical ions were observed: WE, $(M + H)^+$; TAG, $(M + H)^+$ and $(M + H - \text{fatty acid})^+$; Chl, $(M + H - H_2O)^+$; CE, $(M + H - \text{fatty acid})^+$; diacylated α,ω -diols (DiAD) were detected as $(M + H)^+$ ions.

Figure 3. Cholesteryl esters of chalazia and meibum

Panels A1 and A2. Reversed phase chromatogram of an equimolar mixture of authentic cholesterol and six CE with varying length fatty acid residues (Panel A1) and a typical mass spectrum of their common analytical ion m/z 369 (Panel A2).

Panels B1 and B2. Reversed phase chromatogram of CE present in chalazion sample #8 (Panel B1) and a typical mass spectrum of their common analytical ion m/z 369 (Panel B2).

Panels C1 and C2. Reversed phase chromatogram of CE of human meibum (Panel C1) and a typical mass spectrum of their common analytical ion m/z 369 (Panel C2).

Figure 4. Time-dependent changes in the free cholesterol/total cholesteryl esters ratios in chalazia

Panels A–C. Chl and total CE of meibum and two chalazion samples (were analyzed using a normal phase HPLC-positive ion mode APCI MS technique. All CE eluted as one HPLC peak with retention time (RT) of around 4 min. Free cholesterol had a retention time of around 7.6 min. The peaks were integrated using the Xcalibur's built-in Avalon routine and the resulting peak areas (AA) are displayed above each peak.

Panel D. Correlation between Chl/CE ratios and the ages of the lesions. Within the tested range (0 to 28 weeks), samples produced a linear dependence of Chl/CE ratios on the age of the lesions with a correlation coefficient $r^2 = 0.94$. Confidence and predicted limits are computed for 0.95. Each dot is labeled with a number that corresponds to the samples in Table 1. Sample 1 was designated as outlier and was not included in the calculation of the regression.

Figure 5. Time-dependent changes in the distribution of cholesteryl esters in chalazia

Panel A. Chl (peak 1) and individual species of CE (peaks 2–16) of normal meibum were analyzed using a reversed phase HPLC-positive ion mode APCI MS technique. Common analytical ion m/z 369 was used to monitor Chl and CE. Peak 2: $C_{14:0}$ - and $C_{16:1}$ -CE; Peak 3: $C_{16:0}$ - and $C_{18:1}$ -; Peak 4: $C_{18:0}$ - and $C_{20:1}$ -CE; Peak 5: $C_{19:0}$ -CE; Peak 6: $C_{20:0}$ - and $C_{21:1}$ -CE; Peak 7: $C_{21:0}$ -CE; Peak 8: $C_{22:0}$ - and $C_{24:1}$ -CE; Peak 9: $C_{23:0}$ -CE; Peak 10: $C_{24:0}$ - and $C_{26:1}$ -CE; Peak 11: $C_{25:0}$ -CE; Peak 12: $C_{26:0}$ - and $C_{28:1}$ -CE; Peak 13: $C_{27:0}$ -CE; Peak 14: $C_{28:0}$ - and $C_{30:1}$ -CE; Peak 15: $C_{29:0}$ -CE; Peak 16: $C_{30:0}$ - and $C_{32:1}$ -CE. The late-eluting peaks with retention times of more than 27 min were those of Chl-OAHFA.

Panels C–D. Effect of the age of chalazia on the distribution of individual CE in the specimens.

Figure 6. Effects of aging of chalazia on the lipid profiles of the lesions

Panel A. Molar distribution of C_{14} – C_{18} -CE (combined fraction; closed circles) and C_{19} – C_{32} -CE (combined fraction; open circles). Numbers correspond to the samples listed in Table 1. The meibum sample is labeled with a capital M.

Panel B. Lack of correlation between the increase in free Chl (closed squares) and changes in the short chain (closed circles) and long chain CE (open circles) during the initial three weeks into the development of the chalazia.

Table 1

Chalazion study population

Patient No.	Gender	Age of patient, yrs	Other diagnoses	Age of lesion, wks	Location, lower eyelid/upper eyelid, LL/UL	Histopathological diagnosis
1	M	27	Blepharitis MGD Benign neoplasm of the eyelid inner canthus	10	LL	Lipogranulomatous inflammation
2	F	41	Blepharitis	16	UL	Lipogranulomatous inflammation
3	F	58	Absent	6	LL	Lipogranulomatous inflammation
4	F	47	Blepharitis	16	LL	Lipogranulomatous inflammation
5	F	32	Blepharitis Allergic conjunctivitis MGD	3	LL	Lipogranulomatous inflammation
6	F	51	Absent	2	LL	Nonspecific inflammatory reaction
7	M	26	Blepharitis	8	UL	Lipogranulomatous inflammation
8	F	23	Absent	28	UL	Lipogranulomatous inflammation
9 [#]	F	52	Blepharitis Acne rosacea	18	UL	Nonspecific inflammatory reaction
10	F	57	Absent	10	LL	Lipogranulomatous inflammation

[#] Patient #9 took lipid-lowering medication simvastatin for unrelated condition

Table 2

Chalazion and meibomian lipids

Lipid class	Chalazion		Meibum
	Nicolaides et al.,1988	This study	Butovich, 2013
Wax esters	0%	0.05–40% (decrease with the age of lesion)	41%
(Chole)sterol esters	9%	20–30% (the ratio of short-chain to long-chain esters increases with age)	31%
Chl-OAHFA	Not reported	0–3% (decreases with age)	3%
Cholesterol	11%	0.6 – 30% (increases with the age of lesion)	0.5%
Triacylglycerols	4 %	Variable ¹	1%
Ceramides	7%	Variable ¹	<0.1%
Phospholipids	40%	Variable ¹	<0.1%

¹ Highly variable because of the uncontrolled presence of fragments of healthy tissues in the samples