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Changes in Human Meibum Lipid with Meibomian Gland Dysfunction using Principal Component Analysis

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Abstract

Changes in the phase transition temperatures and conformation of human meibum lipid with age and meibomian gland dysfunction have been quantified but with analysis of less than 1% of the infrared spectral range. The remaining 99 % of the spectral range was analyzed with principal component analysis (PCA) and confirms our previous studies and reveal further insights into changes that occur in meibum with age and disease.

Infrared spectra of meibum from 41 patients diagnosed with meibomian gland dysfunction (Md) and 32 normal donors (Mn) were measured. Principal component analysis (PCA) was used to quantify the variance among the spectra and meibum protein was quantified using the infrared carbonyl and amide I and II bands.

A training set of spectra was used to discriminate between Mn and Md with an accuracy of 93 %. This shows that certain spectral regions (eigenvectors) contain compositional and structural information about the component (variable), meibomian gland dysfunction. The spectral features of the major eigenvector indicate that Md contains more protein and relatively less CH₃ and *cis*=CH band intensity compared to Mn. The amount of protein was confirmed from relative infrared band intensities.

Our study supports the idea that compositional differences result in meibum that is less fluid and more viscous with meibomian gland dysfunction so that less lipid flows out of the meibomian gland orifice as observed clinically. This study also demonstrates the power of the combination of infrared spectroscopy and PCA as a diagnostic tool that discriminates between Mn and Md.

Keywords

meibum; infrared spectroscopy; lipids; principal component analysis; meibomian gland dysfunction

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INTRODUCTION

Relationships between meibomian gland dysfunction and dry eye symptoms have been reviewed.[Foulks, et al 2003, Foulks 2007] Numerous changes in meibum composition have been documented that may contribute and be diagnostic of dry eye symptoms. McCulley and Shine have shown that meibum from donors with meibomian keratoconjunctivitis, contains waxes and cholesterol esters,[Shine and McCulley 1991] and triglycerides [Shine and McCulley 1996] that have a higher percentage of saturated and a lower percentage of monounsaturated [Shine and McCulley 2000] and branched acyl chains compared with meibum from normal donors (Mn). In contrast, Joffre et al.,[Joffre et al 2009] have shown that meibum from donors with meibomian gland dysfunction (Md) contains a lower percentage of saturation and higher percentage of branched acyl chains. Md also contains higher levels of phosphatidylcholine [Shine and McCulley 1998] phospholipid unsaturation, [Shine and McCulley 2004] and wax esters, [Mathers and Lane 1998] and lower levels of phosphatidylethanolamine, sphingomyelin,[Shine 1998] cholesterol, and triglycerides. [Mathers and Lane 1998] the polar lipids. Discrepancies may depend on the analytical methods used, sample collection techniques and contamination.[Butovich et al 2007a, Butovich et al 2007b, Butovich 2008, Butovich et al 2009a, Butovich 2009b]

Infrared spectroscopy has been used to study human meibum. One advantage of infrared spectroscopy is that no lipid extraction or preparation is necessary. Using infrared spectroscopy our group found that near physiological temperature human meibum disorder increases with an increase in temperature.[Borchman et al 2007a] We also showed that meibum order and phase transition temperature decrease with age,[Borchman et al 2007b] and increase with meibomian gland dysfunction.[Foulks et al 2010] However, less than 1 % of the infrared spectral range was used in analysis of our previous studies.[Foulks et al 2010, Borchman et al 2007a, Borchman et al 2007b] In this study, the remaining 99 % of the spectrum was analyzed to confirm our previous studies and for insights into changes that occur in meibum with meibomian gland dysfunction. To calculate all the possible variations in the spectra, we used a technique called principal component analysis (PCA). [Malinkowski and Howery 1980, Malinowski 1987, Sutter et al 1992] PCA is a type of chemometric study, performing calculations on measurements of chemical data, that have been used for the last three decades. A data base survey of the health sciences publications show that the number of publications involving principal component analysis is over 19,000. Types of chemometrics include principal component, partial least squares and principal component regression analysis. The remarkable advantage of PCA is that subtle changes in infrared spectra that often cannot be discerned by a trained spectroscopist can be quantified and correlated to such factors as disease. As Shlens states, "With minimal additional effort, PCA provides a roadmap for how to reduce a complex data set to a lower dimension to reveal the sometimes hidden, simplified structure that often underlie it." (Shlens, J. A Tutorial on Principal Component Analysis, web page, <http://www.snl.salk.edu/~shlens/pub/notes/pca.pdf>, 2005, accessed February 5, 2010)

METHODS

Materials

Lipids, lysozyme from chicken egg white and solvents were obtained from Sigma Chemical Co., St Louis MO. Silver chloride windows for infrared spectroscopy were obtained from Crystran Limited, Poole, United Kingdom.

Validation of PCA with Lipid Standards

Mixtures of standards encompassing a range of lipid composition expected for human meibum were made to determine if PCA could be used to discriminate between differences in the meibum composition of CH_3 and $=\text{CH}$. The ranges of compositions tested in mole percent were: wax 10-100, cholesterol ester 0-80, glycerides 4-10, phospholipids 5-15 and hydrocarbon 10-30 (Table 1). An equal molar mixture of stearylpalmitate and oleyloleate were used for the wax standard and cholesterylpalmitate was used as a cholesterol ester. An equal molar mixture of triolein and 1 stearyl-*rac*glycerol was used for the glycerides and dimyristoylphosphatidylcholine and *n*-palmitoylsphingomyelin were used for phospholipid standards. *n*-Tetradecane was used as a hydrocarbon standard. No plastic was used in the procedure. Lipids were kept in an atmosphere of argon by frequently blowing argon into the vials containing lipid. Standard lipids were lyophilized for 18 hours to remove residual water before weighing on a recently calibrated Delta Range® analytical balance sensitive to 0.01 mg (Mettler-Toledo, Columbus OH). Tetrahydrofuran/methanol (THF/MeOH), 3/1, v/v, was bubbled with argon for 10 minutes to remove dissolved oxygen. Standard solutions were made by dissolving the lipid in approximately 1 mL THF/MeOH to make 0.1 M stock solutions. The stock solutions were sonicated in an ultrasonic bath (Branson 1510, Branson Ultrasonics, Danbury, CT) for 10 minutes under an argon atmosphere. The solutions were placed into 9 mm micro vials with a teflon cap (Microliter Analytical Supplies Ind., Suwanee, Georgia). The stock solutions were mixed together to give the lipid compositions listed in Table 1 and stored in 9 mm micro vials with a teflon cap (Microliter Analytical Supplies Ind., Suwanee, Georgia) purged with argon gas.

Standards for the estimation of meibum protein levels from infrared spectroscopy

Protein/wax mixtures were made by lyophilizing lysozyme for 18 hours to remove residual water before weighing on a recently calibrated Delta Range® analytical balance sensitive to 0.01 mg (Mettler-Toledo, Columbus OH). Approximately 60 μL oleyloleate was added on top of the lysozyme in 9 mm micro vials with a teflon cap (Microliter Analytical Supplies Ind., Suwanee, Georgia) and weighed. The standards were sonicated in an ultrasonic bath (Branson 1510, Branson Ultrasonics, Danbury, CT) for 10 minutes under an argon atmosphere. A glass capillary tube was used to transfer a small portion of the wax-lysozyme mixture to a AgCl infrared window. Infrared spectra were recorded using a Nicolet 5000 Magna Series Fourier transform infrared spectra were recorded infrared spectrometer (Thermo Fisher Scientific, Inc., Waltham MA) at a resolution of 1 cm^{-1} and averaging 50 scans. The areas of the carbonyl band near 1740 cm^{-1} , and the Amide I and II bands near 1650 and 1550 cm^{-1} were measured from curve fitting the infrared spectra of the standards using GRAMS/386 software (Galactic Industries, Salem, NH).

Diagnosis of Human meibomian gland dysfunction

Normal status was considered when there was no evidence of keratinization or plugging with turbid or thickened secretions and without dilated blood vessels on the eyelid margin of patent meibomian gland orifices. None of the donors had ever experienced dry eye symptoms or blepharitis.

The diagnosis of meibomian gland dysfunction was made according to the criteria of Foulks and Bron.[Foulks and Bron 2003] Plugging of the meibomian glands of at least 5 out of 10 orifices in the central portion of the upper eyelid was required for diagnosis. The character of meibomian gland expressed secretion had to be turbid, turbid with clumps, or paste-like. Inflammation of the eyelid margin, as evidenced by swelling of the eyelid margin and 2+ vascular injection of the posterior lid margin, was necessary for diagnosis. The presence of telangiectasia of the posterior eyelid margin was confirmatory of chronic disease but not

required for entry. Tear film stability was determined by instillation of sodium fluorescein into the tear film and tear breakup time was less than 5 seconds for all subjects sampled.

Collection of Meibum

Meibum was obtained from normal living subjects as described in Kilp et al., 1986, except that meibomian gland excreta were collected gland expression was done by compressing the eyelid with a platinum spatula. Meibomian eyelids were expressed and about 1 mg of meibum was between cotton-tipped applicators with strict attention to avoid touching the eyelid margin during expression. All four eyelids were expressed and about 1 mg of meibum was collected per individual for direct spectroscopic study. The expressate was collected with a platinum spatula and immediately spread onto the AgCl window. All samples were frozen under argon gas until analysis. Analyses were performed within three weeks of collection of the sample. Written informed consent was obtained from all donors and protocols and procedures were approved by the University of Louisville Institutional Review Board as well as the Louisville Veterans Affairs Institutional Review Board and procedures were in accord with the Declaration of Helsinki.

Fourier Transform Infrared Spectroscopy

Lipid standards were added (~ 1mg) to a AgCl window with care to keep the maximum infrared absorbance between 0.6 and 1. The lipid standards were added a few micro liters at a time and evaporated under a stream of nitrogen gas. The samples were lyophilized for 18 hours to remove the solvent. Infrared spectra of the standards were measured within two days of starting the preparation. Storage of the sample on AgCl windows for over two months under argon did not affect the sample [Oshima et al., 2009].

Infrared spectra were measured using a Nicolet 5000 Magna Series Fourier transform infrared spectrometer (Thermo Fisher Scientific, Inc., Waltham MA). The meibum was placed on a AgCl window and without any additional preparation, it was inserted into the instrument sample chamber. One hundred and fifty 150 scans with a resolution of 1 cm^{-1} were averaged to obtain each spectrum.

Principal component analysis—Principal component and infrared data analysis were performed with GRAMS/386 software (Galactic Industries, Salem, NH). PCA analyzes the variations between a group of spectra called a training set. PCA finds the variations the variations between a group of spectra called a that appear to be synchronized proportionally and extracts them to produce eigenvectors that often look like infrared spectra. Eigenvectors also called loading spectra or factors represent a component that changes in concentration from sample to sample. The group of spectra used to generate eigenvectors is called a training set. The training sets may be eventually used to model how the eigenvectors relate to a component such as age or disease. A training set of infrared spectra from mixtures of standards were made to determine if PCA could be used to discriminate between differences in the meibum composition of CH_3 and $=\text{CH}$. PCA was also used to detect subtle differences in spectral variation and to assess whether or not spectra of meibum from normal donors (Mn) differ from those of donors with meibomian gland dysfunction (Md). Cross validation was used for each spectrum, removing a spectrum from the training set and using the remaining training set samples to perform the decomposition and calibration calculations. For each spectrum, two constituents were used, age and 'meibomian gland dysfunction/normal score'. The training set was set up by assigning Mn spectra a 'meibomian gland dysfunction/normal score' of 0 and Md spectra a 'meibomian gland dysfunction /normal score' of 100. The training set encompassed the CH and OH stretching region from 3612 to 2490 cm^{-1} and the fingerprint region from 1814 to 676 cm^{-1} . The total number of samples used in the training set was 73, 41 Md and 32 Mn samples.

The first step in obtaining eigenvectors from a training set of data is to determine the number of eigenvectors to use. Too many eigenvectors and the calibration model will be over fit introducing “noise” vectors. Too few eigenvectors and the calibration will be under fit and inaccurate. Choosing the number of eigenvectors is generally non-subjective.

A Prediction Residual Error Sum of Squares (PRESS) plot may be used for choosing the ideal number of eigenvectors. The smaller the PRESS value the better the model is able to predict the concentrations of the calibrated constituents, age, and ‘meibomian gland dysfunction/normal scores’. The chosen number of eigenvectors determined by the PRESS plots can be confirmed by plotting the eigenvector number versus the % of total variance it accounts for.

A mathematically “reconstructed” spectrum can be obtained by adding all the products of each score associated with an eigenvector times the eigenvector. If the principal component analysis model is good, that is if the spectral variations are caused by the principal component (in this case meibomian gland dysfunction) the reconstructed spectrum will be very similar to the original spectrum. The scores for an eigenvector plotted against the scores of another eigenvector were used to determine which eigenvectors were the most important to modeling components.

Statistics

Data are presented as the average $\pm$ the standard error of the mean. Significance was determined using the Student's t test or the correlation coefficient from the linear regression best fit. Values of $p < 0.01$ were considered statistically significant.

RESULTS

Lipid Standards—PCA was successful in discriminating between differences in the composition of CH_3 and $=\text{CH}$ over a range of lipid compositions reported for human meibum. Eigenvectors 1 and 2 grouped the standard samples into two classical elliptical Mahalanobis boundaries (Fig. 1 A and B). [Mahalanobis and Mahalanobis 1936] The group with negative eigenvector 1 and 2 scores (Fig. 1 A) grouped samples with a $=\text{CH}/\text{CH}_2$ ratio between 0.0656 to 0.0674 and the group with a positive score was grouped from 0.0696 to 0.0721 $=\text{CH}/\text{CH}_2$. PCA could discriminate between samples that were different by 2 % and perhaps even less. Similarly, the lower group in Fig. 1B was grouped from 0.684 to 0.0962 CH_3/CH_2 whereas the upper group was grouped from 0.1081 to 0.1579 CH_3/CH_2 . The protein standards were less complex than the lipid standards, none-the-less most of the samples were grouped with a negative eigenvector 1 score excluding two points with higher protein values above 6.635 moles amide/mole wax (Fig. 1 C).

The primary purpose of this study was to discriminate between groups and to note differences as a guide to what groups may be important to quantify in future studies. Quantification will be reported with ^1H - nuclear magnetic spectroscopy results in future papers. Figure 2 shows that PCA with cross validation for each spectrum could also be used to quantify infrared spectra in relationship to small changes in $=\text{CH}/\text{CH}_2$ (Fig. 2 A and B), CH_3/CH_2 (Fig. 2 C) and protein to lipid ratios (Fig 2 D). The linear regression analyses for the standard curves in Fig. 2 are presented in Table 2. All of the standard curves were linear with $p < 0.005$.

Human Material

Thirty-two human donors of Mn and 41 donors of Md were recruited from the members and associates of Dr. Borchman's laboratory and patients of Dr. Foulks at the Kentucky Lions

Eye Center and the Veterans Affairs Hospital, Louisville Kentucky. Donors with eye make up were excluded. The donor profile is presented in Table 3.

The number of eigenvectors—Average infrared spectrum of human meibum is shown in Figure 3. Band assignment and compositional and physical properties derived from meibum infrared spectra have been published.[Borchman et al 2007b] Because it is difficult to visually detect differences between the average spectra of Md and Mn, PCA was used to extract subtle spectral variations (eigenvectors) and used them later to discriminate whether a spectrum belongs in the Mn or Md group based on meibomian gland dysfunction being the principal component causing these difference.

Three eigenvectors were necessary to describe most of the variance in our infrared spectra for the constituent, age (Fig. 4A). For the constituent 'meibomian gland dysfunction /normal score', 6 vectors were needed (Fig. 4B). Eigenvectors 1, 2, 3, 4, 5 account for 68, 15, 8, 2 and 2 % of the variance in the meibomian gland dysfunction /normal constituent scores, respectively (Fig. 5).

Spectral Discrimination—The training set discriminated between Mn and Md with accuracy of 93 % (Fig. 6A) and predicted the age of the donors of Mn but not Md (Fig. 6B). Because we are able to discriminate between Mn and Md, the Eigenvectors must contain compositional and structural information about the changes that occur with meibomian gland dysfunction. The following sections discuss the results related to determine the compositional and structural changes that occur in meibum with meibomian gland dysfunction. There was no correlation between the predicted and actual ages of the Md donors (Fig. 6 B). The predicted values for the age of Md donors were more variable, ± 20 y, and slightly higher than those for Mn (Fig. 6B).

Scores and Mahalanobis Boundry Groupings—Eigenvector 1 grouped the Mn spectra of adolescent normal donors, 14 to 23 years of age, in a classical elliptical Mahalanobis boundry (Fig. 7A, X's, left).[Mahalanobis and Mahalanobis 1936] The spectra of the other normal adults and MD were clustered together so eigenvector 2 and 3 when plotted against eigenvector 1 did not discriminate between Mn and Md (data not shown). When eigenvector 4 was plotted against eigenvectors 1 (Fig. 7A), 2 (Fig. 7B) and 3 (Fig. 7 E,F), most of the Mn and Md spectra were grouped with minimal overlap. Eigenvector 2 was also significant in discriminating between Mn and Md (Fig. 7 B,C,D). The average eigenvector score for Mn was positive and for Md was negative. (Table 4). Negative scores for eigenvectors 2, 3 and 4 generally favored the grouping of Mn whereas positive scores favored the grouping of Md (Fig. 7).

Eigenvector spectra—The 4 major eigenvector spectra for the constituent 'meibomian gland dysfunction /normal score', are shown in Figures 8 and 9. The eigenvectors 1-4 accounted for 94 % of the variance in the spectra of Md and Mn. Some of the spectral characteristics of the eigenvectors that correspond to infrared band assignments are listed in Table 5.

Eigenvector 1, 2 and 4 scores for Mn were generally negative (Table 4), so eigenvector 1, 2 and 4 were generally subtracted to account for the variance. The opposite was true for Md. Eigenvector 1, 2 and 4 contain infrared amide I and II and other bands that are due to protein vibrations (Table 5). The major difference between Mn and Md, based on the eigenvector 1, 2 and 4 scores, is the elevated level of protein in Md compared to Mn. This can be seen in the result of subtracting eigenvector 1 (Fig. 10B) from Mn (Fig. 10A) resulted in a spectrum (Fig. 10C) that was very similar to that of a pure wax (Fig. 10D) with a small contribution

from cholesterol ester at 1377 cm^{-1} . The higher protein content of Md compared to Mn is seen in the difference spectrum (Fig. 11, middle).

Eigenvector 2 reveals additional subtle differences between Mn and Md. Adding eigenvector 2 to the average Md spectrum because Md spectra generally have positive scores (Table 4) results in larger protein amide A, I and II bands and a smaller cholesterol ester band at 1377 cm^{-1} , a slightly higher C-O band at 1179 cm^{-1} (Fig. 12 B) and much less intensity for the =CH stretching band at 3010 cm^{-1} and CH_3 stretching intensity at 2954 cm^{-1} (Fig. 12A). The opposite is true in subtracting eigenvector 2 from Mn. The eigenvectors are subtracted because Mn generally has a negative score (Table 4). Therefore, from the subtraction results, the intensity of the =CH and CH_3 infrared bands is relatively higher for Mn spectra compared to Md spectra. Mn contains a higher percentage of cholesterol esters and protein than Md.

Eigenvector 3 accounts for shifting the C=O band in Md spectra from 1749 to 1737 cm^{-1} and eigenvector 4 accounts for shifting the CH_2 bending band in Mn from 1467 to 1473 cm^{-1} .

Quantification of Meibum Protein

The PCA data indicating that one of the major differences between Mn and Md is the amount of protein lead us to investigate the protein band region in more detail. Amide A, I and II bands are resolved in the infrared spectra of human meibum (Fig. 13). The amide bands could arise from proteins or sphingolipids. Based on the spectra in Figure 13 bottom, the source of the amide bands is probably due to protein since the sphingomyelin phosphate symmetric PO_2^- stretching band at 1085 cm^{-1} and -C-O-P-O-C diester stretch at 1055 cm^{-1} (Lamba et al., 1991) are not resolved in the spectra of meibum. All three amide bands are relatively less intense in the average infrared spectrum of Mn compared with Md.

To quantify the amount of amide I and II bands relative to the intensity of the carbonyl band at 1737 cm^{-1} the region was curve fit (Fig. 14). The relative intensity of the amide I and II bands was related to the phase transition temperature (Fig. 15) and meibum order (Fig. 16). The infrared carbonyl band bandwidths were 19 cm^{-1} for both Mn and Md and the carbonyl frequencies were $1737.61 \pm 0.09\text{ cm}^{-1}$ and $1737.4 \pm 0.1\text{ cm}^{-1}$, for Mn and Md respectively. The amount of protein in meibum was determined by interpolating the meibum amide I/C=O intensity ratio from the standard curve measured from mixtures of the protein lysozyme and the wax oleylolate. The analysis was validated based on our previous study (Navarro et al., 1984). The standard curve gave a slope of 0.246 moles amide/mole C=O, $r = 0.922$. The amount of meibum protein from the four groups of meibum spectra is given in Table 6. To estimate a weight ratio from the molar ratio (amide/C=O), the molar ratio was multiplied by 0.2191.

DISCUSSION

In the present study PCA was used to discriminate between Mn and Md with an accuracy of 93 %. This could be important for diagnostic purposes, but more importantly, the ability to discriminate between Mn and Md indicates that subtle differences in the infrared spectra occur between Mn and Md that distinguish the two groups of spectra. The possible compositional and conformational differences that lead to the infrared spectral differences are discussed below.

The 7 % of the spectra of Mn and Md that were not assigned to the correct group could be due to many factors. One factor is that contaminants, constituents that are shared by other causes or spectral aberrations that do not appear at the selected group wavelengths of the

other samples and match the wavelengths of the group, could cause the sample to be falsely classified. It is also possible that some of the outlying data were from donors with other systemic factors that complicated their diagnosis or that experienced transient signs and symptoms of meibomian gland dysfunction or were transiently closer to normal at the time of collection. In future studies, the level of inaccurate discrimination will diminish because the accuracy of PCA discrimination becomes more accurate as the training set becomes larger.

The accuracy of the predicted age compared to the actual age of the Md group was slightly higher and the variation twice as large, ± 20 years, as the Mn group reported [Borchman et al 2010]. It is possible that some of the spectral variations that were used to discriminate meibomian gland dysfunction, interfered with the spectral variations associated with age. It is also possible that the 'spectral characteristics' or 'biological' age of the meibum associated with donors with meibomian gland dysfunction appeared higher than for the Mn group. Lipid order is higher in younger Mn compared to older Mn [Borchman et al 2010] yet aged-matched Mn is more ordered than Md [Foulks et al 2010] so the conformational changes and probably the compositional changes of meibum from patients with meibomian gland dysfunction is probably not an exacerbation of the aging process. A negative score for eigenvectors 1 and 3 (Fig. 7 A) made it possible to discriminate between a group of Mn aged 14 to 23 years from all of the other groups.

The Mahalanobis grouping and eigenvector scores for Md and Mn suggest that Md has more protein, and slightly less cholesterol esters, and relatively less *cis* =CH and CH₃ groups. The latter two differences would be expected to order the hydrocarbon chains of Md. Indeed, using almost all of the samples in this study, we have shown that Md is more ordered and exhibits a higher phase transition temperature than Mn.[Foulks et al 2010]

McCulley and Shine showed that in one form of meibomian gland dysfunction called meibomian keratoconjunctivitis (MKC), the wax and cholesterol esters [Shine and McCulley 1991] and the triglycerides [Shine and McCulley 1996] of Md contained a lower percentage of unsaturated and branched fatty acids (and therefore less CH₃ groups) compared to Mn. Other groups of Md such as seborrheic, alone or seborrheic with meibomian seborrhea did not show a significant difference in the fatty acid chain composition of Md compared with Mn. [Shine and McCulley 1991] These results are contrary to those of Joffre et al.[Joffre et al 2009] who show a 60 % decrease in saturated fatty acids and a 50 % increase in hydrocarbon chain branching in Md. No quantification was performed in this study, and we can not be certain that the differences in the intensity of the infrared CH₃ and *cis* =CH bands were due to just lipid and not protein or other compounds in meibum. At present, our results support the Shine and McCulley study. The changes observed by Joffre et al., are well above the detection limit of infrared spectroscopy yet we did not observe them. A careful quantitative analysis of the extracted lipid in our samples using infrared, and ¹H-nuclear magnetic spectrometers and matrix assisted laser desorption mass spectrometry is almost completed which may help to resolve this issue.

We found that the relative amount of amide I and II bands in meibum increased with decreasing age in Mn and with MGD (Fig. 15). The amide I and II bands are associated with proteins. The changes were similar for both the amide I and II bands, so it is unlikely that the =CH bending band that underlies the amide I band contributes to this relationship since the =CH band does not interfere with the amide II band. Our finding of protein in meibum is not novel. Over 90 proteins have been identified in human meibum (Tsi, et al. 2006). Md was found to contain cellular debris (Korb and Heriquez 1980; (Terada et al., 2004). The cellular debris, presumably proteins composed 60 % of the meibum (Terada et al., 2004).

The protein keratin was found to be 10 % higher in Md compared to Mn and was suggested to initiate MGD by occluding the meibomian gland orifice (Ong et al., 1991).

In the present study the relative amount of protein in meibum was linearly related to meibum lipid order (Fig. 16) and phase transition temperature (Fig. 15). It is reasonable that, as meibum proteins and other factors such as saturation increase with meibomian gland dysfunction, the lipids become more ordered and more viscous. Viscosity has been assessed indirectly by visual appearance (Mathers and Lane, 1998; Ong 1996), but this assessment may be due more to protein in the meibum and the color of the meibum rather than its viscosity, which was measured with infrared spectroscopy as lipid order (Borchman et al., 2007, 2010b; Foulks et al., 2010). Correlation does not necessitate cause, but the relationships between the amount of protein, meibum phase transition temperature and order, age and meibomian gland dysfunction are reasonable and not likely to be due entirely to chance or coincidence.

Does meibum protein contribute to the etiology of dry eye?

The etiology of dry eye symptoms related to MGD is beyond the scope of the current paper (Knob and Knob, 2009). Dr. Foulks points out in his review article (Foulks, 2007) that reduced delivery of meibum to the lid margin and to the tear film may lead to increased tear evaporation. What makes Md more ordered (viscous) than Mn? Our infrared spectra of meibum show that Md has more protein than Mn and that the amount of protein is related to, lipid order (viscosity) and phase transition temperature. This relationship could be coincidental or causative. It is reasonable that, as the lipids become more ordered and more viscous as observed, less lipid flows out of the meibomian gland orifice and more casual lipid is present on the lid margin. The changes in meibum composition and physical properties with meibomian gland dysfunction as well as a decrease in the amount of meibum available to form the tear film lipid layer could lead to a thinner tear film lipid layer, greater osmolarity and higher rate of evaporation and decreased breakup time all leading to dry eye symptoms and inflammation.

The age paradox

Loss of meibum delivery with meibomian gland dysfunction can reasonably explain the destabilization of the tear film with meibomian gland dysfunction as discussed above. This is not true with age related changes in meibum. With increasing age, meibum lipid order and phase transition temperature decrease (Borchman 2010 and Fig. 7) and the amount of meibum on the lid margin increases [Chew et al., 1993a]. More meibum with age should stabilize the tear film lipid, yet paradoxically, with increasing age the stability of the tear film decreases, opposite of what would be expected from our arguments for meibomian gland dysfunction. For instance tear break-up time (Chow and Yap, 1993), keratinization and drop out (Hyken and Bron, 1992) osmolarity (Chow and Yap, 1993; Mathers et al., 1996) evaporation rate (Chow and Yap, 1993; Mathers et al., 1996) increase although some studies show no change in the rate of evaporation with age (Craig and Tomlinson, 1998; Rolando and Refojo, 1983; Tomlinson and Giestorecht, 1993). Craig and Tomlinson, 1997, noted that “where the human lipid layer is absent, or is not confluent, and the tear film is unstable, tear evaporation is increased.” However, where there is a stable, intact lipid layer, regardless of lipid thickness, tear evaporation is retarded.” Yokoi et al., 1999, and Chew et al., 1993a, came to a similar conclusion that there is an ample reservoir of meibum on the lid margin to provide lipid to cover the tear film. Yokoi et al., 1999 states that at some point, when the reservoir of meibum on the lid reaches a critically low amount, there will not be enough to cover the tear film. This critically low amount may not be reached with the normal aging population. It is reasonable that the amount of lipid delivered is not a factor in tear film stability at lower ages especially since with age the amount of lipid on the lid

margin is larger yet tear film stability is lower. The quality of the meibum such as the degree of saturation, branching and compositional differences as well as other factors such as tear film protein-tear film layer lipid interactions may be important for explaining both age and meibomian gland dysfunction and tear film stability observations. McCulley and Shine [Shine and McCulley 1991] noted that compared to their studies of human meibum, rabbit meibum [Tiffany 1979] is more unsaturated and presumably more disordered which may lead to a more stable tear film.

It is reasonable that with meibomian gland dysfunction, as meibum lipids become more saturated and contain less branched chain hydrocarbons and more protein they will be more viscous and more ordered as observed. [Oshima et al 2009] It has been suggested that ordered, more viscous lipids, would impede the delivery of meibum to the lid margin. [Borchman et al 2007a, Nagymihalyi et al 2004] Our study supports the idea that compositional differences result in meibum that is less fluid and more viscous with meibomian gland dysfunction resulting in less lipid flow out of the meibomian gland orifice. This study demonstrates the power of the combination of infrared spectroscopy and PCA as a diagnostic tool that discriminates between Mn and Md. Some of the major moieties that change with meibomian gland dysfunction were revealed so that future studies are planned to quantify the changes using infrared and ¹H-nuclear magnetic spectroscopes and matrix assisted laser desorption mass spectrometry.

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Figure 1. Mahalanobis groupings of standards for eigenvectors 1 versus 2 calculated by principal component analysis for A) $=CH/CH_2$, standards in Table 1, B) CH_3/CH_2 , standards in Table 1. C) Protein standards composed of lysozyme and wax.

Figure 2.

Principal component analysis was used on a training set of standards in Table 1 and lysozyme protein mixtures to predict A) =CH/CH_2 , B) CH_3/CH_2 , and C) protein amide/wax C=O . The actual and predicted values are graphed.

Figure 3.

Average Infrared spectra of human meibum (□ □ , bold) Md. (□ □, gray) Mn.

Figure 4.
'PRESS' plots showing the optimum factors to use (arrow). A) Age component. B)
Meibomian Gland Dysfunction and Normal Score constituent.

Figure 5.

The % of total variance for each eigenvector (factor) related to the 'meibomian gland dysfunction /normal score' constituent.

Figure 6.

A) A training set was used to discriminate between the spectra of meibum from normal donors and the spectra of meibum from donors with meibomian gland dysfunction. This shows that the infrared spectra must contain compositional and structural information about the changes that occur with meibomian gland dysfunction. A score above 50 (vertical line) is considered a sample with meibomian gland dysfunction. B) The same training set used for 4A was used to predict the age of the donors with meibomian gland dysfunction within ± 20 y at a 95 % confidence limits. (●) Mn group from: Borchman et al 2010. (○) Md group. (□□) Linear regression fit and (---) 95 % confidence limits of Mn group data from: Borchman et al.

Figure 7.
Mahalanobis groupings for select pairs of eigenvectors from the principal component analysis of meibomian dysfunction scores.

Figure 8.

Infrared spectra of A) Cholesterol palmitate. B) Oleylolate C) Eigenvector 1 for the constituent 'meibomian gland dysfunction /normal score'. D) egg white lysozyme. Numbers are infrared band assignments: 1) Amide A, 3282 cm^{-1} 2) Amide 1, 1656 cm^{-1} 3) Amide 2, 1525 cm^{-1} 4) 1380 cm^{-1} 5) C-C twist, 720 cm^{-1} .

Figure 9.
Infrared spectra of A) Eigenvector 4, B) Eigenvector 3, C) Eigenvector 2.

Figure 10. Infrared spectra of A) Average spectrum of Mn age greater than 50 years. B) Eigenvector 1. C) Difference spectra of the average spectrum of Mn age greater than 50 years minus eigenvector 1. D) Spectrum of the wax oleyloleate.

Figure 11.

There was very little difference between the average Md spectrum (top bold) and the average Mn spectrum (top thin). (second from bottom) difference spectrum average Md minus average Mn. The difference spectrum resembled that of a protein. (bottom) Infrared spectrum of anhydrous lysozyme.

Figure 12. Infrared spectra of the carbonyl, amide I and bands for average human meibum Md (□ □ , bold, upper) and curve fit (□ □, gray, lower).

Figure 13.

Infrared spectra at 24.8 °C on a AgCl window. Top: A) Average of human meibum (□ □ , bold, upper) Md. (□ □ , gray, lower) Mn. B) Bovine sphingomyelin. Bottom: Fingerprint region for A) Oleyloleate B) Average of human meibum (□ □ , bold, upper) Md. (□ □ , gray, lower) C) Bovine sphingomyelin D) lysozyme.

Figure 14.
Infrared spectra of (thin gray) average Md, (bold black) average Md plus eigenvector 2.

Figure 15.

The relationships between the relative amide band intensity and phase transition temperature of human meibum. Lipid phase transition temperatures for normal samples were taken from Borchman et al., 2010. Lipid phase transition temperatures for Md were taken from Foulks et al., 2010. Values are average $\pm$ the standard error of the mean. Age ranges were : normal child, 13 years and below; normal adolescent, 14 to 32 years; normal adult 33 years and above. The same samples were used to measure the lipid phase transition temperature and the relative intensity of the amide bands.

Figure 16.

The relationships between the relative amide band intensity and lipid order of human meibum. Lipid order for normal samples were taken from Borchman et al., 2010. Lipid order for Md were taken from Foulks et al., 2010. Values are average $\pm$ the standard error of the mean. Age ranges were : normal child, 13 years and below; normal adolescent, 14 to 32 years; normal adult 33 years and above. The same samples were used to measure lipid order and the relative intensity of the amide bands.

Table 1

Standards used in this study.

Molar Percentage of Standard Lipids				
WAX	Cholesterol Ester	Glycerides	Phospholipids	Hydrocarbons
80	20			
80	11	4	5	
80	0	5	15	
20	40	6	4	30
32	40	8	10	10
70	20			10
70	10			20
86				14
100				
90				10
90		10		
90	10			
80				20
80		10	10	
80	20			
70				30
60	40			
10	80	10		

Table 2

Regression analysis for standard curves in Figure 2.

	Correlation coefficient	Intercept	Slope	p
=CH/CH ₂ , Fig 2 A	0.9855	0.0112	0.843	< 0.005
=CH/CH ₂ , Fig 2 B	0.8576	0.0104	0.853	< 0.005
CH ₃ /CH ₂ , Fig 2 C	0.8837	0.0178	0.781	< 0.005
Protein/lipid, Fig 2 D.	0.9798	0.115	0.960	< 0.005

Table 3

Donor Data

	Normal	Meibomian-gland dysfunction
Average Age (y)	33 ± 22	66 ± 15
Median Age (y)	20	69
Age Range (y)	14 – 88	29 – 87
Male (%)	72	63
Caucasian (%)	84	81
Black (%)	0	7
Hispanic (%)	0	2
Asian (%)	16	0
Unknown	0	10

Table 4

Eigenvector Scores.

	Eigenvector Score (Average $\pm$ Standard Deviation)			
Eigenvector number	1	2	3	4
Normal Group (Mn)	-1.6 ± 4.8	-0.7 ± 1.0	-0.3 ± 0.6	-0.4 ± 0.7
Meibomian Gland Dysfunction Group (Md)	1.1 ± 2.4	0.5 ± 1.4	0.2 ± 1.1	0.3 ± 0.9

Table 5

Eigenvector number	Eigenvector Band Intensity ^c (Average $\pm$ Standard Deviation)			
	1	2	3	4
OH Bend, 3456 cm ⁻¹	n.c.	n.c.	n.c.	+
Amide A, 3296 cm ⁻¹	+	n.c.	n.c.	+
CH ₂ , symmetric/asymmetric stretch, 2918/2849 cm ⁻¹	+	+	+	–
C=O, 1737 cm ⁻¹	+	–	shift ^a	–
Amide 1 and C-H bend, 1656 cm ⁻¹	+	+	–	+
Amide 2, 1525 cm ⁻¹	+	+	–	+
CH ₂ bend, 1487-1466 cm ⁻¹	+	–	+	Shift ^b
CH ₃ deformation, 1377 cm ⁻¹	+	–	n.c.	n.c.
C-O asymmetric bend, 1179 cm ⁻¹	+	–	n.c.	n.c.
1150/1108 cm ⁻¹	n.c.	n.c.	–	+
C-C rock, 721 cm ⁻¹	+	–	n.c.	+

n.c. stands for no change

^a Shift from 1749 cm⁻¹ to 1737 cm⁻¹

^b Shift from 1467 cm⁻¹ to 1473 cm⁻¹

^c (+) Intensity of eigenvector band is above 0. (–) Intensity of eigenvector band is below 0.

Table 6

Infrared amide I band intensity and amount of meibum protein.

	Infrared Band Intensity Ratio (Amide I/ C=O)	Molar Ratio Amide I/ C=O	Meibum Protein (% by weight)^a
Normal Child Group 3 to 13 years old	0.8 ± 0.1 (6)	3.3 ± 0.4	42 ± 5
Normal Adolescent Group 14 to 33 years old	0.59 ± 0.07 (10)	2.4 ± 0.3	34 ± 4
Adult Group above 34 years old	0.40 ± 0.07 (9)	3.9 ± 0.7	26 ± 5
MGD Group	1.0 ± 0.1 (36)	3.3 ± 0.3	46 ± 5

^a weight % calculated from the amide I/ C=O band intensity ratio, assuming a wax molecular weight of 532.9 g, and 0.95 % pure lysozyme, 129 amide groups per mole, and a lysozyme molecular weight of 14309 g/mole.