Spectroscopic measurements of meibum compositional, structural, and functional relationships to elucidate the role of meibum in dry eye.

Anthony Chigozie Ewurum

University of Louisville

Follow this and additional works at: https://ir.library.louisville.edu/etd

Part of the Analytical Chemistry Commons, Eye Diseases Commons, and the Investigative Techniques Commons

Recommended Citation

https://doi.org/10.18297/etd/3814

This Doctoral Dissertation is brought to you for free and open access by ThinkIR: The University of Louisville's Institutional Repository. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of ThinkIR: The University of Louisville's Institutional Repository. This title appears here courtesy of the author, who has retained all other copyrights. For more information, please contact thinkir@louisville.edu.
SPECTROSCOPIC MEASUREMENTS OF MEIBUM COMPOSITIONAL, STRUCTURAL, AND FUNCTIONAL RELATIONSHIPS TO ELUCIDATE THE ROLE OF MEIBUM IN DRY EYE

By

Anthony Chigozie Ewurum
B.S., Indiana University Southeast, 2016
M.S., University of Louisville, 2020

A Dissertation
Submitted to the Faculty of the
College of Arts and Sciences of the University of Louisville
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy
in Chemistry

Department of Chemistry
University of Louisville
Louisville, Kentucky

May, 2022
Copyright 2022 by Anthony Chigozie Ewurum

All rights reserved
SPECTROSCOPIC MEASUREMENTS OF MEIBUM COMPOSITIONAL,
STRUCTURAL AND FUNCTIONAL RELATIONSHIPS TO ELUCIDATE THE
ROLE OF MEIBUM IN DRY EYE

By

Anthony Chigozie Ewurum
B.S., Indiana University Southeast, 2016
M.S., University of Louisville, 2020

A Dissertation Approved on

March 25, 2022

By the following Dissertation Committee:

Dissertation Chair
Dr. Douglas Borchman

Dissertation Co-chair:
Dr. Muriel Maurer

Dr. Frank Zamborini

Dr. Xiang Zhang
DEDICATION

This dissertation is dedicated to God almighty who has made it at all possible for me to accomplish this

To my marvelous and lovely parents Mr. Ephraim Emeka Pueme and Mrs. Ngozi Blessing Pueme, who always encouraged me and gave a lot for me to pursue higher education in the sciences,

my former research advisor late Prof. Cecilia .M. Yappert for spurring on my passion in analytical chemistry,

and to all my supportive family household and Church on the Rock family.
ACKNOWLEDGEMENTS

In the name of God almighty, I reflect on the course of this work, and to his name, I ascribe the utmost blessings and laudation for completion.

From the outset, I am exceedingly indebted to my major professor, Prof. Douglas Borchman for taking up the helm of my Ph. D research and allowing me to be a part of his bioanalytical lab during an uncertain scholastic transition time. I truly value his patience in training me speedily in the science and machinations of dry eye disease research. I am forever thankful to him for helping my professional career by giving me the opportunities to attend conferences and make career-building acquaintances. I consider him a spectacular advisor and an ardent personal growth influencer even till today.

I want to extend my sincerest appreciation to Dr. Muriel Maurer for being an excellent co-advisor and committee member who has made sure I fulfill the chemistry requirements of my Ph.D. I am grateful for your ever-timely updates, extensive help, and insights throughout all my time at the University of Louisville. I would like to personally thank the remainder of my committee members: Dr Xiang Zhang for his invaluable insights into analytical chemistry, his coursework which introduced me to the beautiful world of analytical instrumentation, and guide tips for stellar scientific presentations. I will not forget to show enormous appreciation to Dr. Frank Zamborini who have always challenged me in the way I think as an analytical chemist and for being an immense
encourager. I would like to submit a deserving thanks to my former fellow researchers in Professor Borchman’s lab. My profound gratitude goes to Dr. Samiyyah Sledge for teaching me a lot about research in the lab and always entertaining every question I had. I would like to say a big thank you to Akhila Ankem and Sravya Veligandla for all their contributions to our research.

I would like to post-humously convey my humblest and deepest thank you to Late Prof. Cecilia for fueling my interest in analytical chemistry and spectroscopy. Every question I had that were the most rudimentary was patiently answered. Her enthusiasm about science was infectious and I am very grateful because I would not know Dr. Borchman without her.

I also extend my appreciation to everyone at the University of Louisville Chemistry Department for their help throughout my program. A special shoutout to my former CGSA crew: Dr. Arghya Pratim Ghosh, Dr. Megan Mckintosh, Kritika Bajaj, Chinmay Potnis for incredible experiences during classes and Derby Lecture Series. A big thanks to Dr. Neal Stolowich for helping me become comfortable with $^1$H-NMR. I extend my regards to Prof. Richard Baldwin for the privilege of being his TA during my time as a Ph.D. scholar.

Last but definitely not the least, I would like to thank all my friends, family members and COTR family for all their encouragement and tenacity in staying with me through all these years. I am grateful for my dearest and priceless parents and their ever-ready eagerness to support in every way they can throughout my studies. My deepest appreciation goes to to my siblings; Nnaemeka and Lucky, my deepest appreciation goes to them for always helping me in any way they can to keep me level headed through
jokes and conversations. I would personally like to give a thankful mention to Mr. Ken Dickson for keeping me in check and lifting me up always. I will not forget Ms. Ursula Lattis and Alex Lattis for all their support personally throughout my program. I would like to extend my gratitude to Emmanuel Awosu, Jessica Harris & Amzi Bock for all their prayers, unquantifiable support, and celebrations for all my milestones; I am hugely grateful to Yves & Monique Beda for their encouragement through everything and willingness to assist me whenever. Thank you all for believing in me; It is done...!!!
ABSTRACT

SPECTROSCOPIC MEASUREMENTS OF MEIBUM COMPOSITIONAL, STRUCTURAL AND FUNCTIONAL RELATIONSHIPS TO ELUCIDATE THE ROLE OF MEIBUM IN DRY EYE

Anthony Chigozie Ewurum

May 14, 2022

The major aim of my dissertation was to investigate the etiology of dry eye disease which affects about 7 million people in the United States, causing symptoms that can lead to visual disturbance. Correlation between dry eye and an abnormal lipid layer of the tear film has been found. Tear film lipids originate mostly from the meibomian glands. Cholesteryl ester (CE) and Wax ester (WE) lipids make up most of the human meibum lipidome and the CE/WE ratio has been shown to decrease in patients with meibomian gland dysfunction. Model studies using synthetic CE and WE, although providing some insight, are weak in their application as human CE and WE contain variable amounts of hydrocarbon chain branching, saturation and chain lengths. It is thus almost impossible to model the diverse composition of human meibum lipids using synthetic WE and CE. In \textit{vitro} model studies of meibum lipids were therefore conducted using adsorption column chromatography, $^1$H-NMR, and FTIR spectroscopies to investigate meibum lipid interactions and the relationships between meibum lipid conformation and composition changes.
Interactions between synthetic minor meibum lipids and hyaluronic acid (HA) were also investigated to identify practicable relevance to dry eye treatment. HA is used in eye drops with other therapeutics for dry eye symptoms mitigation. Data suggest physiological levels of HA, PC and SM may be related eye drop therapy. Human meibum CE and WE were also completely separated and spectroscopically verified. Phase transitional data of CE/WE mixtures indicated that CE changes the phase characteristics of meibum depending on whether it is more or less ordered than WE. Changes in the meibum CE/WE ratio could therefore explain changes in meibum order with age and meibomian gland dysfunction (MGD) which may influence tear film stability. CE/WE ratios from the meibum of donors with Sjögren syndrome were also compared to meibum from donors without dry eye to better understand the relationships between dry eye disease and meibum compositional differences.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................ iv
ABSTRACT .......................................................................................................................... vii
LIST OF TABLES .................................................................................................................. ix
LIST OF FIGURES .............................................................................................................. xiv

CHAPTER

I. INTRODUCTION .............................................................................................................. 1

1.1. TEAR FILM LAYERS .................................................................................................... 1
    1.1.1. Mucin layer ........................................................................................................... 2
    1.1.2. Aqueous layer ..................................................................................................... 3
    1.1.3. Lipid layer .......................................................................................................... 7
        1.1.3.1. Sources of lipids ........................................................................................ 7
        1.1.3.2. Classes and distribution of tear – film lipids .............................................. 9

1.2. TEAR FILM STUDIES AND DRY EYE DISEASE .................................................... 12
    1.2.1. Etymology and definition ................................................................................... 12
    1.2.2. Types of dry eye disease and causes ................................................................. 14
    1.2.3. Lipid layer and dry eye disease ......................................................................... 15

1.3. INSTRUMENTATION USED TO STUDY MEIBUM ............................................. 16
    1.3.1. NUCLEAR MAGNETIC RESONANCE (NMR) ............................................... 16
        1.3.1.1. Principles of NMR ................................................................................... 16
        1.3.1.2. Pulsed fourier transform (FT) NMR ......................................................... 17
        1.3.1.3. Some chemical information from NMR ................................................. 20
1.3.1.4. NMR equipment..................................................................................................24
1.3.1.5. Previous $^1$H - NMR meibum studies............................................................25
   1.3.1.5.1. Lipid composition.........................................................................................25
   1.3.1.5.2. Lipid hydrocarbon chain saturation.............................................................26
   1.3.1.5.3. Lipid chain branching..................................................................................26
1.3.2. CHROMATOGRAPHIC SEPARATION TECHNIQUES...........................................27
   1.3.2.1. Liquid chromatography (LC).........................................................................30
      1.3.2.1.1. Basic requirements for LC....................................................................30
      1.3.2.1.2. Previous meibum chromatography studies and reasons for this study........34
1.3.3. FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)...........................35
   1.3.3.1. FTIR instrumentation.....................................................................................36
   1.3.3.2. Reasons for this study...................................................................................39
   1.3.3.3. Parameters obtainable from meibum FTIR studies.........................................39
   1.3.3.4. Saturation and age-related studies.................................................................42
   1.3.3.5. CE studies.....................................................................................................46

CHAPTER
II. HYALURONIC ACID – LIPID BINDING
  2.1. INTRODUCTION........................................................................................................49
  2.2. MATERIALS AND METHODS...............................................................................53
     2.2.1. Chemicals and reagents..................................................................................53
     2.2.2. Sample preparation and binding assay.........................................................53
     2.2.3. NMR analysis................................................................................................53
  2.3. RESULTS....................................................................................................................55
  2.4. DISCUSSION.............................................................................................................56
  2.5. CONCLUSION............................................................................................................63
CHAPTER

III. A SPECTROSCOPIC STUDY OF THE COMPOSITION AND CONFORMATION OF CHOLESTERYL AND WAX ESTERS PURIFIED FROM MEIBUM

3.1. INTRODUCTION ............................................................................................................. 64

3.2. MATERIALS AND METHODS ...................................................................................... 68

3.2.1. Chemicals and standards ......................................................................................... 68

3.2.2. Meibum collection .................................................................................................... 68

3.2.3. Column chromatographic separation of CE and WE from human meibum ................. 69

3.2.4. Combining CE and WE .............................................................................................. 71

3.2.5. NMR spectroscopy ................................................................................................. 71

3.2.6. Measurement of lipid phase transitions using FTIR spectroscopy ......................... 73

3.2.6.1. Lipid phase transitions ......................................................................................... 73

3.3. RESULTS ....................................................................................................................... 76

3.3.1. Enrichment of CE and WE ...................................................................................... 76

3.3.2. Hydrocarbon chain branching ............................................................................... 79

3.3.3. Phase transitional parameters ............................................................................... 79

3.4. DISCUSSION ................................................................................................................. 84

3.4.1. Enrichment of WE and CE from human meibum ...................................................... 84

3.4.2. Question addressed in aim 2. Does the complexity of the hydrocarbon chains found in human meibum WE and CE influence how changes in the ratio of WE and CE ester influence hydrocarbon chain conformation? ....................................................... 89

3.4.2.1. Conformational changes when pure smetic CE is mixed with WE ...................... 89

3.4.2.1.1. Phase transition temperature and hydrocarbon chain order ................................ 90

3.4.2.1.1.1. Potential biological implications of changes in the phase transition temperature and hydrocarbon chain order ................................................................. 90

3.4.2.1.2. Maximum and minimum frequency of the phase transition ................................ 90

3.4.2.2. Conformational changes when pure lamellar WE is mixed with CE .................. 94
**LIST OF TABLES**

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Major TF lipids</td>
<td>10</td>
</tr>
<tr>
<td>2. $^1$H chemical shift assignments for hyaluronan</td>
<td>57</td>
</tr>
<tr>
<td>3. Hyaluronan – lipid binding parameters from figure 16</td>
<td>58</td>
</tr>
<tr>
<td>4. Standard curves for the calculation of the mole % CE</td>
<td>74</td>
</tr>
<tr>
<td>5. Assignments for meibum $^1$H - NMR resonances as numbered in figures 20, 21, and 26</td>
<td>80</td>
</tr>
<tr>
<td>6. Hydrocarbon chain branching for donors’ CE and WE purified from meibum</td>
<td>81</td>
</tr>
<tr>
<td>7. Phase transition parameters for purified wax and cholesteryl esters from infrared spectroscopy</td>
<td>83</td>
</tr>
<tr>
<td>8. $P$-values from Student’s t-test for data from fig. 22 and fig. 23</td>
<td>92</td>
</tr>
<tr>
<td>9. Linear regression analyses for fig. 22 and fig. 23</td>
<td>93</td>
</tr>
<tr>
<td>10. Meibum composition from $^1$H-NMR and FTIR spectra</td>
<td>105</td>
</tr>
<tr>
<td>11. Parameters of meibum from infrared spectroscopy</td>
<td>108</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Tear film layers</td>
<td>5</td>
</tr>
<tr>
<td>2. Anatomy of the lacrimal gland of the human eye</td>
<td>6</td>
</tr>
<tr>
<td>3. Locations of the lipid glands</td>
<td>8</td>
</tr>
<tr>
<td>4. Structures of some major meibum lipids</td>
<td>13</td>
</tr>
<tr>
<td>5. Spin types in $^1$H NMR and NMR theory</td>
<td>19</td>
</tr>
<tr>
<td>6. $^1$H NMR pulse demonstration</td>
<td>22</td>
</tr>
<tr>
<td>7. Carbon numbering associated with CE$s$ proton resonance and a typical NMR spectrum from a 31-year old male caucasian donor</td>
<td>28</td>
</tr>
<tr>
<td>8. Schematic diagram of a Michelson interferometer</td>
<td>37</td>
</tr>
<tr>
<td>9. Gauche conformer and trans conformer of 1,2 - disubstituted ethane</td>
<td>42</td>
</tr>
<tr>
<td>10. Models for ordered all trans rotamer and disordered gauche rotamer</td>
<td>43</td>
</tr>
<tr>
<td>11. Lipid order changes with dry eye disease and phase transition temperature changes with dry eye disease</td>
<td>45</td>
</tr>
<tr>
<td>12. Changes in hydrocarbon chain order with age</td>
<td>47</td>
</tr>
<tr>
<td>13. Changes in cholesteryl esters with MGD calculated using FTIR and $^1$H-NMR</td>
<td>48</td>
</tr>
<tr>
<td>14. Hyaluronic acid polymeric structure</td>
<td>50</td>
</tr>
<tr>
<td>15. Typical $^1$H-NMR spectrum of high molecular weight hyaluronan in D$_2$O</td>
<td></td>
</tr>
</tbody>
</table>
at 25 °C and effect of increasing dipalimitoyl phosphatidyl choline on the $^1$H-NMR spectra of hyaluronan .......................................................... 54
16. HA- Lipid binding profiles .................................................................................. 60
17. The structure of the wax ester oleyl oleate ...................................................... 65
18. A picture of ILUX instrument for meibum collection...................................... 70
19. A typical lipid phase transition of a meibum mixture of wax ester and cholesteryl ester from the younger donor containing 7.6 mol % cholesteryl ester ......................... 74
20. $^1$H-NMR spectra of fractions from MgO column ........................................ 77
21. $^1$H-NMR spectra of total wax ester and cholesteryl ester separated from meibum ...................................................................................... 78
22. Phase transition parameters for meibum from older donor ............................ 85
23. Phase transition parameters for meibum from younger donor .............. 86
24. Standard curves used to convert CE/WE from total yielded to moles.......... 88
25. Simulated pictograms of meibum lipid packing ........................................... 95
26. $^1$H-NMR spectra of a 42 - year-old caucasian female with Sjögren’s syndrome ..... 103
27. Cholesteryl ester/wax ester molar ratios calculated from the nuclear magnetic resonance spectra of meibum ............................................................ 104
28. A typical infrared spectrum of meibum from a 36-year-old female hispanic donor with Sjögren’s syndrome ............................................................... 107
CHAPTER I
INTRODUCTION AND BACKGROUND

1.1 TEAR FILM LAYERS

Human tears were generally stratified into three layers in 1946: lipid, aqueous and the mucinic layers. The lipid layer is located at the anterior region of the tear film, while the mucinic layer is positioned at the posterior region of the tear film, above the cornea. The aqueous layer lies in between the lipid and mucinic layer. Tears were later classified into five layers: non-polar lipid, polar lipid, aqueo-mucinic sol, aqueo-mucinic gel, and the glycocalyx layer (membrane-bound mucin). The lipid and aqueous components of human tears come from different glands: the exocrine and the endocrine glands. Endocrine glands secrete chemical substances straight into body tissues and cells while the exocrine glands secrete substances through ducts atop the surface of a part of the body. The exocrine glands can be subdivided into the apocrine, holocrine and the merocrine glands. Discharges of apocrine glands’ seep into their cellular luminal apex for temporary storage. Secretion cycle is completed by the fracture of the cellular membrane and the subsequent diversion of the granular effluent with pieces of the membrane into the lumen of the gland, to be later distributed. The lipid – producing Moll gland is an example of such gland. The holocrine glands cells secrete into the
cytoplasm and expression due to exocytosis is initiated when the cytoplasm is saturated with the product. The cell involved undergoes complete lysis and the stored secreted product is expelled, alongside the entirety of the cellular components. Some examples of the holocrine glands are the Zeis glands and the Meibomian glands. The third subclass of exocrine glands, the merocrine glands, also make granules in their luminal apex just like the apocrine glands. Similarly, the exocytotic action of the cellular membrane discharges the sole contents to the surface of the body through a duct. Example of such glands is the lacrimal gland.

1.1.1. Mucin layer

Mucin glycoproteins are found in mucus. Mucus overlay most cells and protects epithelial surfaces from harm by catalyzing cell growth, ensuring connectivity of cells to the extracellular matrix, among other functions. Mucin proteins are secreted onto the ocular conjunctiva conjoining the corneal epithelium. Mucin-secreting endocrine glands were discovered in 1837 and found to be on sac structures on the conjunctiva, which is presently known to be the seat of goblet cells. More mucin proteins have also been suggested to come from nominal acinar cells in the serous lacrimal glands or from the goblet cells in tubular ducts. Over the years, mucins of different kinds have been discovered but the conjunctival mucins have been studied extensively. Their glucoprotein framework contain large hydrocarbonate components. The protein moiety of mucins consists of docked cysteine groups with viable sulfhydryl groups, in a long - chain fashion. With a thickness of 2.5 µm – 5 µm (Fig. 1), the mucin layer is also made up of sublayers whose member mucins have been divided into three categories: the sol-producing, gel-producing, and the transmembrane type. The sol-forming mucin is
the least dense of the three types. Comprised of MUC7, MUC9 and the recently discovered MUC8 and MUC11, they are the most soluble type of mucin found in the aqueous layer and even further toward the conjunctiva. The gel-forming type of mucin is the most abundant form, lying closer to the corneal epithelium than the sol-forming type and extends close to the lipid layer. Although this variety of mucin includes MUC2, MUC5AC, MUC5B and MUC6, the most prevalent type is MUC5AC. A decrease in the amount of MUC5AC has been associated with dry eye disease (DED). The third type of mucin, the transmembrane mucins, are produced on the epithelial surface of different tissues. In the eye, they are manufactured on the surface of the cornea and the conjunctiva. These types of mucins are glycosylated glycoproteins and are also naturally polyanionic. They are made up of MUC1, MUC3A, MUC3B, MUC4, MUC12, MUC13, MUC15, MUC16, and MUC17. The glycocalyx network of the corneal base epithelium consists of the MUC1, MUC4 and MUC16 mucins. Mucins serve many functions, acting as surface tension modulators for efficient tear flow, increase water conservation, maximize viscosity in tears via sol-gel equilibrium in addition to decreasing refractive irregularity. They also help to coordinate ocular immunologic responses, prevent ocular UV damage and improve refractivity.

1.1.2. Aqueous layer

The aqueous layer, also known as the aqueous-serous layer, originates from the main lacrimal gland and the accessory lacrimal (Krause and Wolfring-Ciaccio) glands. The main lacrimal gland has two adjacent lobes, the palpebral and orbital, both lying laterally at the edge of the aponeurosis of levator palpebrae superiors (Fig. 2). The
orbital lobe, twice the size of the palpebral lobe, sits in the fossa lacrima on the anterior orbital area.\textsuperscript{15}

The palpebral lobe, conjoining the conjunctiva superior and lateral fornix, is affixed below the aponeurosis unit of the levator palpebrae superioris.\textsuperscript{15} Both of these lobes house excretory ducts extending into the superior conjunctival fornix.\textsuperscript{15} The accessory lacrimal gland is a category of smaller glands situated in the conjunctival lamina propia within the conjunctiva.\textsuperscript{15} These type of glands are the glands of Krause and glands of Wolfring-Ciaccio, and they have ducts opening unto the conjunctival surface.\textsuperscript{15} While the main lacrimal gland largely produces tears due to ocular stimulation, these accessory glands help to maintain the secretion of basal tears to maintain ocular hydration.\textsuperscript{3} The main lacrimal gland also assists in the lubrication of the cornea as well as keeping the cornea hydrated.\textsuperscript{3} The amount of the accessory glands in humans can vary, because while the main lacrimal gland is a singular dual-lobed structure in each eye, the number of accessory glands have been reported to fluctuate between 8 to 20 across individuals.\textsuperscript{3} The transcorneo-conjunctival sources of tears include all the excretion products from the lymphatic and blood vessels of the conjunctival and corneal epithelia.\textsuperscript{3} With a depth of \textasciitilde 4 - 10 µm (Fig. 1), the aqueous layer contains polar and insoluble compounds such as metabolites, electrolytes, proteins as well as the sol- and gel-mucin in its anterior and posterior regions respectively (Fig. 1).\textsuperscript{16,17} These elements give this layer its more appropriate ‘aqueous-serous mucin layer’ designation. Secreted at a rate of \textasciitilde 1.2 µL/min, this aqueous layer has many functions:\textsuperscript{18} It is very instrumental in maintaining the refractive property of the eye and eliminating particulate sedimentation in the eye by
Figure 1. Tear film layers (Source: Reprinted from Bland HC, Moilanen JA, Ekholm FS, Paananen RO. Investigating the role of specific tear thin films connected to dry eye syndrome: A study on O-Acyl-o-hydroxy fatty acids and ceramides. [Langmuir, 2019; 35(35): 3545-3552. https://pubs.acs.org/doi/10.1021/acs.langmuir.8b04182]. Further permission related to the material excerpted should be directed to the ACS.)
controlled constant flow.\textsuperscript{16} It equally prevents ocular dehydration and resulting discomfort in between blinks.\textsuperscript{16} With an antimicrobial proteome of $\sim 1500$, this layer

ensures sufficient microbial inhibition by the action of enzymes such as lysozymes, lipocalin and lactoferrin. This layer also gives nourishment to the corneal epithelium as it glides across the surface of the eye. The Third layer of the human tear, significantly out of reach of most of the dispersed mucins in the film, is the lipid layer.

1.1.3. Lipid layer

This layer is the thinnest layer in tear film. With its 0.015 – 0.160 µm thickness (Fig. 1), it is composed of ~ 256 lipid classes. Comprised of non-polar and polar lipids, most of the lipids come from the meibomian glands, with miniscule amounts from the Glands of Zeis, Moll, and controversially, the Gland of Harder.

1.1.3.1. Sources of lipids

The meibomian glands are the most prolific of the lipid producing glands. First described in 1666, meibomian glands are a type of sebaceous gland located in the tarsal plates of the upper and lower eye lids (Fig. 3). With ~25 – 40 on the upper eyelid and ~20 – 30 in the lower, these glands secrete meibum, the main source of tear lipids. Meibomian oil is steadily produced, flowing into the meibomian gland duct. It is expressed onto the tear film surface upon blinking and spreads across the tear film surface by the maragoni effect. The upward movement of the upper eyelids during blinking spreads the lipid over the aqueous surface of tears. The lipids in meibum decrease the surface tension of tear film (TF) and give stability to human tears. Even though most of the tear film lipid layer (TFLL) come from human meibum, cellular fragments from the ocular surface, conjunctiva, cornea and lacrimal glands have been suggested to introduce more lipids to the tear film. To a greater extent, phospholipids, a significant component of the polar subphase between the non-polar lipids and aqueous layer, have been found to be much higher in the TF than in meibum.
**Figure 3.** Locations of the lipid glands. (Source: Rynerson JM, Perry HD. DEBS - a unification theory for dry eye and blepharitis. *Clinical ophthalmology (Auckland, NZ)* 2016;10:2455-2467. Originally published by and used with permission from Dove Medical Press Ltd.)
More so, the concentration of phospholipids is higher in reflex and flushed tears compared with basal tears. The glands of Zeis first described in 1835, are a collection of sebaceous glands in the front half of the eyelid margin (Fig. 3). Their holocrine secretion system stems from the degeneration of the cell, releasing its protoplasmic contents which include glycerides, esters, sterols, paraffins and squalene. The second minor lipid-producing glands are the glands of Moll. These sebaceous, apocrinic glands function as pheromonic structures and they are strategically located in the eyelid margin and caruncula (Fig 3). The glands of Moll produce pheromonic pungent signals which have been suggested to have evolved to prevent the spill-over of tears over the eyelid margin. Their secretion products include largely lipids, and significant amounts of carbohydrates, ammonia and proteins. The gland of Harder is another gland with a secretion harboring some lipid content for vertebrates that possess it. The glands of Harder are rare in the animal kingdom, so it has been less studied compared with the other glands and the data on the definite make up and surface characteristics of the secretion product.

1.1.3.2. Classes and distribution of tear-film lipids

The TF and meibum contain numerous classes of lipids that have been quantified by extensive HPLC MS and Tandem Mass spectrometry (MS/MS) (table 1.1). (O-acyl)-ω-hydroxy fatty acids (OAHFAs), acylglycerols (AGs), phospholipids (PLs), cholesteryl esters (CEs) and wax esters (WEs), free fatty acids (FA), cholesterol (Cho) and glycerols have also been quantified in tears and meibum. Tear lipids can be classified based on their polarities, complexities and ionic properties.
Table 1. Major TF lipids. (Source: Borchman D. Lipid conformational order and the etiology of cataract and dry eye. *J Lip Res* 2021;62)

<table>
<thead>
<tr>
<th>Lipid species</th>
<th>Major human Tear Film Lipids (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dihydrosphingomyelin&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>Sphingomyelin&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.3</td>
</tr>
<tr>
<td>Phosphatidylcholine&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.2</td>
</tr>
<tr>
<td>Phosphatidylethanolamine (&lt;i&gt;l&lt;/i&gt;-&lt;i&gt;O&lt;/i&gt;-alkyl ether)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>Phosphatidylethanolamine&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.5</td>
</tr>
<tr>
<td>Phosphatidylerserine&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.3</td>
</tr>
<tr>
<td>Phosphatidylinositol&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.8</td>
</tr>
<tr>
<td>Phosphatidic Acid&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.9</td>
</tr>
<tr>
<td>Lyso phosphatidylethanolamine&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.8</td>
</tr>
<tr>
<td>Lyso phosphatidylcholine&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.0</td>
</tr>
<tr>
<td>Lyso phosphatidylerserine&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.6</td>
</tr>
<tr>
<td>Ceramide&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.2</td>
</tr>
<tr>
<td>Cholesterol&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.5 – 2.1&lt;sup&gt;13&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phospholipids&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.2</td>
</tr>
<tr>
<td>CEs&lt;sup&gt;b&lt;/sup&gt;</td>
<td>44.8</td>
</tr>
<tr>
<td>WEs&lt;sup&gt;b&lt;/sup&gt;</td>
<td>35.2</td>
</tr>
<tr>
<td>Triacylglycerides&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.8</td>
</tr>
<tr>
<td>Diacylglycerides&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.3</td>
</tr>
<tr>
<td>O-acyl-&lt;i&gt;ω&lt;/i&gt;-hydroxy-fatty acid&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.5</td>
</tr>
</tbody>
</table>

<sup>a</sup>Molar percent of phospholipids.  <sup>b</sup>molar percent of all lipids
Complex lipids vary from simple lipids in that simple lipids are usually in their most basic forms, requiring no hydrolysis. Cho and free fatty acids (FFAs) are some common examples of these simple lipids. The complex lipids are comprised of acylglycerols, PLs, WEs, CEs, ceramides, phosphatidylglycerols, etc. The polar (non-ionic) lipids with long acyl chains include hydroxy ceramides, complex alcohols such as diacylglycerols (DAGs), and PLs. They are considered as being amphipathic as they do not have permanent charges but have significant electronegative groups that confer polarity. Ionic polar lipids found in TF such as glycerophospholipids, lysophospholipids, phosphatidylcholine (PC) and short-chain FAs are polar due to their permanent charges. Amphiphilic polar lipids such as some DAGs, oxysterols, and some classes of ceramides are also present in meibum. Amphipathic lipids can also be zwitterionic in nature with two opposite charges on one molecule. TF polar lipid molecules are located with their hydrophilic moieties next to the aqueous layer while their hydrophobic moieties face toward the bulk lipid layer. The bulk of the lipid layer extending away from the aqueous layer toward the air, are poorly soluble in water and they havae extremely long acyl chains compared with the polar lipids. Long-chain ceramides, CEs, WEs and triacylglycerols (TAGs) compose the bulk lipid layer and make up more than 80% of tear film lipids. (Table 1) CE hydrocarbon acyl chains are unusually long, (30 - 45 mol %) long – chained C22:1 – C34:1. WE hydrocarbon acyl chains (30 – 50 mol %) are composed of C18:1 – C30 alcohol - based long chains. Meibum is composed of ~ 4 mol % of (O-acyl)-ω-hydroxy fatty acids (OAHFAs), Cho below 0.5 %, ~ 2 mol % TAGs, and 0.01 mole % PLs. The TFLL has a wider range of lipids and much more PLs but the same types with varying ratios have
been quantified.\textsuperscript{31,43} As expected, CEs and WEs make up the bulk of the TF lipids, 44.8 mol \% and 35.2 mol \%, respectively.\textsuperscript{37} Interestingly, the PL concentration of $\sim 8.2 - 10$ mol \%\textsuperscript{31,37,40} is three orders of magnitude greater than found in meibum. The polar lipids are also likely to contribute to the surfactant properties of tears.\textsuperscript{31,37,40} Sphingomyelins (SM), lysophospholipids, and glycerophospholipids were found to make up the bulk of the TFLL phospholipids.\textsuperscript{37}

\textbf{1.2. TEAR FILM STUDIES AND DRY EYE DISEASE}

Because the TFLL maintains the surface properties of the TF, many studies have been focused on the role of the TFLL in dry eye disease. Dry Eye disease (DED) (or Keratoconjunctivitis) is a chronic and continuous ocular ailment that has been estimated to affect more than 16 million adults in the United States and up to 30\% of people greater than 50 years of age, worldwide.\textsuperscript{21,44} The estimated annual financial burden of DED is up to $55$ billion in the U.S alone.\textsuperscript{45} Although prevalence indisputably increases with age up to 30\% of the worldwide age group above 50 years,\textsuperscript{21,44} it is also very significant in adults between 18 – 34 years old.\textsuperscript{46}

\textbf{1.2.1. Etymology and definition}

DED was described in 1995 as a “disorder of the tear film due to tear deficiency or excessive tear evaporation which causes damage to the interpalpebral ocular surface and is associated with symptoms of ocular discomfort”.\textsuperscript{47}
**Figure 4**: Structures of some major meibum lipids
DED is multifactorial as its incidence could be caused by irregularities in the ocular surface of the tear film.\textsuperscript{48} The etiology of DED includes the malfunctioning of part(s) of the ocular anatomy that leads to ocular inflammation, decreased tear stability between blinks, hyperosmolarity and visual disruption.\textsuperscript{49-55}

1.2.2. Types of dry eye disease and causes

There are two types of dry eye disease: aqueous-deficient DED (ADDE) and evaporative (ED).\textsuperscript{29, 56} Aqueous tear-deficient DED which affects 10\% of DED patients\textsuperscript{57} can either be caused by Sjögren’s syndrome (SS) or other non-Sjögren’s factors.\textsuperscript{56} It is caused mainly by inadequate lacrimal gland output.\textsuperscript{56} Hampered lacrimal gland efficiency due to non-Sjögren factors can be a result of the occlusion of the meibomian glands, reduced gland reflex, or drugs.\textsuperscript{48} ED is brought about by high water loss from the eye surface despite normal tear output\textsuperscript{29} and even though each of the components of either layers of tear film confer characteristics that are important in eye homoeostasis, the most prevalent type of DED, the evaporative type, is attributed largely to meibomian gland dysfunction (MGD).\textsuperscript{29} The International Workshop on Meibomian Gland Dysfunction states that MGD is a result of the occlusion of the ocular terminal duct, leading to changes in the quality/quantity of the glandular secreted substance.\textsuperscript{56} As the lipid layer is the most outward layer in the eye and by consequence, the last line of acute tear evaporation prevention, many studies were performed on the components of the TFLL and their role in mitigating abnormal rate of tear evaporation. Because most of the lipid layer (80\%) comes from human meibum, abnormalities in the meibomian gland were initially studied. In concert, there are other ocular, medication-induced or structural conditions in the etiology of MGD.\textsuperscript{56} Some of the ocular factors are underlying
conditions of blepharitis, use of contact lens and ocular parasite inhabitation.\textsuperscript{56} Systemic conditions can include cholesterol levels, psoriasis, Sjögren’s syndrome, hypertension, menopause, aging, rosacea, and other factors.\textsuperscript{56} Medications such as antidepressants, menopausal hormones, progestins, may also exacerbate the occurrence of MGD.\textsuperscript{56} Several types of therapies have been employed over the years in the management of DED. Most of these therapies include medications with anti-inflammatory agents, steroids, topical lubricants, fatty acids and secretagogues.\textsuperscript{56} More invasive routes include acupuncture and tear draining-occlusive procedures.\textsuperscript{58} Nonetheless, an effective long term cure for DED has yet to be determined due to varieties of DED syndrome severity, nonexistence of a universal yardstick for curative outcomes, and the non-specificity of some published treatments to DED.\textsuperscript{58} Therefore, the aim of one of our projects was to determine how changes in the ratio of CE/WE known to occur with DED, relate to conformational changes shown to be related to DED.

1.2.3. Lipid layer and dry eye disease.

\textit{In-vitro} Langmuir trough studies associated with DED and tear film stability have suggested that OAHFAs limit the evaporation of the TF.\textsuperscript{59} The OAHFAs partition in the polar sublayer on the distal part of the TFLL adjacent to the aqueous layer.\textsuperscript{59} An inverse relationship between the severity of dry eye symptoms and TFLL thickness has been shown. DED affects meibum lipid composition directly and/or indirectly. Even though inconsistencies in meibum composition due to sampling method, instrumentation, and contamination possibility may be inevitable,\textsuperscript{60-64} cautious conclusions are still warranted. With MGD, meibum has negligible amounts of phosphatidylcholines,\textsuperscript{65} higher amounts
of wax esters,\textsuperscript{36} and negligibly lower quantities of phosphatidylethanolamine, sphingomyelin,\textsuperscript{65} triglycerides and cholesterol\textsuperscript{36} have been documented.

1.3. INSTRUMENTATION USED TO STUDY MEIBUM

1.3.1. NUCLEAR MAGNETIC RESONANCE (NMR)

1.3.1.1. Principles of NMR

First discovered, described and investigated in 1938,\textsuperscript{66} NMR is an excellent tool for compositional, structural, and conformational analyses of samples. This technique takes advantage of the atomic nucleus, which is a small compact region of an atom that houses positively-charged protons and uncharged neutrons.\textsuperscript{66} The inherent electrostatic nature of this nuclei gives it a spin on its axis.\textsuperscript{66} Spinning charges introduce a small magnetic property to the nuclei which moves from the north pole direction of a magnet to the south pole direction.\textsuperscript{66} The collective magnetic moment of the spin is oriented in the arbitrary “north” pole of the nucleus.\textsuperscript{66} The magnetic moment varies for different nuclei and isotopes due to neutrons, and these differences separates the NMR-active nuclei from the NMR inactive nuclei.\textsuperscript{66} High resolution output requirement however, limits the resonant signals to nuclei with $\frac{1}{2}$ spin.\textsuperscript{66} On a three - dimensional coordinate plane and in the absence of an external magnetic field, the direction of the nucleus’ local magnetic field due to its axial spin is random.\textsuperscript{66} When an external magnetic field, $B_0$, is introduced in the direction of the Z-axis, the nucleus may or may not try to align its spin axis to the axis of the applied magnetic field.\textsuperscript{66} As a result of the extra angular momentum of the nuclear spin, nonetheless, the force exerted by the external magnetic field causes the nucleus to teeter around the axis or in misalignment with the axis, at a particular
frequency known as Lamour or resonant frequency. Mathematically, this frequency is linearly related to the strength of $B_o$, and the magnetogyric ratio ($\gamma$) which is the native magnetic field generated by the spin of the nucleus:

$$\nu = \frac{\gamma B_o}{2\pi}$$

where $\nu$ is the resonant frequency. As a result of this, an increase in magnetic power increases the resonant frequency and vice versa.

1.3.1.2. Pulsed fourier transform (FT) NMR

Different types of atoms and their isotopes have NMR-active spins. However, proton -NMR is easily the most ubiquitous type of NMR used because of a proton’s inherently superior magnetogyric ratios over other active nuclei. The $^1$H signal is four times stronger compared with $^{13}$C, ten times stronger compared with $^{15}$N, and even seven times stronger than $^2$H. In addition, proton natural abundance also improves its general preference over other molecules. A proton nucleus has a spin quantum number one-half and according to the selection rule $2l + 1$ for determining spin states, protium has two states. During the nuclear interaction with $B_o$, a lower energy spin state ($\alpha$ spin) can be created if the spin aligns with the axis ($45^\circ$ to the $B_o$) of the applied magnetic field, or a higher energy spin state ($\beta$-spin) can be birthed if it does not align ($135^\circ$ to the $B_o$) (Fig. 5). Even though this native energy quantization of spins exists to a very minute degree in a nuclear magnetic field, the introduction of an external magnetic field greatly widens the spins’ energy difference ($\Delta E$). The stronger (higher MHz) the magnet, the greater the energy difference. With a collection of protons in a sample to be analyzed by NMR, there are more spins to be distributed between the two energy states. The precessions of each group of spin states create two opposite conical path distributions, based on the two
different energy levels and the disorder of each spin group precession.\textsuperscript{66} The external field notwithstanding, ultimately gives excess $\alpha$-spins at the low energy level due to the energy disparity.\textsuperscript{66} In spite of the fact that a group of net excess alpha spins are realized due to most of the opposing spins canceling out, their same-frequency precession will be oriented out of phase with one another, giving a static net magnetization vector in the middle of the conical distribution of the $\alpha$-spins.\textsuperscript{66} This net magnetization vector of the excess $\alpha$-spins would be in the direction of the $z$-axis because of the direction of the external magnetic field, and it is this vector quantity that is manipulated and subsequently measured by NMR.\textsuperscript{66} Because a magnetic moment has to precess on the $x$ – $y$ axis to be measured, a multi-wavelength radiofrequency (RF) pulse is able to force all magnetic moments from individually-teetering spins to originate from the same axis, thereby syncopating their precession.\textsuperscript{66} This pulse also gives extra energy to some of the spins to transition to the $\beta$-spin energy level.\textsuperscript{66} A RF pulse can be applied at different angles of the cartesian coordinates, but the most commonly used are 45\textdegree, 90\textdegree and sometimes, 180\textdegree.\textsuperscript{66} A 90\textdegree RF pulse is short, but strong enough to affect the spins already precessing around $B_o$ towards a new but brief $B_1$ along the $x$-$y$ plane, moving each spin 90\textdegree towards the $x$-axis.\textsuperscript{66} This shifts the funnel-shaped precession pathway of the net spins from the $z$-axis onto the $x$-direction counter-clockwise and for a brief moment, the net magnetization is concentrated in the $x$-direction.\textsuperscript{66} In order not to violate the quantization rule of the $B_o$ field, some of the net spins on the left side of the cone with the pulse are consequentially at approximately 135\textdegree to the $B_o$ field, while the ones on the right side become oriented 45\textdegree to the $B_o$ field on the $x$-$y$ plane.\textsuperscript{66}
**Figure 5:** Spin types in $^1$H NMR and NMR theory. $\Delta E = $ energy difference; $h =$ plank’s constant; $\nu =$ frequency; $B_o =$ external magnetic field; $\alpha =$ low energy spins; $\beta =$ high energy spins. (With kind permission from source: Jacobsen, NE. Fundamentals of NMR Spectroscopy in Liquids. *NMR Spectroscopy Explained*: 1-38.)
The cohesive net magnetization magnifies the sinusoidal voltage registered from the averaged rotating magnetic vector after the pulse stops, hitting the detector on the y-direction after each revolution, from the positive y-direction to the negative y-direction. This activity is recorded as a magnetization vs time signal, known as a free induction decay (FID) signal. A Fourier transform mathematical operation of the signal converts the signal into a magnetization vs frequency distribution plot. Even though the sensitivity of an NMR instrument can be improved exponentially by increasing the strength of the external magnet, the dependence on just the extra α-spins to be excited makes NMR less desirable when compared to other analytical instruments. However, its advantages are numerous.

1.3.1.3. Some chemical information from NMR.

Chemical shift: Fundamentally, the idea of chemical shift comes from the net effect of the combination of the native magnetic field created by the flow of bonding and non-bonding electrons, on the nucleus according to Lenz’s law, when present in an external magnetic field. This induced magnetic field will oppose the external magnetic field, reducing the eventual net field felt by the nucleus. As a result, it is difficult to ensure that each nuclei of interest experiences the actual strength of the applied magnetic field. Chemical shifts can be further exacerbated by the pull mechanism of a neighboring atom’s high electronegativity on the electrons of an atom with ½ spin nuclei, exposing it more and causing a higher registered magnetic field in the vicinity of the NMR-active nuclei. As a nuclei’s resonating frequency is affected by the magnitude of the magnetic field applied or experienced by the pertinent nuclei, the resonant frequency is more accurately related to the net field felt by the nuclei according to the equation:
\[ \nu = \gamma B_{\text{eff}} / 2\pi, \text{ and } B_{\text{eff}} = B_0 (1 - \sigma) \]

\( B_{\text{eff}} \) is the effective magnetic field, \( \gamma \) is the magnetogyric ratio, and \( \sigma \) is the shielding factor.\(^{67}\) Because of the differences in NMR instruments, chemical shifts, rather than the classical numerical nuclear resonating frequencies, have become a more universal measurement of chemical environment monitoring and structural elucidation in a NMR spectrum.\(^{67}\) Chemical shifts are measured in parts per million (ppm) which is a millionth \( (10^{-6}) \) of the resonating frequency of the NMR nuclei.\(^{67}\) This is so because the resonating frequency of a type of nucleus will not vary much in a magnetic field.\(^{67}\) The small variances have to be in a unit large enough to be quantified and spread out to reflect the range of frequencies possible for a nucleus in several types of environments.\(^{67}\)

Denoted by \( \delta \), chemical shifts vary in range depending largely on the atomic nuclei due to the amount of bonding and non-bonding electrons possible with that atom, ultimately leading the magnification of the effect of one group of electrons over the other.\(^{67}\)

**Spin-spin splitting:** Spin-spin splitting is a scalar coupling interaction between a closely bonded group of nuclei.\(^{66}\) It occurs because of the interference of the magnetic field of an adjacent NMR-active nucleus on the resonant frequency of a particular nuclei through their bonds.\(^{66}\) Based on the proximity of a bonded nuclei to another, the magnetic field of a nuclear spin can upset the magnetic field of the bonded electrons’ spins.\(^{66}\) This progressively affects the magnetic field encountered by an adjacent nuclei.\(^{68}\) Concisely, the total external magnetic field experienced by a nucleus includes the additive, substractive, and shielding effects of the magnetic field of the neighboring nuclei.\(^{66}\) The substractive magnetic field effect comes when the \( \beta \)-spins of this adjacent atom opposes
Figure 6. $^1$H NMR pulse demonstration, where $\vec{M}$ = vector sum of magnetization. (With kind permission from source: Jacobsen, NE. Fundamentals of NMR Spectroscopy in Liquids. *NMR Spectroscopy Explained*: 1-38.)
the external magnetic field, decreasing the net field encountered by the nuclei of interest. The additive magnetic field effect happens when the $\alpha$-spins of the nearest nuclei attached to a different atom aligns with the native magnetic field, which slightly increases the overall net magnetic field perceived by nuclei of interest. These two simultaneous events give more than one resonance very close to one other, with slightly different chemical shifts. The difference between the shifts is the coupling or $J$-value. This phenomenon helps identify the type of atoms surrounding different NMR nuclei by bonding.

\textit{T}_1 \textit{ and } \textit{T}_2 \textit{ relaxation:} These two types of relaxation provide information related to the size and the molecular dynamics of each molecular spin. Following the termination of the RF pulse, as molecules are within the external magnetic field, the spins begin to fall out of cohesion as they lose energy and return to thermal equilibrium. The first type of relaxation is called the spin-lattice relaxation or longitudinal relaxation ($T_1$). This phenomenon involves the transfer of nuclei energy to the surrounding lattice, restoring the normal distribution of the $\alpha$ and $\beta$ spin types by the stimulated change of $\beta$ spins to $\alpha$ spins. Longitudinal relaxation is affected significantly by lattice mobility due to tumbling, and as a result of this, larger or more viscous solutions register shorter $T_1$ relaxation times as long as the lattice motion frequency is equal to the larmor frequency of the nucleus of interest. $T_2$ refers to the time it takes for the loss of coherence for the excess $\alpha$ spins, due to relaxation. This occurs due to differences in the native magnetic field surrounding the spins, that diversifies the precession rate of each nuclei in a molecule. The resulting effects due to changes in the magnetic field phase of each nuclei spin remains mostly insignificant as long as the molecular motion (such as
tumbling) is rapid. A slower molecular motion gives ample time for differing precession frequencies to be recorded, giving rise to broadened resonances. This phenomenon decreases with increases in molecular weight of samples.

*Nuclear Overhauser effect:* This is a dipolar coupling interaction between two nuclei that occurs through space with a distance of 5Å or less. When one nucleus is irradiated with a weak electromagnetic pulse, the pulse equalizes the population of the spins on both energy levels. This leveling however perturbs the distribution of the spins of nearby nuclei by decreasing or increasing the NMR signal of the proximal nucleus due to the coupled relaxation or consequential excitation. The extent of nuclear overhauser effect experienced by nuclei can be used to approximate the 3D structures of large molecules.

### 1.3.1.4. NMR equipment

The NMR instrument is comprised of a superconducting magnet which has a closed loop of Nb/Ti alloy wire all submerged in a liquid helium bath. The loop carries a current which produces a constant magnetic field. The helium container has an insulating vacuum jacket with another layer of liquid nitrogen for additional cooling. A wire coil wound around the NMR sample produces and receives a radiofrequency pulse. The readout device controls the relay of strong and short RF pulses to the probe. The free induction decay (FID) signal is intercepted by the probe after the pulse. The analog-to-digital converter amplifies and converts the weak FID signal to an audio frequency signal and spreads them across time intervals, giving rise to a numerical digital FID signal. The readout computer sorts through the intensities and times of the pulse outputs presented by a transmitter, as well as accepting and processing the digital FID.
The computer’s final digitized output is a Fourier transform spectrum that can be stored to analyzed further. 66

1.3.1.5. Previous $^1$H - NMR meibum studies

While many methods such as TLC,32, 35 GC,32, 69-71 and MS62, 63, 70-76 have been used in meibum lipid analyses, these techniques have their shortcomings for consecutive meibum studies: Mass spectrometers are destructive to the sample and their spectra can be very convoluted.77 TLC requires both high resolution and precision.77 Detection using TLC can also be problematic due to high risk of contamination and sample degradation.77 Derivatization or sample labeling in GC can alter the sample being investigated such as hydrolysis and transesterification, introducing more complexity during bottom-up analyses.77 The high temperatures of GC also can cause the destruction or unwarranted isomerization too in samples.77 NMR spectroscopy is very informative, circumventing these issues of analyzing meibum lipids and is one of the spectroscopy apparatus that has been used for decades to study the composition of ocular lipids.42, 78-82

1.3.1.5.1. Lipid composition

As meibum has been established as a complex niche of lipids, different resonances are especially apparent in $^1$H-NMR for the identification of meibum components such as waxes, cholesteryl esters and triglycerides. Rabbit meibum and TF phospholipids were studied using NMR spectroscopy in 1996.83, 84 The level of terpenoids in meibum have also been probed.85 $^1$H- NMR and HSQC experiments have been employed to identify most of the proton resonances of cholesteryl esters and total human meibum resonance assignments.42, 78 Squalene levels in meibum were quantified
using HSQC spectroscopy. The 4 ppm to 4.8 ppm region for meibum contains resonances corresponding to the ester groups of CE, WE, and glycerides. The resonances at 4.1 to 4.3 ppm have been assigned to the glyceride/glycerol backbone CH$_2$ protons while the resonances at 4.6 ppm have been assigned to the carbon 3 proton of cholesterol (Fig. 7). The resonances at 0.66 ppm and 1 ppm regions have been assigned to CE while the triplet at 4.0 ppm has been used to quantify WE. Quantification by NMR spectroscopy is possible because the characteristic CE and WE resonances are the same regardless of chain branching, saturation or hydrocarbon chain length. The intensities of the resonances at 4.0, 4.6, 1 and 0.66 ppm were used to quantify the CE/WE ratio in meibum. The advantage of NMR spectroscopy over spectrometry techniques is that the molecular weights, ionization efficiency, or response factor of each type of CE and CE is not necessary for the measurements of over 30,000 combinations of lipid species in meibum.

1.3.1.5.2. Lipid hydrocarbon chain saturation

NMR spectroscopy is very advantageous because the level of meibum lipid saturation can be measured. Therefore, the prevalence of CH = CH double bonds and/or CH$_2$–CH$_2$ single bonds as a measure of unsaturation or saturation, respectively, can be determined using assigned $^1$H-NMR resonances.

1.3.1.5.3. Lipid chain branching

Hydrocarbon chain branching reduces the ability of hydrocarbon chains to pack closely together, thus increasing lipid fluidity and decreasing Van der waal interactions between chains. Straight chain, anteiso-, and iso- branchings in human meibum have
been explored\textsuperscript{78} as well as their resonance assignments\textsuperscript{62} verified.\textsuperscript{78, 89} Using heteronuclear single quantum coherence, branching resonance assignments,\textsuperscript{35, 42, 91} quantification of branching was made for meibum. It was found\textsuperscript{42, 78} that CH\textsubscript{3} moieties from anteiso-branched lipids impede CH\textsubscript{2} hydrocarbon-hydrocarbon interactions, leading to more disordered hydrocarbon chains.\textsuperscript{78} \textsuperscript{1}H – NMR studies showed a low prevalence of CH\textsubscript{3} moieties in meibum from patients with MGD compared with meibum from patients without dry eye.\textsuperscript{92, 93} Meibum from donors with MGD had more iso-branching compared with normal meibum from donors without dry eye.\textsuperscript{78}

1.3.2. CHROMATOGRAPHIC SEPARATION TECHNIQUES

Chromatography includes all the techniques used to separate components of a mixture, based on their interaction-driven distribution on a stationary phase as they are propelled by a solvent mobile phase. The mobile phase is usually a gas or a liquid. The stationary phase is commonly a liquid laced inside a capillary tube, liquid immobilized on solid particles packed in a wound cylindrical column, or a sole solid support. The equilibration between the mobile phase and stationary phase is the driving force for the separation and differential mobility of chromatographic separations. The mobility of solutes in the mobile phase is measured by the retention time which is the time it takes for a solute to leave a column. Since its first use in 1906, this technique has grown in diversity and popularity.\textsuperscript{23} There are five classes of chromatography: adsorption, ion-exchange, molecular exclusion, affinity and partition chromatography.\textsuperscript{94} Adsorption is the oldest class of chromatography. It uses a solid stationary phase as well as a gaseous or a liquid mobile phase, and the solute is separated by its adsorption and movement on the solid particles’ surfaces due to the flow of the mobile phase. This type of chromatography
Figure 7: (a) Carbon numbering used in (b) and (c) associated with CEs proton resonance. (b,c) A typical NMR spectrum from a 31-year old male caucasian donor. The resonance near 4.0 due to WEs is a triplet. The resonance due to cholesterol number C21 is a doublet. Resonances for straight chain CH₃ and anteiso-CH₃ moieties are composed of two major resonances, and the iso-CH₃ moieties are composed of two major and one major (left shoulder) resonance. The resonances due to cholesterol number C26 and 27 are not resolved but rather, are buried under the straight chain resonances. (With kind permission from Borchman D, Ramasubramanian A, Foulks GN. Invest Ophthalmol Vis Sci. 2019; 60: 2286-2293.)
is based on attraction between species and includes thin-layer chromatography (TLC) and column chromatography. Another class of chromatography is ion-exchange chromatography, which as the name suggests, involves the covalent linkage of ions on a stationary phase resin solid support. The pull of electrostatic attraction brings solutes with opposite ionic moieties together with the stationary phase as they move through a liquid mobile phase. Molecular (size) exclusion, gel filtration or gel-permeated chromatography involves a mobile phase that moves through a porous gel with a small pore size, allowing the passage of small solute molecules through it and leaving behind the larger ones to go around the porous gel. The smaller molecules that fit through the porous gel travel through the whole path length of the porous gel, taking a longer time to elute than the larger molecules. Affinity chromatography is the most selective class of chromatography. This most recent type of separation class uses very specific interactions between a component of a mixture flowing through the column and another molecule attached covalently on the stationary phase. After all the solutes have flowed through the column, the captured solute left on the stationary phase is released by varying the pH or ionic strength of the mobile phase. Partition chromatography, another attraction-based separation, has the stationary phase immobilized on the solid support surface as a thin film. The mobile phase is either a liquid or a gas and the analytes partitions between both phases. Some of this type of chromatography are gas chromatography and all forms of liquid chromatography. Our study involved the use of liquid chromatography which will be discussed in the next sections:
1.3.2.1. Liquid chromatography (LC)

Traditionally, liquid chromatography is any separation procedure where the mobile phase is a liquid and includes adsorption, partition, ion-exchange and molecular exclusion chromatography. The earliest forms of LC are paper chromatography, thin-layer (TLC), and classical open- column (adsorption). The simplicity and affordability of TLC made it an advantageous alternative to open column chromatography. However, increases in the complexity of samples and the need for automation, efficient quantification and a low detection limit brought about improvements in liquid chromatography. Even though gas chromatography is popular due to its speed and automation, the analyte high temperature stability and overall sample volatility requirements reduce the wider applications of this method. Modern LC uses many types of detectors compared with gas chromatography. Open-column (adsorption) chromatography, also referred to as liquid – solid chromatography, is often used as a more refined apparatus for separation conditions already identified and established using TLC. This technique was primarily used in our research for separating meibum lipids with high recovery. On the other hand, modern forms of LC involve closed high-pressure columns equipped to separate picograms and nanogram quantities of analytes, giving rise to high performance LC (HPLC) and ultra performance LC (UPLC).

1.3.2.1.1. Basic requirements for LC

Column: The column is a rudimentary requirement of a separation apparatus. The column is usually in a rigid glass or steel cylinder as in LC, or a winding tubular contraption as in GC. In adsorption separation, the columns are fitted with appropriate
flow adaptors to prevent undue mixing of the mobile phase and sample. The columns may have a water jacket to regulate the temperature of the sample.

**Stationary phase:** This is one of the two most important part of any separation. This component can be a solid packing of polar silica gel, which is the most common, or it can also be covalently attached groups on a silica gel support with reaction-deactivated silanol groups. The diameter and the topography of the solid support can be manipulated to yield the best separation chromatograms. The polarity of the stationary phase can be optimized by modifying the bonded moieties on the solid support.

**Mobile phase (MP):** The mobile phase can either be a liquid, as in LC, or a gas as in GC. For better separation, different levels of polarities can be achieved by a combination of two or even three solvents to match based on separation ability, or the opposing polarity of the stationary phase. For HPLC, a polar mobile phase and non-polar stationary phase results in a reverse-phase HPLC (RP-HPLC) system and a non polar mobile phase with a comparable polar stationary phase gives a normal-phase HPLC (NP-HPLC).

**Detectors:** Separated solute signals are measured by a detector. The detector should ideally be sensitive to low concentrations of every analyte being separated, be unaffected by temperature and solvent-flow changes, express no band-broadening characteristics, have no destructive effect on solute, and have its signal exhibit a direct relationship with solute concentrations. A class of LC detectors known as bulk property detectors measure changes in general physical properties such as the refractive index of solvent and solute mixture (eluate). Compared with physical property detectors, the solute property type of detectors discriminate well because they are more sensitive to
changes in a chemical property of the solute, e.g., UV absorption. The UV-Vis photo/spectrophotometer detector is the most frequently used detector because that can be used to detect many compounds. The UV or visible absorption of a sample is based on the Beer-Lambert law, relating absorbance to concentration according to the relationship:

\[ A = \log \frac{I_o}{I} \epsilon bc, \]

where \( A \) represents absorbance, \( I_o \) is incident light intensity, \( I \) stands for the transmitted light intensity, \( C \) is sample concentration, \( \epsilon \) is sample molar absorptivity and \( b \) is the path length of sample cell (cm). This absorbance detector sensitivity is relatively high with \( \sim 10^5 \) dynamic range. As a result, it is a useful tool as long as the absorption of the mobile phase is very low.

Another type of detector, the refractive index detector, is marginally used. With applications of interferometry, deflection physics, or laws of reflection, it records the refractive index difference(s) between the mobile phase as an eluent, and as part of the eluate. As a result of a signal contingent on a refractive index difference between the mobile phase and the solute, this type of detector is less sensitive than a UV-VIS detector and is usually not suitable for trace analyses.

A fluorescence detector is a more selective and sensitive detector compared with other detectors. It detects and measures fluorescence energy emanating from UV-excited solutes. This type of detector is only employed when other sensitive detection methods are not available or do not work.

For both general and selective signal monitoring, infrared photodetectors can be used based on the absorption of IR radiation, with a limitation to solvents with little to zero absorption tendencies. An additional benefit of this type of detector is its stability at elevated temperatures (up to 150°). In addition, they can be employed in certain cases
of gradient elution unlike some bulk property detectors because bulk property detectors
are usually sensitive to changes in temperature and MP composition. IR detectors can
reach a detection limit as low as 1 µg of solute for certain types of functional group IR
bands.

Detection of compounds that can undergo oxidation or reduction by electricity is
possible with electrochemical (amperometric) detectors. This type of detector measures
the current between a reference electrode and another polarizable one, as a function of the
voltage applied. This detector is very useful especially for compounds that cannot be
detected using UV-VIS. Despite the possibility of detection predicated upon the
presence of an electrically conductive mobile phase, conductivity can be increased by the
addition of salt to increase the MP ionic strength, with the utmost care to not compromise
the separation. Radioactivity detectors are another highly specific group of detectors
that are used to observe radio-labeled compounds. Common radioactive isotopes
employed are $^{35}$S, $^{125}$I, $^{14}$C and $^{3}$H. These isotopes emit photons detected by a chemical
scintillator. Despite its high specificity, these types of detectors can cause band
broadening and peak tailing due to the size of the sample cell aimed to increase sample
and eluent contact time with the scintillator. Sometimes, the common isotopes are also
harder to detect. However, these detectors can be excellent for purification when sample
recovery is not a high priority. Conductivity detectors are used to measure the
conductivity of mobile phases as they come in contact with ionic solutes. They are very
preferable especially when a solute does not have sufficient UV chromophore. However, the effect of temperature on the registered conductivity is very significant. In
addition, mobile phase flow rate can sometimes influence their performance.
Nevertheless, they are very good for ionic organic and inorganic molecules. Perhaps the most sensitive common detector to date is mass spectrometry. Other less common LC detectors utilize chemiluminescence, light-scattering, optical rotation, and photoionization.

1.3.2.1.2. Previous meibum chromatography studies and reasons for this study

HPLC was first used to study human meibum in 1979. Since then, it has become even more popular in tear film research. HPLC-UV was initially used to separate and study meibum phosphatidylethanolamine and sphingomyelin composition as possible indispensable polar lipids that aid in the prevention of keratoconjunctivitis and blepharitis. The same separation technique was used to probe the types and structures of the polar lipids in meibum. Differences in polar lipids for patients with meibomianitis were investigated using HPLC-UV. More accurate quantification and identification of phospholipids was probed using NP-HPLC-MS. HPLC-MS has equally been employed in investigating lipid profile differences in sex, age, patients before and after treatment with drugs affecting lipid metabolism, and pre and post-menopausal women. The fact that CEs and WEs make up about 80% of meibum lipids means they are a good lipid class to be investigated. Due to their close proximity in non-polarity, separation and accurate molar yield of both species have proven to be difficult. Despite this, CE and WE, alongside other lipids, have been separated using TLC and HPLC, and UPLC-MS. Part of our ensuing CE/WE studies required complete chromatographic separation, with sufficient molar yields to accommodate recombination in different ratios whilst spectroscopically allowing detection.
1.3.3. FOURIER TRANSFORM INFRARED SPECTROSCOPY

Infrared radiation (IR) was discovered in 1800. The wavelength range of IR is 0.78 – 1000 µm. IR is divided into three regions: near-IR from 800 nm to 2.5 µm, mid-IR from 2.5 µm to 25 µm, and far-IR from 25 µm to 1 mm. IR spectroscopy can be applied to solids, liquids or gases. The modern form of IR spectroscopy is Fourier transform infrared spectroscopy (FTIR). FTIR is an absorption technique used to study organic and inorganic materials based on the excited vibrational movement of functional groups attached to their molecules when IR is absorbed. Specific parts of a molecule like functional groups or aliphatic chains have vibration modes such as stretching (symmetric and asymmetric), bending, wagging, twisting or scissoring modes. Each type of vibration is associated with a specific IR frequency, just like a tuning fork that is vibrated by a certain sound frequency. These vibrational modes absorb specific IR wavelengths, inducing their movements. The total amount of vibrational modes depends on the geometry of the molecule. For a linear molecule, 3n-5 denotes the formula for the calculation of vibrational modes, where n is number of atoms. A non-linear molecule has a total number of 3n-6 vibrational modes. The wavelength of IR absorption bands rarely reflect the calculated basic vibrational modes because the only modes to be detected are the ones that cause an overall change in dipole moment of the molecule. Additionally, more bands are introduced due to molecular vibrational overtones which are caused by the transition of a molecule from a ground state to the second excited state. Coupling and combination bands can also occur due to interactions between basic vibrations or between overtones and fundamental modes. Thus, each compound provides a unique IR fingerprint that can be used for
compositional and qualitative analysis.\textsuperscript{114, 115} In particular, vibrations of covalent bonds such as C-H, C-O, C-N can be detected.\textsuperscript{23}

1.3.3.1. FTIR instrumentation

The modern FTIR spectrometer consists of a radiation source, a Michelson interferometer, and a detector (Fig. 8).\textsuperscript{113, 114} Even though radiation sources have not changed for decades, FTIR radiation sources are frequently water-cooled to enhance experimental stability.\textsuperscript{114} The interferometer replaces the monochromator in a conventional dispersive IR instrument as it is more efficient.\textsuperscript{113, 114} For better resolution and faster experiments, the interferometer works by splitting the beam from the radiation source and concomitantly produces an optical path difference between sister beams.\textsuperscript{114} In essence, incoming radiation beam from a broadband IR source (with multiple frequencies) is divided by a beamsplitter\textsuperscript{114} which exhibits a 50% reflectivity and zero absorption.\textsuperscript{113} One of the beams hits a static mirror and the other hits a moving mirror. As both beam return, they re-converge at the beam splitter and cause interference patterns.\textsuperscript{114} Thereafter, the re-converging radiation is split again back to the radiation source while the rest goes through the sample in the sample holder and onto the detector.\textsuperscript{114} The two mirrors begin equidistant from the beamsplitter, and for each frequency, the in-phase split sister beams create a constructive interference wave pattern that increases the amount of radiation available to be absorbed by the sample.\textsuperscript{114} As the moving mirrors change position, interference patterns are created, with more destructive patterns developing as the mirror moves away from its starting position.\textsuperscript{114} The result is a complex array of superimposed interferograms for each frequency, showing detected photo-intensities versus optical path difference based on mirror positions.\textsuperscript{114}
transform mathematical operation gives a spectrum covering the range of frequencies introduced by the radiation source. A reference interferogram is also obtained by the interferometer beforehand and combined with the interferograms of the sample to better detect absorbed wavelengths. This background interferogram is used by the interferometer to correct for the remaining presence of ambient air and its constituents after the instrument has been purged with an inert gas. FTIR detectors are usually deuterated triglycine sulfate or mercury cadmium telluride. These detectors possess very fast scan times of about 1 sec or less. Mercury cadmium telluride detectors are more sensitive and faster than deuterated triglycine sulfate detectors because they measure photons that are quantum in nature. However, mercury cadmium telluride detectors require cooling to 77K which is liquid nitrogen temperature. Deuterated triglycine sulfate detectors with pyroelectric functionality, measure temperature changes and function primarily at room temperature. As an advantage, FTIR deals with the low energy associated with IR on the electromagnetic radiation scale by providing a high throughput and higher signal-to-noise ratio compared to a conventional IR instrument, as interferograms are added together to boost the signal-to-noise ratio ratio. In addition, very minimal radiation is lost as a result of the absence of a slit. Different meibum lipids have been detected by this technique and their phase transition behavior studied. The structure and hydrocarbon chain conformation have also been probed using FTIR. With complex samples such as meibum, ‘global’ differences in lipid composition can be measured.
1.3.3.2. Reasons for this study

The global aspect of measuring hydrocarbon chain composition is advantageous over mass spectrometric measurement. For instance, to measure lipid saturation using mass spectroscopy, the saturation level of all the 30,000 types of molecules found in meibum would have to be added together. Standards for each molecule would be necessary for quantification. With FTIR, the C=C stretching band intensity and position is the same for all hydrocarbon moieties and standards for the 30,000 molecules found in meibum are not necessary. Compositional analysis using FTIR may also be complemented using other spectroscopic techniques such as NMR as the sample is not destroyed or altered using spectroscopic instrumentation as it is with mass spectrometric analysis.23

1.3.3.3. Parameters obtainable from meibum FTIR studies

Minimum and Maximum Frequency ($\tilde{\nu}_{\text{sym}}$) are two vibrational quantities employed to estimate most disordered (maximum) and most ordered states (minimum).88,127 Phase transition temperature is another parameter obtained in FTIR temperature studies. The geometry of meibum lipids are orthorhombic at a lower temperature than the transition temperature, and monoclinic when temperature is above the phase transition temperature.131 Prior to our studies, there were no studies on meibum CE and WE to ascertain their packing properties when they are mixed together. X-ray crystallographic studies have investigated the structures and packing of standard CE and WE to understand the minimal energy requirement a mixture of the two lipids may have to maximize their hydrophobic chain interactions.90
In our studies, temperature is used to cause meibum lipids to go from an ordered phase to a disordered phase. The phase transition temperature is the temperature where half of lipid molecules go through a phase change.\textsuperscript{88, 127} Because the phase transition temperature of a meibum lipid is near its physiological temperature, the higher the phase transition temperature, the more ordered a lipid will most likely be at physiological temperature, and vice versa. From the phase transitions, one can measure the strength of lipid-lipid interactions, and the order of lipids at the temperature of the meibomian glands at 36 °C and on the surface of the eye at 33.4 °C.\textsuperscript{132} Lipid order is an important parameter to measure\textsuperscript{22, 33, 77, 88, 92, 117-120, 124-129, 131, 133-139} as it is related to tear film stability.\textsuperscript{89, 134} The CH\textsubscript{2} - CH\textsubscript{2} symmetric stretching band is important because lipid saturation relates to the conformation of meibum.\textsuperscript{131} Conformation refers to the orientation of bonds.\textsuperscript{131} This arrangement of lipid hydrocarbon chain bonds is affected by temperature, which leads to rotamerization. Traditionally, rotamers are defined by their axial rotation about a chemical bond.\textsuperscript{131} After this rotation, bonds may or may not cause the molecules to undergo steric strains. In a more confined solid state, the presence of steric effects are more likely to lead to instability, and conformational changes are noticeable.

Traditionally, \textit{trans} and \textit{gauche} isomers refer to the orientation of molecules about a double bond. \textit{Trans} isomers referring to molecules across the double bond, and \textit{cis} isomers, on the same side of the double bond. The same nomenclature used for isomers has classically been applied to rotamers. More appropriately, the terms “staggered”, “anti”, and “eclipsed” conformations should be applied to rotamers. Throughout the thesis, we used the terms “\textit{trans}” and “\textit{gauche}” rotamers that have been historically used (Fig. 9). When lipids are ordered, the hydrocarbons are arranged in a
trans conformation (Fig. 10). This allows the lipid hydrocarbon chains to pack tightly together, maximizing van der Waal’s interactions between chains. When the hydrocarbon chains are disordered, the number of gauche rotamers increases, the lipids pack less tight, and minimal van der Waal’s interactions are recorded. Therefore, at higher temperatures, meibum lipids contain 18% trans and 82% gauche rotamers, and are in a more fluid, liquid-crystalline phase. At lower temperatures, the hydrocarbon chains of meibum lipids form more trans rotamers, with 72% trans: 28% gauche rotamers. Trans rotamerization creates more ordered hydrocarbon chains which are said to be in a more ordered gel phase. The CH₂ - CH₂ symmetric stretching band frequency therefore, may be used to measure trans/gauche rotamer content by extrapolating the CH₂ symmetric stretching band frequency at 33.4 °C from the fit of a transition curve and subsequently converting the frequency to the percentage of gauche and trans rotamers. This conversion is done using a linear curve equation derived from the CH₂ symmetric stretching band vibrational frequencies at 33.4 °C, of known very ordered and disordered compounds. A detailed account of the measurement of lipid phase transitions using FTIR is provided in section 3.2.6. FTIR investigations have linked meibum lipid order to saturation, branching, phase transition temperature, cooperativity, and CE levels. Differences between lipid ordering in native tear lipids and meibum lipids have also been investigated. Meibum from donors with dry eye are more ordered and have higher phase transition temperatures compared with meibum from donors without dry eye (Fig. 11).

Relative cooperativity, another FTIR verifiable parameter, is a measure of the degree to which the melting or ordering of a lipid influences a neighboring lipid. As
the phase transition curve gets broader, the relative cooperativity decreases. Cooperativity is also related to the heterogeneity of the system as a more heterogenous mixture has a lower cooperativity compared with a more homogeneous mixture. All the phase transitional parameters were used to estimate the change in entropy (ΔS) and change in enthalpy (ΔH).

1.3.3.4. Saturation and age-related studies

Saturation studies using FTIR have provided insights into the possible link between unsaturation and reduction in meibum lipid order in individuals 20 years or older. Saturation has also been linked to an increase in the phase transition temperature of native and synthetic lipids. Comparing adult and adolescent meibum, FTIR studies revealed that adolescents 16 to 23 years old and adults 32 to 61 years old had similar saturation levels. Yet, the order of their meibum decreased (Fig. 12). Therefore, factors other than saturation must have caused the decrease in order with age. Similarly, rise in lipid order with age for meibum from donors 1 and 20 years old due to increase in saturation failed to be substantiated because adult and adolescent meibum have been shown to be less saturated compared with children. Thus, the relationship between meibum lipid stiffness and saturation is inconclusive. Notwithstanding, the level of CE/WE ratio in meibum was to be probed to perhaps, reconcile the dip in lipid order above 20 years and the increase in order between 1 and 20 years.
Figure 9: Gauche and trans conformer of 1-bromo-2-chloroethane. (Reprinted by permission from Ponnadurai Ramasami: Springer Nature. Theoretical gas phase study of the gauche and trans conformers of 1-bromo-2-chloroethane: Springer Nature. Copyright © 2007. doi: 10.1007/978-3-540-72586-2_42)
**Figure 10.** Models for a.) Ordered *trans* rotamers and b.) Disordered *gauche* rotamer. Arrows show changes in chain arrangement that lead to bending commonly associated with gauche rotamers. (Source: Borchman D, et al. Physical changes in human meibum with age as measured by infrared spectroscopy. *Ophthalmic Res* 2010;44: 34-42. Copyright © 2010 Karger Publishers, Basel, Switzerland)
Figure 11. Lipid order changes with dry eye disease (Left) and phase transition temperature changes with dry eye disease (Right). (With kind permission from source: Foulks GN, Borchman D. Meibomian gland dysfunction: the past, present, and future. Eye Contact Lens 2010;36:249-253. DOI: 10.1097/ICL.0b013e3181e0d37)
1.3.3.5. CE studies

Studies investigating the role of CE in the decline in hydrocarbon chain order with age bolsters the idea that changes in CE with age affects tear structure and stability.\textsuperscript{42, 149} An increase in the mole percent of CE was associated with CE-WE mixture phase transition temperature.\textsuperscript{42} A small increase of 5 mole percent saturated CE gave rise to a 65 °C change in the phase transition temperature of oleyl oleate, an unsaturated WE.\textsuperscript{149} The phase transition temperature of WE soared by 14 °C upon the addition of 50% CE.\textsuperscript{42} Infrared spectroscopic investigations of CE changes with DED have shown that in MGD – induced dry eye\textsuperscript{42, 78} and dry eye due to Parkinson’s,\textsuperscript{150} the patients’ meibum had CE levels ~ 70% lower than normal meibum (Fig. 13). A decrease in CE with DED did not correlate to a decrease in lipid order. Meibum lipid order increased with dry eye compared with meibum from normal donors.\textsuperscript{116, 117, 120, 128} Factors other than just CE levels alone could therefore influence the higher order of meibum from donors with DED (Fig. 11).\textsuperscript{150} As a result, model studies might not be enough to describe the relationship of the CE and WE in the complex meibum where the hydrocarbon chains are branched and contain various levels of saturation.\textsuperscript{150} Other macromolecular changes besides hydrocarbon chain conformational changes may play a role in the observed ordering of meibum with DED.\textsuperscript{150}
Figure 12. Changes in hydrocarbon chain order with age. (Source: Borchman D, Foulks GN, Yappert MC, et al. Physical changes in human meibum with age as measured by infrared spectroscopy. Ophthalmic Res 2010; 44:34-42. Copyright © 2010 Karger Publishers, Basel, Switzerland.)
Figure 13. Changes in cholesteryl esters with MGD calculated using FTIR (open bars) and $^1$H-NMR (filled bars). (Source: Hetman ZA, Borchman D. Concentration dependent cholesteryl-ester and wax-ester structural relationships and meibomian gland dysfunction. Biochemistry and Biophysics Reports 2020; 21:100732.)
CHAPTER II

HYALURONIC ACID – LIPID BINDING

2.1 INTRODUCTION

Hyaluronic acid (HA) is a polysaccharide with repeating units made up of glucuronic acid (GlcA), and an amino sugar, glucosamine (N-GlcNA), linked via a glycosidic bond (Fig. 14). It was first detected in 1934 in bovine vitreous humor (VH). The structure of HA was determined in 1950. The length of the HA chain varies by species and region. For example, rabbit VH has 2000-3000 kDa HA strands but bovine HA strands are shorter at 500-800 kDa. Along the polysaccharide chains, hydrogen bonds cause the strand to twist, forming a helical 'ribbon' with hydrophobic and hydrophilic regions between the 1-3 and the 1-4 linkages. About half of the HA found in the human body is present in the skin, the remainder found is in synovial fluid, the vitreous body, the umbilical cord, and places where friction occurs such as joints, tendons, sheaths, pleura, and the pericardium. HA was used to replace vitreous during ocular surgery in the 1950’s. Other applications of HA include: visco-supplementation in joints and restoration of synovial fluid, vocal cord therapy and sinus surgery, filling of facial wrinkles and depressed scars, and drug delivery. Of importance to the current study, HA is also used in eye drops to treat dry eye. One disaccharide unit of HA can interact with 15 water molecules to increase its
Figure 14. Hyaluronic acid polymeric structure. Protons in the hydrophobic face are in red. (Source: Ewurum A., Alur A.A., Glenn M, Schnepf A, Borchmann D. Hyaluronic acid–lipid binding. BMC Chemistry 2021;15:36.)
molecular weight up to 1000 fold. As discussed below, HA-phosphatidylcholine (PC) interactions have been well characterized. However, HA-phospholipid (PL) interactions involving other PL have not. The intensity of the resonances’ of the methyl protons on the N-GlcNA decrease upon interacting with PLs giving rise to resonance broadening due to shielding. Furthermore, NMR data, in addition to gel permeation chromatography and multi-angle laser-light scattering, suggest possible interactions between the PC tails and the hydrophobic patches of HA. In general, PC assembly involves assembly aggregation around HA strands, but HA molecular weight and calcium cations can affect this organization. The historical importance of structural changes to HA induced by PLs in synovial fluids have been reviewed. It has been suggested that HA-lipid hydrophobic interactions in synovial fluids contribute to lubricating and protecting joint cavities. The importance of HA in relation to providing an elastic hydrostatic cushion and retaining water in the synovial fluid in the cavity of diarthrodial joints was recognized in 1953. HA is a major constituent of synovial fluid, 2 to 4 mg/mL. PLs are also major constituents of synovial fluid, 0.14 mg/mL. PC is the major PL in synovial fluids at 61% of the PL. SM and Cho account for 19 % and 15 % of the lipids, respectively. Other lipids such as phosphatidyl ethanolamine, phosphatidyl inositol, Cho, monoglycerides, diglycerides, triglycerides, and FFA are also present. PLs have been found in human and porcine VH. The lipids in the anterior region of the VH come from the lens and the lipids in the posterior region of the VH come from the retina. Elevated levels of lipid are observed in the VH of humans with diabetes and it is speculated that these lipids contribute to vitreous liquefaction. Liquefaction is the result of the collapse or contraction of the collagen/hyaluronan network, where collagen
can be considered to act as the scaffold and HA fills the spaces in between.\textsuperscript{151, 176} It has been speculated that lipids binding to HA, a major component of the VH other than water,\textsuperscript{156, 167, 176} could disrupt HA-collagen interactions leading to vitreous liquefaction and retinal detachment. The liquefaction of the VH, which is associated with aging, begins as early as the second decade of life and almost 50\% of the VH is liquefied by the 8\textsuperscript{th} and 9\textsuperscript{th} decades of life.\textsuperscript{176} The age-related changes that cause this liquefaction are not known at the molecular level. Free radicals, generated by metabolic processes or by photosensitizized reactions, have been shown to induce depolymerization and conformational changes in HA.\textsuperscript{177} Additionally, diabetic patients develop VH degeneration earlier in life. Liquefaction and thinning of the VH weaken the adhesion between the posterior VH cortex and the inner limiting membrane of the retina which can ultimately lead to posterior vitreous detachment, whereby the vitreous separates from the retina.\textsuperscript{151, 178} HA-lipid binding could also be relevant to dry eye treatment, as HA is used with other therapeutics in eye drops to treat dry eye.\textsuperscript{153, 163-166} A thin layer of lipid coats the surface of tears and contributes to tear film stability.\textsuperscript{29, 89, 134, 179} The tear film lipid layer (TFLL) consists of PL\textsuperscript{29} and OAHFA that partition at the interface region between the TFLL and tear aqueous (See section 1.2.3).\textsuperscript{89} Above the PL is the bulk lipid layer consisting of WE and CE over 17 molecules thick. Only PC-HA binding has been qualitatively measured thus far. Other lipids such as PE, SM and Cho are present in the VH, and synovial fluid and other PL and WE are present in the TFLL. As HA is used in eye drops as a therapy for dry eye, and PL-HA binding especially with diabetes, could contribute to vitreous liquefaction, the aim of the current study therefore, was to quantify PL-HA, Cho-HA and Wax-HA binding.
2.2. MATERIALS & METHODS

2.2.1. Chemicals and reagents

Polymeric HA sodium salt from Streptococcus equi \([(α-ΔGlcU-(1→3)-GlcNAc)n]\) (MW=1500 to 1800 kDa), D_2O and palmitoyl sphingomyelin (pSM), dipalmitoyl phosphatidylcholine (DPPC), dipalmitoyl phosphatidic acid (DPPA), palmitoyl palmitate (PP), palmitoyl glyceride (PG) and cholesterol (Cho) were purchased from Sigma-Aldrich (St. Louis, MO).

2.2.2. Sample preparation and binding assay.

Stock solutions of HA and lipids were prepared in D_2O at a concentration of 10 mg/mL. They were homogenized using a microprobe sonicator (Branson, Ultrasonics Co., Danbury, CT, USA) three times for 30 secs with a two-minute rest period between sonication, at a probe setting of 4. Samples for the binding study were prepared with a final volume of 0.5 mL with a final HA concentration of 5 mg/mL (13.15 µmoles dimer). Samples were allowed to equilibrate for 30 hours under argon gas (analyzed, ultra-pure; Welders Supply, Louisville, KY) to avoid oxidation. The samples were placed in NMR tubes for analysis.

2.2.3. NMR analysis

NMR spectra were obtained on a 700 MHz NMR spectrometer equipped with a 5 mm \(^1\text{H} \ ^{13}\text{C}\) enhanced PFG cold probe (Palo Alto, CA). All \(^1\text{H}\) spectra were acquired with a minimum of 250 scans, 45° pulse width, and a relaxation delay of 1.000 second. All spectra were obtained at 25°C unless stated otherwise. Spectra were manipulated and quantified using GRAMS/386 software (Galactic Industries, Salem, NH). A typical spectrum of HA is shown in Figure 15.
Figure 15. a) Typical $^1$H-NMR spectrum of high molecular weight hyaluronan in D$_2$O at 25 °C. U = d-glucuronic acid moiety, N = N-acetyl glucosamine moiety. b) Effect of increasing dipalmitoyl phosphatidyl choline on the $^1$H-NMR spectra of hyaluronan. (Top to bottom) Increasing concentrations of dipalmitoyl phosphatidyl choline as in Fig. 16a. The absolute intensities of the resonances of the HA–PL mixtures change from run to run due to instrumental tuning differences and the number of scans, so the data in Table 3 and in Fig. 16 were calculated and plotted relative to the intensity of the D$_2$O resonance at 4.79 ppm. (Source: Ewurum A, Alur AA, Glenn M, Schnepf A, Borchman D. Hyaluronic acid–lipid binding. BMC Chemistry 2021;15:36.)
In some instances such as with SM, a sharp lipid band appeared and was removed using the ‘zap’ function. The intensity of the HA resonances was measured between 3.1 and 4.2 ppm. The absolute intensity of the resonances of the HA-PL mixtures changes from run to run due to instrumental tuning differences and the number of scans, so the data in Table 3 and in the Figures were calculated relative to the intensity of the D$_2$O resonance at 4.79 ppm. The following equation was used to measure the % decrease in HA resonance intensity, a lower level measure of the % lipid bound where I is the resonance intensity:

$$\left[ \frac{(I_{HA}/I_{D2O})_{no\ lipid} - (I_{HA}/I_{D2O})_{plus\ lipid}}{(I_{HA}/I_{D2O})_{no\ lipid}} \right] \times 100 / 0.6$$

The constant 0.6 was calculated from 6 hydrophobic protons that bind to lipid / 10 total protons in a HA dimer (Fig. 14). Data are presented ± the standard error of the mean.

2.3. RESULTS

A typical $^1$H-NMR spectrum of high molecular weight HA is shown in Figure 15a. The region of the $^1$H-NMR spectrum encompasses protons that are in the hydrophobic lipid-binding region and hydrophilic region of the glycerides (Fig. 14). When lipids bind to HA, the intensities of the proton resonances decrease (Fig. 16a and b) suggesting HA-lipid interactions broaden the resonance and shield protons from the magnetic field. The decrease in the HA intensity with increasing lipid concentration (Fig. 15b) is a lower level of HA-lipid binding since lipid could bind to HA and not decrease the intensity of the HA resonance. The absolute intensity changes from run to run due to instrumental tuning differences and number of scans so data in Table 2 and in Figure 16 were calculated relative to the intensity of the D$_2$O resonance at 4.7 ppm. Cho weakly
binds to HA (Fig. 16b), followed by PG, and PP < DPPC, and thereafter, DPPA and pSM. Extrapolating the best-fit linear regression line to 100 % bound, the maximum amount of phospholipid bound was 14 ± 1 µmoles, close to the value of 13.15 µmoles HA dimers in the assay (Figure 16, Table 3). This indicates a 1 to 1 molar ratio of bound lipid to HA dimer. Two to three times more PG and the wax PP were however needed to reach 100 % bound HA when compared to the other phospholipids, suggesting that they have a higher binding constant. The two choline containing phospholipids, DPPC and pSM both had a maximum binding ratio of 1:1 HA:PL (Table 3), however, pSM initially decreased the intensity of the HA resonances much more than DPPC (Fig. 16a). DPPA without a choline head group but with the same acyl chains as DPPC both had a maximum binding ratio of 1:1 HA:PL, however, DPPA initially decreased the intensity of the HA resonances much more than DPPC (Figs. 16a and b).

2.4. Discussion

The binding of PC to HA has been well characterized and reviewed. The current study is unique in that HA-lipid interactions were measured with a range of lipid concentrations using a variety of lipids such as PC, sphingomyelin and wax. The lipid studied are relevant to vitreous liquefaction, tear film stability and lubrication of synovial joints. HA-cholesterol binding was relatively weak. A unique finding of our study is that HA does bind to WE and CE, two lipids found in tears. The 1H-NMR lipid-HA binding assay presented in the current study is novel and could be used in future studies to quantify the binding characteristics of lipids to HA.
Table 2. $^1$H chemical shift assignments for hyaluronan. (Source: Ewurum A, Alur AA, Glenn M, Schnepf A, Borchman D. Hyaluronic acid–lipid binding. *BMC Chemistry* 2021;15:36)

<table>
<thead>
<tr>
<th>Proton number (see figure 14)</th>
<th>Experimental high-molecular weight polymer (ppm)</th>
<th>Literature$^a$ low-molecular weight polymer (ppm)</th>
<th>Literature$^b$ HA₅ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Acetyl glucosamine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1</td>
<td>4.55</td>
<td>4.55</td>
<td>4.55</td>
</tr>
<tr>
<td>H2</td>
<td>3.83</td>
<td>3.83</td>
<td>3.84</td>
</tr>
<tr>
<td>H3</td>
<td><em>(3.71)</em></td>
<td><em>3.73</em></td>
<td>3.71</td>
</tr>
<tr>
<td>H4</td>
<td>3.51</td>
<td>3.51</td>
<td>3.52</td>
</tr>
<tr>
<td>H5</td>
<td>3.48</td>
<td>3.47</td>
<td>3.48</td>
</tr>
<tr>
<td>H6</td>
<td>3.9</td>
<td>3.91</td>
<td>3.91</td>
</tr>
<tr>
<td>H6'</td>
<td><em>(3.71)</em></td>
<td>3.71</td>
<td>3.76</td>
</tr>
<tr>
<td>H-CH₃</td>
<td>2.02</td>
<td>2.01</td>
<td>2.02</td>
</tr>
<tr>
<td>D-Glucuronic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1</td>
<td>4.45</td>
<td>4.46</td>
<td>4.46</td>
</tr>
<tr>
<td>H2</td>
<td>3.33</td>
<td>3.34</td>
<td>3.34</td>
</tr>
<tr>
<td>H3</td>
<td>3.58</td>
<td>3.58</td>
<td>3.58</td>
</tr>
<tr>
<td>H4</td>
<td><em>(3.71)</em></td>
<td>3.74</td>
<td>3.74</td>
</tr>
<tr>
<td>H5</td>
<td><em>(3.71)</em></td>
<td>3.72</td>
<td>3.70</td>
</tr>
</tbody>
</table>

$^a$ In D₂O at 30 °C $^{181}$

$^b$ In 10% D₂O/H₂O at 24 °C $^{182}$

$^c$ unresolved resonance
Table 3. Hyaluronan—lipid binding parameters from Figure 16. (Source: Ewurum A., Alur AA, Glenn M, Schnepf A, Borchmann D. Hyaluronic acid—lipid binding. BMC Chem 2021;15:36)

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Phosphatidylcholine</th>
<th>Sphingomyelin</th>
<th>Phosphatidic acid</th>
<th>Palmitoyl palmitate</th>
<th>Monolaurate</th>
<th>Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA concentration (%)</td>
<td>13 ± 3</td>
<td>13 ± 6</td>
<td>17 ± 5</td>
<td>41 ± 12</td>
<td>57 ± 13</td>
<td>110 ± 42</td>
</tr>
<tr>
<td>Number of ions (nmol)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X-axis intercept (nmol)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slope</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% bound / (nmol Lipid)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HA concentration was 13.15 µmoles in 0.5 mL D2O ± 95% confidence interval.
The basis for the NMR assay is that when lipids interact with HA, the HA protons involved in the interaction spin slower, causing the resonances to become broader and less intense. Our assay used a concentration of 5 mg/mL HA, close to the physiological value found in synovial fluids, 0.2 mg/ml.\textsuperscript{154} The molecular weight of HA does influence lipid-HA interactions.\textsuperscript{168-171} The molecular weight of the HA used in our assay was between 1,500 and 1,800 kDa, similar to that found physiologically.\textsuperscript{154} PL titration of HA with different molecular weights has not been done. It is reasonable to speculate that small differences in the molecular weight of HA does not affect lipid binding as the hydrophobic face of the polymeric structure presented to the lipid for binding is the same for all molecular weight HA near to the MW of the HA used. By interpolating the binding from our plots in Figure 19, we calculate that physiological levels of HA, PC and SM\textsuperscript{172} would bind only 4% of the hydrophobic hydrogens of HA. Our data suggest that the cholesterol in synovial fluids would not influence HA at all. Whether HA–lipid hydrophobic interactions in synovial fluids contribute to lubricating and protective properties in joint cavities at a level of 4% binding has yet to be determined. HA-lipid hydrophobic interactions would be even less in arthritic joints in vivo considering that HA is degraded to low-molecular weight moieties that interact with lipid less than high molecular moieties of HA.\textsuperscript{171} Perhaps higher levels of PL, especially SM, could lubricate and protect joint cavities therapeutically. The adult vitreous humor HA concentration is approximately 0.2 to 0.3 mg/mL (\sim 0.5 \mu moles HA diamers) in the human VH depending on age.\textsuperscript{154} One may question if
Figure 16. HA - Lipid binding profiles. a) Phospholipid profile with a choline headgroup. b) Phosphatidylcholine. c) Lipids found in the tear film. (Source: Ewurum A, et al. Hyaluronic Acid – Lipid Binding. BMC Chemistry 2021;15:36.)
there is enough PL in the VH to bind to HA to contribute to VH liquefaction. PL synthesis and types have been quantified in animals.\textsuperscript{175, 183, 152, 184-186} The concentration of PLs in the VH of cows, sheep, rabbits, rodents and dogs ranges approximately from 1 to 130 nmoles/mL.\textsuperscript{184, 185, 187, 188} Based on our binding studies, at the highest concentration of PL in the VH of animals, assuming 1:1 binding, 24\% of the total HA would be expected to bind to PL. However, even less HA–PL binding would be expected in human VH since humans have a lower amount of PL in the VH (\textasciitilde 3 nmoles/mL)\textsuperscript{188} compared to animals. No significant differences between the concentrations of HA in the VH of diabetic and non–diabetic patients have been measured.\textsuperscript{187} Therefore with diabetes, only an increase in PL concentration in the VH could potentially increase HA-PL binding. Relatively higher levels of PLs and glucose in the VH of diabetic patients compared to VH from non-diabetics has been observed\textsuperscript{174} and is believed to be caused by a decrease in the blood-retinal barrier with diabetes.\textsuperscript{189} Quantitative studies to determine PL concentrations in the VH of diabetics are needed to determine if PL contributes to increased VH liquefaction in diabetic patients.

HA-PL binding could be important to trap PL entering the VH to keep them from forming light scattering micelles. Lack of cholesterol-HA binding could be why there is little cholesterol found in the VH\textsuperscript{175} as it is likely to pass through the VH unimpeded. Therapies for complicated retinal detachments often includes the removal of the VH (vitrectomy), and its replacement with an endotamponade such as perfluorocarbons, air, gases and silicone oils and hydrophilic, hydrogel-based systems such as hyaluronic acid, has been reviewed.\textsuperscript{190} The strategy behind the tamponade is to stabilize the retina. HA tamponades such as HA offer the advantage over hydrophobic tamponades in that they do
not leave a small amount of liquid at the opposite pole of the buoyancy vector wherein growth factors accumulate and promote pro-inflammatory processes. Hydrophobic tamponades also lead to complications such as emulsification, cataract formation, and the need for revision surgery. HA used as a vitreous substitute provides optical, viscoelastic, and biocompatible properties. It would be interesting to see how lipid binding to HA, if it occurs over time, affects the biocompatible properties. As discussed in the Introduction, HA-lipid binding could also be relevant to dry eye treatment as HA is used as a component of artificial tears eye drop for dry eye alleviation, ranging between 0.1 – 0.3 %. The current study indicates that HA in eye drops could interact hydrophobically with both the bulk lipid layer of the TFLL consisting of wax and cholesteryl esters and the monolayer of phospholipids at the aqueous interface of the TFLL. PL head group, the molecular weight of HA and calcium interactions are likely to be involved in PL-HA interactions. The finding that HA interacts with the wax PP and mono-glyceride, PG, is relevant in that waxes and glycerides do not have a hydrophilic charged head group as PL do, so the lipid hydrocarbons chains can be involved in the interaction with HA, as a head group is not necessary for HA-lipid interactions. However, head group interactions may be involved with HA-lipid interaction and the current study shows PL bind much tighter with a lower maximum PL/HA ratio compared with wax/HA suggesting head group involvement. pSM initially decreased the intensity of the HA resonances much more than DPPC indicating that the hydrocarbon sphingosine back bone of pSM and the acyl linked hydrocarbon chains of DPPC influenced the interaction characteristics as the head group choline moieties are the same for both lipids. DPA without a choline head group but with the same acyl chains as
DPPC both had a maximum binding ratio of 1:1, however, DPA initially decreased the intensity of the HA resonances much more than DPPC, indicating that head group binding could be also important. Despite the possible involvement of hydrocarbon chain-HA interactions, the interaction of PC with HA only minimally affects the enthalpy of the hydrocarbon phase transition,\(^{170}\) and both head groups and hydrocarbon chain moieties influence HA-lipid interactions. The PL mono-layer of the TFLL is composed of 72 % PC and 10 % SM, both shown in the current study to interact with HA.\(^{37, 41, 192}\) One could speculate that strands of HA could lay flat on the posterior and anterior surface of the TFLL, ‘holding’ adjacent lipid molecules together, inhibiting tear breakup and increasing tear film stability. The amount of HA in tears is complicated by tear film clearance and whether HA-PL binding is reversible. Future studies involving native tear lipids, rheological studies and in vivo measurements are warranted. HA-lipid binding could be important for topical skin-care products where evaporation of water from the product applied to the surface of the skin could lead to relatively high concentrations of HA. Our data show that the HA would be expected to bind to the sebaceous wax and cholesteryl esters and subcutaneous PL.

2.5. Conclusion

Physiological levels of HA, PC and sphingomyelin would result in 4% of the hydrophobic hydrogens of HA to be bound. HA–PL binding interactions could be important for therapeutic use of HA in eye drops to treat dry eye and to trap PL entering the VH to keep them from forming light scattering micelles. HA–lipid binding may also be relevant to the therapeutic effects of topical skin-care products. Both head group and hydrocarbon chain moieties influence HA–lipid interactions.
CHAPTER III

A SPECTROSCOPIC STUDY OF THE COMPOSITION AND CONFORMATION OF

CHOLESTERYL AND WAX ESTERS PURIFIED FROM MEIBUM

3.1. INTRODUCTION

WE and CE are found in: sebum, on the surface of skin, meibum, a major source of tear lipids; the cuticle of plants; the spermaceti of whales, and the exoskeleton coating of insects. Little is known about how CE and WE interact with one another. The focus of the present study is to bridge this gap of knowledge. CE became widely studied after a seminal publication showed that CE contributes to plaque formation in human aortic intima, contributing to atherosclerosis. In humans, CE transports cholesterol to specific organs, a process that could be important to ocular lens clarity as cholesterol levels are extremely high in human lens, and ocular cholesterol levels are related to lifespan and cataract formation. Sebum contains higher amounts of CE in patients with sensitive scalp compared with those without the condition. WE serves many functions: For some desert insects, WE on their exoskeleton reflects sunlight and prevents water loss. Plants are protected from transpiration, pathogens and ultraviolet radiation by epicuticular wax. Wax in the human ear protects the eardrum from debris. In many marine species, WE is a storage depot for lipids, plays a role in the reception and
Figure 17. a) The structure of the wax ester oleyl oleate. The arrow shows protons that were used to quantify wax esters at 4.0 ppm using \(^1\)H-NMR spectroscopy. The proton on carbon #18 and #19 at 0.66 ppm and 1 ppm, respectively, were used to quantify cholesteryl esters with \(^1\)H-NMR spectroscopy. The \(^1\)H-NMR resonance assignments were made for the numbered carbons. (b) The structure of a cholesteryl ester where R is a hydrocarbon chain. The protons on carbon #18 and #19 at 0.66 ppm and 1 ppm, respectively, were used to quantify cholesteryl esters using \(^1\)H-NMR spectroscopy. (Source: Ewurum A, Ankem A, Georgiev G, Borchman D. A spectroscopic study of the composition and conformation of cholesteryl and wax esters purified from meibum. Chemistry and Physics of Lipids 2021; 238:105088.)
transmission of sound, and provides buoyancy. Maintenance of the ratio of CE/WE in human tear lipids may be important to tear film stability and the prevention of dry eye. CE and WE make up ~ 80% of human meibum. Two populations of donors have been observed, with lower and higher ratios of CE/WE. The CE/WE ratio has been shown to decrease in patients with meibomian gland dysfunction and increase between birth and 19 years of age. It is unclear whether changes in CE/WE ratio are related to tear film stability or are markers for and/or contributes to dry eye disease. Nor is it known how CE influences the structure of meibum. The key to understanding how CE and WE are involved in natural and pathological processes is to elucidate the compositional, structural and functional relationships of these lipids. Vibrational spectroscopy has been a valuable tool to measure lipid hydrocarbon chain conformation (structure). For instance, a seminal study showed that Cho increases the phase transition temperature and broadness of PL phase transitions, increases the order of disordered hydrocarbon chains, and decreases the order of ordered hydrocarbon chains. The relationships between lens membrane cholesterol content, membrane lipid conformation and cataract has been reviewed. The variable hydrocarbon chains found in CE and WE adds a layer of complexity to the relationships between CE and WE interactions that Cho does not have. Vibrational spectroscopy has also been used to study straight chain hydrocarbon WE and CE interactions. Like Cho, CE dramatically increased the phase transition temperature of pure WE. However, unlike cholesterol, CE increased the order of ordered WE, indicating the acyl chain of CE impacts WE hydrocarbon chain order. In the current study, we used infrared spectroscopy to analyze native WE and CE fractions from meibum to determine if or how CE influences WE hydrocarbon chain
conformation and vice-versa. Human meibum was used as a source of CE and WE because meibum is composed of mostly CE and WE, and the esters contain complex hydrocarbon chains. The current study adds a layer of complexity to previous cholesterol, CE and WE studies as native CE and WE acyl chains have variable lengths, saturation and branching whereas Cho and model synthetic CE and WE do not.

The three major aims of the study were to answer the following questions:

1. Could human meibum WE and CE be completely separated?

2. Does the complexity of the hydrocarbon chains found in human meibum WE and CE influence how changes in the ratio of WE and CE ester influence hydrocarbon chain conformation?

3. Why is WE and CE often present together? And how does WE influence CE hydrocarbon chain conformation and vice-versa?

To answer question one, we used adsorption column chromatography with a MgO solid phase to separate CE from WE from human meibum. To answer questions two and three, we compared the phase transitions of meibum WE and CE mixtures that have complex hydrocarbon chains, to those of simpler synthetic WE and CE from previous studies. $^1$H-NMR spectroscopy was used to characterize the separated CE and WE, and from infrared spectra, lipid structure and conformation were determined similar to previous studies.
3.2. MATERIALS AND METHODS

3.2.1. Chemicals and standards

HPLC-grade solvents and chemicals were acquired from Sigma Chemical Co., St Louis MO.

3.2.2. Meibum collection

Written consents were obtained from the two volunteers, and all protocols were in accordance with the tenets of the Declaration of Helsinki. Protocols and procedures for the current retrospective study were approved by the University of Louisville Institutional Review Board (# 11.0319, August 2016). The donors did not complain of DED and their meibomian gland orifices showed no evidence of keratinization or plugging with turbid or thickened secretions, and no dilated blood vessels were observed on the eyelid margin. Meibum collection was done by medically qualified personnel. Meibum was expressed from four eye lids of each volunteer using an ILUX instrument (Fig. 21) according to the manufacturer’s instructions after a mild anesthesia with proparacaine hydrochloride ophthalmic, 0.5 % drops (Bausch and Lomb, Bridgewater, NJ) was introduced in each eye. The ILUX instrument delicately clamps on each eye lid, warming them for ~90 s, and then applies gentle pressure on the eye lid to express the visible meibum magnified by ILUX. Approximately 0.5 mg of meibum lipid was obtained at each collection per donor. Meibum from two donors, was collected five times over a period of a month. The meibum from each donor was separately pooled. Each expressate was collected with a platinum spatula and immediately dissolved into 0.5 mL of CDCl₃ in a 9-mm glass microvial with a Teflon cap (Microliter Analytical Supplies Ind., Suwanee, GA). Samples
were stored in the freezer until their use in separation. The samples were never exposed to any plastic to avoid plasticizers. Control CDCl₃ spectra were run with the meibum samples to ensure no impurities were present in the CDCl₃.

3.2.3. Column chromatographic separation of CE and WE from human meibum.

A glass column of 1.7 cm inside diameter and 43.5 cm length (excluding cotton wool sitting) was made. A slurry of MgO and chloroform was used to make the MgO stationary phase. The solution of the pooled meibum (1 mL) was layered onto the column. Separation of CE and WE was achieved with a 13.1 cm column slurry height and varying elution solvent volume for both donors. Consecutive 25 mL aliquots of 250 mL hexane initiated the younger donor meibum lipids’ separation gradient course with 37 percolated fractions for ~ 3.25 h, and then added aliquots of 100 % chloroform were used, yielding 26 eluate portions for ~1.75 h. The final forty fractions, including those suspected to contain unseparated CEy and WEy, were ran twice again by gradient elution. For the older donor meibum lipids’ separation, 25 mL aliquots of 175 mL hexane were added in the beginning, and then 25 mL aliquots of binary eluent mixtures of 95:5, 93:7, 90:10, 85:15, 70:30, 50:50, 0:100 hexane: chloroform (vol: vol) were introduced sequentially. Fractions were collected in a beaker each hour for the first 120 min, with subsequent collections in 27 test tubes for 73 min, followed by a 77-minute final collection in a beaker. All fractions were left in ambient conditions for 5 days in a fume hood to evaporate solvent(s). CE and WE separated from the older donor are abbreviated C EO and W EO. CE and WE separated from the younger donor are abbreviated CEy and WEy.
**Figure 18.** A picture of ILUX instrument for meibum collection. (Source: Tauber J, Owen J, Bloomenstein M, Hovanesian J, Bullimore MA. Comparison of the iLUX and the lipiFlow for the treatment of meibomian gland dysfunction and symptoms: A randomized clinical trial. *Clinical Ophthalmology (Auckland, NZ)* 2020;14: 405-418. Originally published by and used with permission from Dove Medical Press Ltd.)
3.2.4. Combining CE and WE

The four samples in 600 µL of CDCl₃, CEo, WEo, CEy and WEy, were mixed proportionally to give a range of CE/WE ratios as in Table 4. A X axis value of 20 % in the figures (20 % CE (% total esters separated)) was obtained by mixing 20 µL of CEo with 80 µL of WEo, or by mixing 20 µL CEy with 80 µL WEy. After mixing the WE with the CE, 500 µL of CDCl₃ was added to each sample as listed in Table 4. Since we were interested only in the molar ratio of WE and CE, we did not calculate the concentrations of purified WE and CE which we estimate to be around 1 mg each. The molar ratio of CE / WE was measured using NMR spectroscopy. CE has a characteristic resonance at 0.66 ppm and 1.01 ppm, while WE has a resonance at 4.0 ppm. Areas under these NMR resonances were quantified, and the molar CE/WE ratio calculated, factoring in the hydrogen resonance contribution by each type of ester.

3.2.5. NMR spectroscopy

A 700 MHz ¹H-NMR was used to analyze every collected fraction. Each of the fractions were reconstituted with 600 µL D-chloroform and analyses were performed with 250 scans, 45° pulse width, a 1.000 s relaxation delay between 0 –11 ppm. Pooled CE and WE samples from both donors, as well as a blank of chloroform, were measured using the same conditions but with 1024 scans. Chemical-shifts were referenced to the CDCl₃ resonance at 7.25 ppm resonance. GRAMS/386 software (Galactic Industries, Salem, NH) was used to analyze all spectra. Hydrocarbon chain branching and the molar ratios of CE to WE were calculated as described previously.

<table>
<thead>
<tr>
<th>X- axis label</th>
<th>Purified WE in CDCl₃ (μL)</th>
<th>Purified CE in CDCl₃ (μL)</th>
<th>CDCl₃ (μL)</th>
<th>CE (mole %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figs. 22, 23 &amp; 24</td>
<td>Meibum from older donor</td>
<td>Meibum from younger donor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
<td>500</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>80</td>
<td>20</td>
<td>500</td>
<td>12.0</td>
</tr>
<tr>
<td>30</td>
<td>70</td>
<td>30</td>
<td>500</td>
<td>15.7</td>
</tr>
<tr>
<td>40</td>
<td>60</td>
<td>40</td>
<td>500</td>
<td>22.2</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
<td>50</td>
<td>500</td>
<td>28.9</td>
</tr>
<tr>
<td>75</td>
<td>25</td>
<td>75</td>
<td>500</td>
<td>37.0</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>100</td>
<td>500</td>
<td>100</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
<td>500</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>80</td>
<td>20</td>
<td>500</td>
<td>7.6</td>
</tr>
<tr>
<td>30</td>
<td>70</td>
<td>30</td>
<td>500</td>
<td>9.0</td>
</tr>
<tr>
<td>40</td>
<td>60</td>
<td>40</td>
<td>500</td>
<td>10.8</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
<td>50</td>
<td>500</td>
<td>14.0</td>
</tr>
<tr>
<td>75</td>
<td>25</td>
<td>75</td>
<td>500</td>
<td>25.0</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>100</td>
<td>500</td>
<td>100</td>
</tr>
</tbody>
</table>
3.2.6. Measurement of lipid phase transitions using FTIR spectroscopy.

Purified CE and WE each in a total volume of 0.6 mL CDCl₃ were mixed as shown in Table 4. Lipid phase transitions were measured as described previously:¹¹⁹ “All of the 500 µL of sample in CDCl₃ was applied to a KCl infrared window. The solvent was evaporated under a stream of Argon gas and the window was placed in a lyophilizer for 4 hours to remove all traces of solvent. Infrared spectra were measured using a Fourier transform infrared spectrometer (Nicolet 5000 Magna Series; Thermo Fisher Scientific, Inc., Waltham MA). Lipid on the KCl window was placed in a temperature-controlled infrared cell. The cell was jacketed by an insulated water coil connected to a circulating water bath (model R-134A; Neslab Instruments, Newton NH). The sample temperature was measured and controlled by a thermistor touching the sample cell window. The water bath unit was programmed to measure the temperature at the thermistor and to adjust the bath temperature so that the sample temperature could be set to the desired value. The rate of heating or cooling (1°C/15 minutes) at the sample was also adjusted by the water bath unit. Temperatures were maintained within ± 0.01°C. Exactly 100 interferograms were recorded and averaged. Spectral resolution was set to 1.0 cm⁻¹. Infrared data analysis was then performed (GRAMS/386 software; Galactic Industries, Salem, NH).”

3.2.6.1. Lipid phase transitions

In the current study, temperature was used to cause meibum lipid to go from an ordered phase to a disordered phase. From the phase transitions, we can measure the strength of lipid-lipid interactions, and the order of lipids at the temperature of the
Figure 19. A typical lipid phase transition of a meibum mixture of wax ester and cholesteryl ester from the younger donor containing 7.6 mol % cholesteryl ester. The larger the Y axis value, the more disordered the hydrocarbon chains. Insets show the conformation of ordered hydrocarbon chains containing all trans rotamers and disordered gauche rotamers. (Source: Ewurum A, et al. A spectroscopic study of the composition and conformation of cholesteryl and wax esters purified from meibum. Chemistry and Physics of Lipids 2021; 238:105088.)
meibomian glands at 36 °C and on the surface of the eye, 33.4 °C. As mentioned earlier in the introduction, lipid order may be an important parameter to measure as it is related to tear film stability. A phase transition plot (Fig. 19) was used to study the relationships between temperature and order. As described previously, the frequency of the symmetric CH2 stretching band near 2850 cm\(^{-1}\) (\(\tilde{v}_{\text{sym}}\)) was used to estimate the content of trans and gauche rotamers in the hydrocarbon chains according to the equation:

\[
\tilde{v}_{\text{sym}} = (\tilde{v}_{\text{sym}})_{\text{minimum}} + \left( (\tilde{v}_{\text{sym}})_{\text{maximum}} - (\tilde{v}_{\text{sym}})_{\text{minimum}} \right) / (1 + (\text{temperature}/T_c)^{\text{hillslope}})
\]

\(\tilde{v}_{\text{sym}}\) is the frequency of the symmetric CH2 stretching band near 2850 cm\(^{-1}\). Tc is the phase transition temperature.

\(\tilde{v}_{\text{sym}}\) was calculated by first baseline leveling the OH - CH stretching region between 3500 and 2700 cm\(^{-1}\). The wavenumber (cm\(^{-1}\)) associated with an infrared band has been historically called the frequency of the band since it is proportional and related to vibronic frequencies. The center of mass of the CH symmetric stretching band was calculated by integrating the top 10% of the intensity of the band. The baseline for integrating the top 10% of the intensity of the band was parallel to the OH-CH region baseline. The change in \(\tilde{v}_{\text{sym}}\) verses temperature was used to characterize lipid phase transitions as described previously. Since rotamers are either in trans or gauche conformations, phase transitions were fit to a two-state sigmoidal equation using Sigma plot 10 software (Systat Software, Inc. Chicago IL). Lipid order at 33.4 °C was calculated by extrapolating the \(\tilde{v}_{\text{sym}}\) at 33.4 °C and 36 °C from the fit of the phase transition and then converting \(\tilde{v}_{\text{sym}}\) to the percentage of trans rotamers, a measure of lipid conformational
order. The data for percentage of trans rotamer were used to calculate the phase-transition enthalpy and entropy from the slopes of Arrhenius plots.” As introduced earlier, meibum exists in a gel form at lower temperatures (Section 1.3.3.3.). Due to the physiological inability of meibum being a solid with 100 % trans, its gel phase allows the hydrocarbon chains to pack tightly together (Fig. 18) with about 72% trans rotamer content.89 At higher temperatures, meibum is in the liquid crystalline phase and therefore, meibum is not a liquid (0 % trans) at those temperatures. Eight parameters which can be measured from phase transitional data obtained from infrared spectra, have been defined previously.88, 127

3.3 RESULTS

3.3.1 Enrichment of CE and WE

CE and WE were separated from meibum donated by two healthy volunteers who did not show signs of dry eye or Meibomian gland occlusion. One donor, was a 66-year-old Caucasian male and the other donor, was a 29-year-old black male. Race has no statistically significant influence on the composition of meibomian samples when the donors were matched by age.216, 217 WE began to elute with 100 % hexane for both donors, after 20 min into the run. CEo began to elute 2.5 h with hexane: chloroform mixture while CEy began to elute after 3.5 h. A few fractions from the younger donor in which CE and WE were not completely separated, were run a second time by gradient elution. Fractions were initially analyzed using 1H-NMR to determine which fractions contained WE or CE (Fig. 20). Fractions with WE or CE were each pooled and analyzed more carefully (Fig. 21). 1H-NMR spectra of CE and WE enriched from meibum are
Figure 20. A) and B) $^1$H-NMR spectra of fractions from MgO column.\textsuperscript{140} a) Human meibum from the older donor prior to loading on the column. b) Wax ester fraction 1 c) Wax ester fraction 2. d) Cholesteryl ester fraction 20. e) Cholesteryl ester fraction 22. Similar fractions to 1 and 2, 20 and 22 were pooled and analyzed more carefully. See Fig. 24 and Table 5 for resonance assignments. (Source: Ewurum A, et al. Chemistry and Physics of Lipids 2021; 238:105088.)
Figure 21. $^1$H-NMR spectra of (a and b) total wax ester and (c and d) cholesteryl ester separated from meibum. A) The = CH and ester region of the $^1$H-NMR spectra. B) The CH$_3$ region of the $^1$H-NMR spectra. (a and c) Meibum from the older donor. (b and d) Meibum from the younger donor. Resonance numbering in (A) and (B) refer to resonance assignments in Fig. 17, and Table 5. (Source: Ewurum A, et al. *Chemistry and Physics of Lipids* 2021; 238:105088.)
presented in Fig. 21. Resonance assignments are presented in Table 5. Note that resonances assigned to CE (Table 5), numbers 1 and 3 in Fig. 21A and numbers 6, 7, 8 and 18 in Fig. 21B, are missing in the WE spectra (Fig. 21a and b) and present in the CE spectra (Fig. 21c and d), indicating that the WE was completely separated from the CE. From the intensity of the ester resonance number 5 (Fig. 21A c and d) relative to the resonances numbered 3, 6, 7, 8, 18 in Fig. 21, we calculate\textsuperscript{90} that the molar ratio of CEo/WEo and CEy/WEy was 2.4 and 1.4, respectively. Compared to the average molar ratio of CE/WE for individuals without dry eye of 0.49, \textsuperscript{90} CEo and CEy were enriched 3 and 5 fold, respectively. CE may even have been completely separated from WE considering 7 % of the resonance # 5 intensity in the spectra of purified CE is likely to be due to cholesteryl diesters (\textomega Type 1-SE)\textsuperscript{39} and not due to unseparated WE.

\subsection*{3.3.2 Hydrocarbon chain branching}

Hydrocarbon chain branching is shown in Table 6. The branching for the younger donor was comparable for CE and WE. The hydrocarbon chain branching for WEo compared with WEy were similar and within the range of previous studies.\textsuperscript{35, 91} The hydrocarbons of CEo contained much less anteiso and more straight chains compared with CEy. Whether this difference is within the level of normal variation is not known as branching in CE has not been measured in other studies. WEo had 16 % less straight-chains and 50 % more iso-chains compared with CEo.

\subsection*{3.3.3 Phase transitional parameters}

FTIR phase transition data (Figs. 22 and 23) were plotted against the % total CE separated relative to the % total WE separated, based on volume according to Table 4.
Table 5. Assignments for meibum $^1$H-NMR resonances as numbered in figures 20, 21, and 26. (Source: Ewurum A, et al. *Chemistry and Physics of Lipids* 2021; 238:105088.)

<table>
<thead>
<tr>
<th>Figure 20 &amp; 21 resonance #</th>
<th>Figure 26 resonance #</th>
<th>Chemical shift (ppm)</th>
<th>Proton resonance assignment. The carbon # refers to the protons on that carbon (Fig. 17b).</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>5.36</td>
<td>Cholesterol Carbon #6 (Fig 17b)</td>
</tr>
<tr>
<td>2</td>
<td>17</td>
<td>5.33</td>
<td>Hydrocarbon =CH- <em>cis</em> (Fig 17b)</td>
</tr>
<tr>
<td>3</td>
<td>18</td>
<td>4.6</td>
<td>Cholesteryl Ester Carbon #3 (Fig 17b)</td>
</tr>
<tr>
<td>4</td>
<td>19</td>
<td>3.9</td>
<td>Wax Ester -CH$_2$-O-(C=O)-</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>3.9</td>
<td>Cholesteryl diester -CH$_2$-O-(C=O)-</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>1.00 to 0.996</td>
<td>Cholesterol Carbon #19 (Fig 17b)</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>0.906</td>
<td>Cholesterol Carbon #21 (Fig 17b)</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>0.897</td>
<td>Cholesterol Carbon #21 (Fig 17b)</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
<td>0.878</td>
<td>Straight-chain</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>0.868</td>
<td>Straight-chain</td>
</tr>
<tr>
<td>11</td>
<td>6</td>
<td>0.858</td>
<td>Straight-chain</td>
</tr>
<tr>
<td>12</td>
<td>7</td>
<td>0.853</td>
<td>Iso-branched</td>
</tr>
<tr>
<td>13</td>
<td>8</td>
<td>0.850</td>
<td>Anteiso-branched</td>
</tr>
<tr>
<td>14</td>
<td>9</td>
<td>0.843</td>
<td>Iso-branched</td>
</tr>
<tr>
<td>15</td>
<td>10</td>
<td>0.839</td>
<td>Anteiso-branched</td>
</tr>
<tr>
<td>16</td>
<td>11</td>
<td>0.829</td>
<td>Anteiso-branched</td>
</tr>
<tr>
<td>17</td>
<td>12</td>
<td>0.821</td>
<td>Anteiso-branched</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>0.799</td>
<td>Not assigned</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>0.789</td>
<td>Not assigned</td>
</tr>
<tr>
<td>18</td>
<td>15</td>
<td>0.663</td>
<td>Cholesterol Carbon #18 (Fig 17b)</td>
</tr>
</tbody>
</table>
Table 6. Hydrocarbon chain branching for donors’ CE and WE purified from meibum.


<table>
<thead>
<tr>
<th></th>
<th>Anteiso branched (%)</th>
<th>Straight chain (%)</th>
<th>Iso branched (%)</th>
<th>Branched (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wax esters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Older donor</td>
<td>8</td>
<td>74</td>
<td>18</td>
<td>26</td>
</tr>
<tr>
<td>Younger donor</td>
<td>18</td>
<td>79</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>Average</td>
<td>13</td>
<td>76</td>
<td>11</td>
<td>24</td>
</tr>
<tr>
<td>Citation$^{50}$</td>
<td>11</td>
<td>83</td>
<td>5</td>
<td>16</td>
</tr>
<tr>
<td>Citation$^{35}$</td>
<td>13</td>
<td>78</td>
<td>9</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cholesteryl esters and di esters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Older donor</td>
<td>3</td>
<td>88</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>Younger donor</td>
<td>15</td>
<td>79</td>
<td>6</td>
<td>21</td>
</tr>
</tbody>
</table>
For example, a value of 20 % in Table 4 represents 20 μL of the total amount of CE separated mixed with 80 μL of the total amount of WE separated. To convert the volume % values in Table 4 to mole % CE, the intensity of resonances at 4, 1 and 0.65 ppm were used as described previously. The standard curves were linear, r = 0.992, 0.985 and slopes of 0.528 and 0.311 were calculated for Fig. 24A and B, respectively. All of the phase transition parameters for the WEy and CEy were higher, P < 0.05, compared with WEO and CEO (Table 7) indicating that WEy and CEy was more ordered compared with the same samples from older donor. The phase transition temperature, order, and maximum frequency of the phase transition of CEO were lower compared with the WEO (Table 7) indicating CEO was more fluid compared with WEO. Conversely, and serendipitously, the phase transition temperature, order, and maximum frequency of the phase transition of CEy was higher compared with the WEy (Table 7). So, by mixing the WE and CE esters from each sample, we could determine how the structure of a WE that was more ordered (stiffer) was influenced by a less ordered CE and vice versa.

Adding 25 % more ordered WEO (compared with CEO) to CEO, significantly, P < 0.05, decreased the hydrocarbon chain order and phase transition temperature (Fig. 22, Table 8). These changes indicate that addition of 25 % WEO to CEO caused a structural rearrangement of molecules leading to a more disordered system. At higher temperatures, when the lipids are maximally disordered, adding 25 % WEO to CEO increased the maximum frequency from 2853.6 ± 0.2 cm\(^{-1}\) to 2854.25 ± 0.06 cm\(^{-1}\) (Table 8), indicating that when WEO is added to CEO, the resulting mixture becomes even more disordered. Increasing amounts of less ordered CEO (compared with WEO) added to more ordered WEO caused the hydrocarbon chain order and phase transition temperature to decrease linearly,
**Table 7.** Phase transition parameters for purified wax and cholesteryl esters from infrared spectroscopy. (Source: Ewurum A, et al. *Chemistry and Physics of Lipids* 2021; 238:105088.)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WEO</th>
<th>CEO</th>
<th>P, CEO vs WEO</th>
<th>WEY</th>
<th>CEY</th>
<th>P, CEY vs WEO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transition temperature (°C)</td>
<td>32.3 ± 0.4</td>
<td>26.4 ± 0.8</td>
<td>&gt;0.0001</td>
<td>33.9 ± 0.3</td>
<td>37.1 ± 0.3</td>
<td>&gt;0.0001</td>
</tr>
<tr>
<td>Cooperativity (hill coefficient)</td>
<td>5.8 ± 0.3</td>
<td>5.7 ± 0.9</td>
<td>&gt;0.5</td>
<td>8.9 ± 0.6</td>
<td>7.3 ± 0.4</td>
<td>0.037</td>
</tr>
<tr>
<td>Order 36.0°C (% trans)</td>
<td>38.0 ± 0.7</td>
<td>32.7 ± 2</td>
<td>0.02</td>
<td>43.2 ± 0.9</td>
<td>57.8 ± 1.2</td>
<td>&gt;0.0001</td>
</tr>
<tr>
<td>Order 33.4°C (% trans)</td>
<td>44.5 ± 0.7</td>
<td>36.3 ± 2.5</td>
<td>0.005</td>
<td>54.2 ± 0.9</td>
<td>67.8 ± 1.2</td>
<td>&gt;0.0001</td>
</tr>
<tr>
<td>Δ enthalpy (kcal/mol)</td>
<td>135 ± 5</td>
<td>122 ± 5</td>
<td>&gt;0.5</td>
<td>186 ± 7</td>
<td>172 ± 8</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>Δ entropy (kcal/mol/degree)</td>
<td>0.44 ± 0.02</td>
<td>0.41 ± 0.02</td>
<td>&gt;0.5</td>
<td>0.61 ± 0.02</td>
<td>0.56 ± 0.03</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>Minimum frequency (cm⁻¹)</td>
<td>2849.54 ± 0.05</td>
<td>2849.2 ± 0.2</td>
<td>&gt;0.5</td>
<td>2848.84 ± 0.06</td>
<td>2848.67 ± 0.05</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>Maximum frequency (cm⁻¹)</td>
<td>2854.19 ± 0.08</td>
<td>2853.6 ± 0.2</td>
<td>0.012</td>
<td>2853.88 ± 0.09</td>
<td>2854.34 ± 0.06</td>
<td>0.0003</td>
</tr>
</tbody>
</table>
excluding 100 % CEo (Fig. 22, Table 9). It is interesting that when the lipids are at low temperatures and maximally ordered, adding less ordered CEo to more ordered WEo causes the minimum frequency to decrease linearly, indicating that the mixture becomes even more ordered with the addition of CEo.

Adding 25 % less ordered WEy (compared with CEy) to CEy, significantly, $P < 0.05$, decreased the hydrocarbon chain order and phase transition temperature similar to adding WEo to CEo above (Fig. 23, Table 8). These changes indicate that like the addition of WEo to CEo, the addition of 25 % WEy to CEy caused a structural rearrangement of molecules leading to a less ordered system. At higher temperatures, when the lipids are maximally disordered, adding 25 % WEo to CEo decreased the maximum frequency slightly but significantly from $2854.34 \pm 0.06 \text{ cm}^{-1}$ to $2854.0 \pm 0.1 \text{ cm}^{-1}$ (Table 8), indicating that when WEo is added to CEo, the resulting mixture becomes slightly more ordered. CEy added to less ordered WEy (compared with CEy) caused the hydrocarbon chain order and phase transition temperature to increase linearly above 20 % CEy, while causing a linear decrease in the $\Delta S$ and $\Delta H$ (Fig. 23, Table 9).

3.4. DISCUSSION

3.4.1. Enrichment of WE and CE from human meibum

The answer to the question addressed in aim 1 is yes: WE and CE could be completely separated. Greater separation of CE from WE could always be made if necessary, by a second passage of select fractions. We used a slurry of MgO and chloroform to prepare a column for the separation of human meibum WE and CE based
Figure. 22. Phase transition parameters for meibum from older donor. X axis data are from mixtures listed in Table 4, where a value of 20 % represents 20 μL of the total amount of CE separated mixed with 80 μL of the total amount of WE separated. The molar ratio of CE/WE is listed in Table 4. Phase transition parameters measured: A) Phase transition temperature, B) Lipid order at 33.4 °C, the temperature of the tear film surface, C) Lipid order at 36 °C, the temperature of the meibomian glands, D) Minimum frequency. Error bars are calculated from standard error of the mean. Mean for each point was averaged from ~14 phase transition measurements. Displayed p – values were calculated from two data points. (Source: Ewurum A, et al. Chemistry and Physics of Lipids 2021; 238:105088.)
Figure 23: Phase transition parameters for meibum from younger donors. X-axis data are from mixtures listed in Table 4, where a value of 20% represents 20 μL of the total amount of CE separated mixed with 80 μL of the total amount of WE separated. The molar ratio of CE/WE is listed in Table 4. A) Phase transition temperature, B) Lipid order at 33.4°C, the temperature of the tear film surface, C) Lipid order at 36°C, the temperature of the meibomian glands, D) Maximum frequency, E) Change in the phase transition enthalpy, F) Change in the phase transition entropy. Error bars are calculated from standard error of the mean for each point. Displayed p-values were calculated from two data points. Error bars are calculated from standard error of the mean for each point. Mean for each point was averaged from ~14 phase transition measurements. Error bars are calculated from standard error: Mean for each point was averaged from ~14 phase transition measurements. Values were calculated from two data points. (Source: Ewurum A, et al. Chemistry and Physics of Lipids 2021;238:105088.)
on previous studies that used a slurry of MgO and water to make thin layer chromatography plates.\textsuperscript{218,219} Our study was unique because we used column chromatography rather than thin layer chromatography. A seminal study of human meibum used this separation technique.\textsuperscript{35} The mixing of MgO with water chemically yields MgOH. Hence, another tenable optimization approach may be to use MgOH instead of MgO, which creates a less porous slurry, possibly facilitating better separation. Like MgO, thin layer chromatography plates of MgOH efficiently separate WE and CE.\textsuperscript{220} Urea adducts of CE and WE improved the separation of CE and WE for rabbit meibum, but could not be used in this study because the adduct could change the physical properties of the esters.\textsuperscript{221} Other oxides and/or hydroxides, besides MgO or MgOH have yet to be explored for the separation of CE and WE. We used a 13 cm high column slurry length which efficiently separated CE and WE, but it is conceivable that using a shorter column may reduce total chromatography time whilst achieving same separation resolution. The significant disparity between the elution times of CEO and CEy was most likely influenced by solvents composition, as more hexane, which is a slower eluent compared to chloroform, was used for our separation of younger donor meibum esters compared to the older donor. This allowed enough time for lipid – stationary phase interactions, facilitating better separation, before introducing 100 % chloroform. The latter separation of older donor meibum esters involved a more fine-tuned protocol facilitated by more solvent gradient, leading to a faster and smoother separation. Gradient elution therefore, gave the optimum separation. $^1$H-NMR which was used to detect CE
Figure 24. Standard curves used to convert CE/WE from total yields to moles. A) Cholesteryl ester (CE). Y axis is listed in Table 4. (Source: Ewurum A, et al. Chemistry and Physics of Lipids 2021; 238:105088)
and WE in the current study is advantageous over rhodamine 6G and UV visualization used previously\textsuperscript{35,218} as NMR spectroscopy does not alter the sample whereas rhodamine could.

3.4.2. Question addressed in aim 2. Does the complexity of the hydrocarbon chains found in human meibum WE and CE influence how changes in the ratio of WE and CE influence hydrocarbon chain conformation?

Changes in the conformation and phase characteristics of CE and WE lipid structure may be grouped into three categories: i) the change when pure smectic CE (Fig. 25f) is mixed with WE; ii) When pure lamellar WE (Fig. 25e) is mixed with CE and iii) when the concentration of CE is varied in a mixture of WE and CE (Fig. 25g). In each category, we considered the situations when CE is more ordered or less ordered than WE. The major finding of the current study is that CE enriched native CE can influence the conformation of native enriched WE, depending on whether the CE is more ordered or less ordered than WE. Many of the infrared measured parameters in Table 7 were different between WEo and WEy and between CEo and CEy. It is uncertain whether these differences are within the normal variation because infrared spectroscopic studies of purified WE and CE have never been performed. Future infrared spectroscopic studies involving the relationships between purified WE and CE and age, race, sex and dry eye types are needed to bridge this gap in knowledge.

3.4.2.1. Conformational changes when pure smectic CE is mixed with WE.

X-ray crystallography of CE shows that at lower temperatures, CE is in the smectic phase (Fig. 25f), with their hydrocarbons, steroid nuclei, terminal methyl and ester carbonyl moieties adjacent to one another with their side chains interdigitated.\textsuperscript{222} An
understanding of what happens to hydrocarbon chain conformation when pure smetic CE is mixed with WE provides insight into aim 3, ‘why is WE and CE often present together and how does WE influence CE hydrocarbon chain conformation, and vice versa?”.

3.4.2.1.1. Phase transition temperature and hydrocarbon chain order

More ordered WEo or less ordered WEy compared with CE, added to CE, decreases the phase transition temperature and hydrocarbon chain order of pure CE. This result is in agreement with model studies in which less ordered (compared with CE) stearyl palmitate or oleyl oleate, was added to cholesteryl behenate.42, 149

3.4.2.1.1.1. Potential biological implications of changes in the phase transition temperature and hydrocarbon chain order

We theorize that hydrocarbon chain order results in increased cohesion between the lipid acyl chains and decreases the spreading of the lipids. Decreased spreading could impact the coating of lipid on the surface of plants and on the exoskeleton of insects. More ordered lipid could decrease the spreading of the TFLL, which in turn may decrease tear film stability.89, 134 As a result, less ordered lipid, like WEo, could be necessary in the TFLL to disrupt the ordered packing of a hypothetical TFLL composed of only CE. Since the trends in the above results are similar for model and native CE, the complexity of the hydrocarbon chains are likely not to be a major influence on the order or phase transition temperature change when WE is added to pure CE.

3.4.2.1.2. Maximum and minimum frequencies of the phase transition.

In terms of tear film lipids, it is somewhat inconsequential to discuss how the addition of WE to CE influences the maximum and minimum frequencies of the phase transitions since these two parameters do not change in meibum from donors with or
without meibomian gland dysfunction, \( P = 0.09 \) and 0.7, respectively.\textsuperscript{117} They do not change despite a large decrease in the CE/WE ratio of meibum from donors with MGD.\textsuperscript{42} However, changes in the minimum and maximum frequencies of the phase transition when WE is added to CE could be important to WE/CE mixtures present in sebum, the cuticle of plants and the exoskeleton coating of insects.

Adding WE that is less ordered compared with CE decreased the maximum frequency of the phase transition. Thus, when the hydrocarbon chains of pure CE are fluid at higher temperatures, the addition of WE causes the packing of the resulting WE/CE mixture to arrange with more disordered hydrocarbon chains. No change in the maximum frequency was observed when adding less ordered (compared with cholesteryl behenate) oleyl oleate or stearyl palmitate was added to cholesteryl behenate.\textsuperscript{42,149} This is evidence that the hydrocarbon chain branching or chain length caused the difference observed between the model and the native WE and CE moieties. Further evidence that the hydrocarbon chain composition influences the hydrocarbon chain conformation of native WE and CE comes from the observation that when WE is more ordered than CE, the maximum frequency increases, opposite of what occurs when WE is less ordered than CE. No model studies have been performed with more ordered WE added to less ordered CE, so no comparisons can be made. We conclude that how the conformation of pure smetic CE in the maximally disordered state changes with the addition of WE depends on whether the WE hydrocarbon chains are more or less ordered than CE and does not depend on merely on the ratio of CE to WE.
Table 8. *P*-values from Student’s t test for data from figures 22 and 23. (Source: Ewurum A, et al. *Chemistry and Physics of Lipids* 2021; 238:105088.)

<table>
<thead>
<tr>
<th>Phase transitional parameter</th>
<th>Samples from (fig. 22)</th>
<th>Samples from (fig. 23)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase transition temperature</td>
<td>&lt;0.0001</td>
<td>0.012</td>
</tr>
<tr>
<td>Order at 33.4°C</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Order at 36°C</td>
<td>&lt;0.0001</td>
<td>0.0015</td>
</tr>
<tr>
<td>Minimum frequency</td>
<td>0.019</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Maximum frequency</td>
<td>&gt;0.05</td>
<td>0.006</td>
</tr>
<tr>
<td>Change in enthalpy (ΔH)</td>
<td>&gt;0.05</td>
<td>0.07</td>
</tr>
<tr>
<td>Change in entropy (ΔS)</td>
<td>&gt;0.05</td>
<td>0.06</td>
</tr>
</tbody>
</table>

CE = cholesteryl ester. Only phase transition parameters with a *P* < 0.05 are significant unless otherwise indicated.
Table 9. Linear regression analyses for figures 22 and Fig. 23. (Source: Ewurum A, et al. *Chemistry and Physics of Lipids* 2021; 238:105088.)

<table>
<thead>
<tr>
<th>Phase transition parameter</th>
<th>Correlation coefficient (r)</th>
<th>( P )</th>
<th>Points examined (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesteryl and wax esters from the older donor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transition temperature</td>
<td>0.947</td>
<td>&lt; 0.01</td>
<td>0 to 75</td>
</tr>
<tr>
<td>Order at 33.4 °C</td>
<td>0.815</td>
<td>&lt; 0.05</td>
<td>0 to 75</td>
</tr>
<tr>
<td>Order at 36 °C</td>
<td>0.780</td>
<td>&lt; 0.05</td>
<td>0 to 75</td>
</tr>
<tr>
<td>Minimum frequency</td>
<td>0.911</td>
<td>&lt; 0.01</td>
<td>20 to 100</td>
</tr>
<tr>
<td>Cholesteryl and wax esters from the younger donor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transition temperature</td>
<td>0.910</td>
<td>&lt; 0.02</td>
<td>30 to 100</td>
</tr>
<tr>
<td>Order at 33.4 °C</td>
<td>0.765</td>
<td>&lt; 0.05</td>
<td>30 to 100</td>
</tr>
<tr>
<td>Order at 36 °C</td>
<td>0.872</td>
<td>&lt; 0.05</td>
<td>30 to 100</td>
</tr>
<tr>
<td>Maximum frequency</td>
<td>0.991</td>
<td>&lt; 0.01</td>
<td>40 to 100</td>
</tr>
<tr>
<td>Change in enthalpy (( \Delta H ))</td>
<td>0.866</td>
<td>&lt; 0.02</td>
<td>0 to 75</td>
</tr>
<tr>
<td>Change in entropy (( \Delta S ))</td>
<td>0.884</td>
<td>&lt; 0.01</td>
<td>0 to 75</td>
</tr>
</tbody>
</table>
It is interesting that when the lipids are at low temperatures and maximally ordered, adding more ordered WEo to less ordered CEo causes the minimum frequency to decrease linearly, indicating that the mixture becomes even more ordered with the addition of WEo. The opposite trend was observed for the model system where oleyl oleate added to cholesteryl behenate caused the minimum frequency of the mixture to increase (order to decrease). Since the trends for model and native systems are different in terms of the minimum frequency of the phase transition, the complexity of the native CE and WE acyl chains are likely to be a major influence on the minimum frequency of the phase transition when WE is added to pure CE.

3.4.2.2. Conformational changes when pure lamellar WE is mixed with CE

A more fluid CE compared with WE decreased the phase transition temperature, order and minimum frequency of WE. Adding more fluid CE compared with WE, to pure lamellar-packed WE had a profound effect on the phase transition parameters, whereas adding more ordered CE compared with WE, to purified WE had little effect. Based on our results, compared with a more ordered CE (relative to WE), a more fluid CE would be expected to have a more profound effect on the hydrocarbon chain conformation of tear film lipid, sebum, the cuticle of plants and the exoskeleton coating of insects composed only of WE.

3.4.2.2.1. Potential biological implications of the conformational changes when pure lamellar WE is mixed with CE

As a tear film lipid layer with more fluid meibum lipid hydrocarbon chains is expected to be more stable, a more fluid CE (compared with WE) added to WE would likely be expected to stabilize the tear film lipid layer compared with a hypothetical tear film lipid layer composed of just WE alone.
**Figure. 25** Simulated pictograms of meibum lipid packing. With kind permission from Borchman D. *Ocul Surf* 2019;17:327-335): a) cholesteryl ester, b) cholesteryl diester, c) wax ester and d) wax diester. CE and WE in a-d are drawn assuming a hydrocarbon chain length of 22 carbons. e) Lamellar packing of pure WE from X-ray crystallography representative of 0 % CE in Fig 20, Fig. 21. (E, top) shows rhombic packing of the hydrocarbon chains. f) Smectic phase packing of CE representative of 100 % CE in Fig 20, Fig. 21. from X-ray crystallography. g) Speculative packing of a WE, CE and phospholipid mixture on an aqueous surface, representative of 20 to 75 % CE in Fig 22, Fig. 23
3.4.2.3. Conformational changes when the concentration of CE changes in a mixture of WE and CE.

Increasing amounts of less ordered CEo (compared with WEo) added to more ordered WEo caused the hydrocarbon chain order at 33.4°C, phase transition temperature, and minimum frequency to decrease linearly. This result is in agreement with model studies in which stearyl palmitate and oleyl oleate were added to cholesteryl behenate. Since the trends in the results are similar for model and native CE in this instance, differences in the complexity of the hydrocarbon chains are likely not to be a major influence on changes in the order or phase transition temperature when more ordered WE is added to less ordered CE. An opposite trend occurred when more ordered CEy (compared with WEy) was added in increments to less ordered WEy. Changes in CE content of WE/CE mixtures undoubtedly changes the hydrocarbon chain conformation and packing of the mixture. The nature of the change depends on the conformation of the WE and CE.

3.4.3. Hydrocarbon chain branching, chain length and saturation of purified WE and CE

Although lipid hydrocarbon chain saturation is a major factor in ‘setting’ the general order of lipids, there is no evidence that hydrocarbon chain saturation changes with MGD. Raman, NMR, and an infrared spectroscopic study all show there is no difference in saturation for meibum from donors with MGD or without MGD. However, hydrocarbon chain saturation may play a role in ordering meibum and tear lipids from patients who had dry eye due to hematopoietic stem cell transplants. To date, there is still no evidence that the hydrocarbon chain length for CE or WE is significantly different for meibum from donors with or without dry eye. The average hydrocarbon chain
branching of WE measured in this study was similar to that measured in other studies.\textsuperscript{35, 91} CEo had significantly less branched chains compared with WEo.

3.4.3.1. Potential biological implications of hydrocarbon chain branching, chain length, and saturation of purified WE and CE

Hydrocarbon chain branching would be expected to contribute to more disordered hydrocarbon chains and could account for why CEo was more disordered than CEy, WEo and WEy.\textsuperscript{222} Hydrocarbon chain branching is unlikely to play a role in ordering meibum from donors with MGD as their meibum contained 14 \% less straight chains compared with meibum from donors without MGD. This would disorder meibum not order it, as observed.

3.5. Conclusions and Implications

i. WE could be separated from CE using MgO column chromatography.

ii. Changes in CE content of WE/CE mixtures undoubtedly modifies the hydrocarbon chain conformation and packing of the mixture. The nature of the effect depends on the conformation of the WE and CE. Differences in the complexity of the hydrocarbon chains are likely not to be a major influence on changes in the order or phase transition temperature when more ordered WE is added to less ordered CE.

iii. The major potential biological implications of our findings is that WE/CE mixtures could be advantageous in nature rather than WE or CE alone because a WE-CE mixture rather than CE alone, could be necessary in the tear TFLL to disrupt the ordered packing of pure CE resulting in better spreading of the lipids and thus to a more stable tear film. WE may also be a necessary component of
sebum, the cuticle of plants and the exoskeleton coating of insects which would be expected to fluidize pure CE allowing the mixture to spread.
CHAPTER IV
A SPECTROSCOPIC APPROACH TO MEASURING MEIBUM LIPID COMPOSITION AND CONFORMATION IN DONORS WITH SJÖGREN’S SYNDROME

4.1 INTRODUCTION

Sjögren's syndrome (SS) is a chronic autoimmune rheumatic disease. It is named after Dr. Henrik Sjögren, who in 1933 described the syndrome.\textsuperscript{229} As many as 4\% of the British population\textsuperscript{230} and higher than 3 million individuals in the United States have SS.\textsuperscript{231, 232} SS involves the lymphocytic infiltration of exocrine glands concomitant with the production of autoantibodies in the blood. There are two main types of Sjögren's syndrome: Primary and Secondary. Primary SS usually occur as a stand-alone case and may involve the collusion of another gland malfunction.\textsuperscript{233} Secondary SS symptoms occurs concomitantly with one or more unambiguous autoimmune disease.\textsuperscript{233} SS affects predominately women (9:1) and has a peak manifestation between 40 and 55 years of age.\textsuperscript{234} Therapies for alleviating the symptoms of SS can include topical application of artificial tear fluid, targeting of appropriate receptors to stimulate secretion of aqueous glandular fluid, inflammation reduction, and management of immune response.\textsuperscript{233} In spite of all these, there is currently no sole regimen for completely curing or ameliorating SS. The clinical aspects of SS have been reviewed.\textsuperscript{235} Relevant to the current study, about 5 – 35\% of people with SS have keratoconjunctivitis sicca (dry eye) and/or dry mouth.\textsuperscript{236, 237}
which may be accompanied by fatigue and musculoskeletal pain. So, ophthalmologists are often the first to diagnose SS. Tear film lipid, the major source of which is from the meibomian glands in the eye lid is important to tear film stability. As tear film lipid composition and structure are related to tear film stability and dry eye, and since tear lipid composition has not been characterized in people with SS, we bridge this gap in knowledge in this study using $^1$H-NMR and infrared spectroscopy.

4.2. MATERIALS AND METHODS

Written informed consent was obtained from all donors. The University of Louisville Institutional Review Board reviewed and approved (#11.0319, August 2016) all protocols and procedures. All procedures were in accord with the Declaration of Helsinki. Written informed consent was obtained from all participants. Meibum from nine donors diagnosed with SS were included in the study. All of the donors were female as SS has a female predominance with a high female to male ratio (9:1). All of the SS patients had moderate to severe dry eye. Meibum was expressed from four eye lids using an ILUX instrument (Alcon, Fort Worth, TX) according to the manufacturer’s instructions. The ILUX instrument, warms the eye lid for about 90 s and applies gentle pressure on the eye lid to express the visible meibum magnified by ILUX (Fig. 21). Approximately 0.5 mg of meibum lipid was collected per individual for direct spectroscopic study. The expressate was collected with a platinum spatula and immediately dissolved into 0.5 mL of CDCl$_3$ in a 9-mm glass micro vial with a Teflon cap (Microliter Analytical Supplies Ind., Suwanee, GA). Argon gas was blown over the samples to prevent oxidation. The sample in the vial was capped and frozen under argon gas until analysis. Analyses were performed within 3 weeks of collection of the sample.
The samples never were exposed to any plastic to avoid plasticizers. Control CDCl₃ spectra were measured with the meibum samples to ensure no impurities were present. Spectral data were acquired using a Varian VNMRS 700 MHz NMR spectrometer (Varian, Lexington, MA) as described in section 3.2.5. After NMR analysis, meibum was layered onto AgCl windows and lipid phase transitions were measured as described previously.⁹¹⁷ “Curves were fit using Sigma plot 10 software (Systat Software, Inc, Chicago, IL), and the confidence levels were obtained from a critical value table of the Pearson product–moment correlation coefficient. Broad phase transitions have a relatively smaller absolute value of the cooperativity. Lipid order was calculated at 33.4°C, the temperature at the surface of the eye, and at 36°C, the temperature of the eyelid”.⁹³ The ocular surface temperature of people with SS was 0.71°C lower compared with people without SS or dry eye.⁹³ Hydrocarbon chain branching and CE/WE ratios were calculated as described previously.⁹⁴,⁹⁵

Data are reported as the mean ± the standard error. Averages were performed using the Student’s t test. A value of $P < 0.05$ was considered statistically significant.

4.3. RESULTS

All of the donors were female as SS has a female predominance with a high female to male ratio (9:1). The age of the Caucasian donors were 42, 42, 63, 71, and 71 years-old. The ages of the two Black donors and one Hispanic donor were 41, 44 and 36 years-old, respectively. The major resonances were well resolved in the ¹H-NMR spectra of the meibum samples (Fig. 26). Resonances for =CH cis, CE and WE were evident in the 4–5.5 ppm region (Fig. 26a, sec 4.3. p115, Table 5). Resonances for cholesterol/CE,
hydrocarbon chain branching and \(^=\text{CCH}_2\) were evident in the 0.6 - 1.2 ppm region (Fig. 26b, Table 5). Meibum from every donor with SS measured contained a significantly (P < 0.01) higher cholesteryl ester/wax ester ratio and more straight chains compared with donors without SS (Fig. 27, sec 4.3. p102, Table 10). The CH stretching region of the infrared spectra were typical of meibum (Fig. 28A). A plot of \(\tilde{\nu}_{\text{sym}}\) verses temperature (Fig. 28B) was used to follow the phase transitions of meibum from an ordered (stiffer) gel phase to a disordered liquid crystalline phase. None of the phase transitional parameters for Mn were significantly different, \(P > 0.05\) for Mss (Table 11). The CH\(_3\)/CH\(_2\) band height ratio may be used to estimate the number of hydrocarbon CH\(_3\) and CH\(_2\) moieties.\(^{150}\) The ratio was not significantly different, \(P = 0.22\) for Mss compared with Mn (Table 11). The spectra for the ratio calculation were taken at an average temperature of 11.8 ± 0.5 °C when the lipid was maximally ordered.

4.4 DISCUSSION

The major findings of the current study are that meibum from every donor with SS measured contained a significantly higher cholesteryl ester/wax ester ratio and more straight chains compared with donors without SS or dry eye. While the influence of cholesteryl ester on meibum function is currently unknown, its effect on meibum structure depends on the hydrocarbon chain composition of the ester.\(^{140}\) Some insights can be drawn from model studies.\(^{42, 149}\) Model studies using the wax oleyloleate\(^{42}\) or sterylpalmitate\(^{149}\) and cholesteryl behenate show that increasing cholesteryl behenate causes the mixture of esters to become more ordered. The application of the model studies’ results to human meibum, again, should be made cautiously as the cholesteryl
Figure 26. $^1$H-NMR spectra of a 42-year-old caucasian female with Sjögren’s syndrome. Numbers indicate the resonance assignments in Table 5. A) The $\equiv$CH and ester region. B) CH$_3$ region. (Source: Ewurum A, Veligandla SR, Swindle JS, Clark JD, Borchman D. A spectroscopic approach to measuring meibum lipid composition and conformation in donors with Sjögren's syndrome. Experimental eye research 2021;210:108713.)
**Figure 27.** Cholesteryl ester/wax ester molar ratios calculated from the nuclear magnetic resonance spectra of meibum. (solid bar) Molar ratios calculated from the intensity of the cholesteryl ester resonance at 4.6 ppm and the wax ester resonance at 4.0 ppm. (open bar) Molar ratios calculated from the intensity of the cholesteryl ester resonances from cholesteryl carbon numbers 18 and 19 and the wax ester resonance at 4.0 ppm. (gray bar) Molar ratios calculated from infrared spectra. MGD is meibomian gland dysfunction previously published (N = 36).\(^\text{149}\) Normal data previously published (N = 27).\(^\text{149}\) Error bars are calculated from standard error of the mean. Sjögren’s syndrome bars (N=8) are from the current study and are significantly higher, P < 0.01, compared with normal and MGD bars (Table 10).\(^\text{239}\) (Source: Ewurum A, et al. *Experimental eye research* 2021;210:108713.)

| Parameter | Meas (n=5) | % Intragen (µ mole/mg) | % Intragen (µ mole/mg) | % Intragen (µ mole/mg) |
|-----------|------------|------------------------|------------------------|------------------------|------------------------|------------------------|
| p         | 0.0805     | 0.96 ± 0.05            | 0.51 ± 0.02            | (31)                   | 66 ± 5                 | 37 ± 1 (65)            | 20 ± 1 (65)            | 23 ± 1 (65)            | 0.4 ± 4                | Iso Branched (%)      |
|           |            |                        |                        |                        |                        |                        |                        |                        |                        |                        |
|           |            |                        |                        |                        |                        |                        |                        |                        |                        |                        |
|           |            |                        |                        |                        |                        |                        |                        |                        |                        |                        |
|           |            |                        |                        |                        |                        |                        |                        |                        |                        |                        |

*Statistically significant from reference 89. Calculated from: (16 +17)/(4+(1+15)/3) where the numbers are the intensities of the resonances numbered in Table 10 except for 3 which is a constant.

a From reference 89. Calculated from: (16+17)/(4+(1+15)/3) where the numbers are the intensities of the resonances numbered in Table 10 except for 3 which is a constant.

b From reference 78.
esters found in human meibum are very long, branched and somewhat unsaturated. Thus, it is almost impossible to duplicate the complex milieu of lipids found in human meibum using model synthetic lipids. Furthermore, we found that the hydrocarbon chain conformation and phase transitional parameters of Mss are not different from Mn despite Mss containing a higher cholesteryl ester/wax ester ratio compared with Mn. Perhaps a higher cholesteryl ester/wax ester ratio causes packing changes that are not reflected in the hydrocarbon chain conformation. Whether a higher cholesteryl ester/wax ester ratio is a marker for or contributes to dry eye related to SS is unclear. Other factors, discussed below could contribute to decreased tear film function related to SS. Previous studies have shown that compared with meibum from donors without dry eye, meibum from donors with MGD contained less, 50 ± 2% straight chains. The larger amount of straight chains would be expected to increase meibum lipid order (rigidity). Meibum hydrocarbon chains that have more conformational order could inhibit the expression of meibum from the meibomian glands, causing an abnormally increased cohesion between the lipid molecules, and inducing formation of abnormally rigid lipid aggregates with limited or heterogeneous spreading at the air/tear surface. The latter is among the major features of the lipid layer in MGD and may result in heterogeneous tear lipid layer with impaired reorganization at blink and limited dilatational elasticity. In the current study, lipid order was calculated at 33.4°C, the temperature at the surface of the eye, and at 36°C, the temperature of the eyelid. The ocular surface temperature is dynamic, ranging from 34 to 35°C, and decreasing over time after blinking at a rate of ~ -0.01 °C/s in healthy eyes.
Figure 28. A) A typical infrared spectrum of meibum from a 36 year-old female hispanic donor with Sjögren’s syndrome. B) A typical phase transition of meibum from a 71 year-old female caucasian donor with Sjögren’s syndrome. Higher values of the CH₂ symmetric stretching frequency indicates a more disordered hydrocarbon chain. Eight phase transition parameters were obtained from the sigmoidal curve fit of the data (○). (Source: Ewurum A, et al. A spectroscopic approach to measuring meibum lipid composition and conformation in donors with Sjögren's syndrome. Experimental Eye research 2021;210:108713.)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mn(^a)</th>
<th>Mss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transition temperature (°C)</td>
<td>30.3 ± 0.4</td>
<td>30.1 ± 0.8</td>
</tr>
<tr>
<td>Cooperativity (hill coefficient)</td>
<td>7.9 ± 0.4</td>
<td>78.2 ± 0.4</td>
</tr>
<tr>
<td>Order 36°C (% trans)</td>
<td>35 ± 1</td>
<td>36 ± 2</td>
</tr>
<tr>
<td>Order 33.4°C (% trans)</td>
<td>40 ± 1</td>
<td>41 ± 2</td>
</tr>
<tr>
<td>(\Delta) enthalpy (kcal/mol)</td>
<td>142 ± 6</td>
<td>144 ± 5</td>
</tr>
<tr>
<td>(\Delta) entropy (kcal.mol/degree)</td>
<td>0.48 ± 0.02</td>
<td>0.48 ± 0.01</td>
</tr>
<tr>
<td>Magnitude (cm(^{-1}))</td>
<td>4.0 ± 0.1</td>
<td>4.0 ± 0.1</td>
</tr>
<tr>
<td>Minimum frequency (cm(^{-1}))</td>
<td>2849.71 ± 0.06</td>
<td>2849.5 ± 0.1</td>
</tr>
<tr>
<td>Maximum frequency (cm(^{-1}))</td>
<td>2853.69 ± 0.09</td>
<td>2853.3 ± 0.1</td>
</tr>
<tr>
<td>CH(_3)/CH(_2) height ratio from FTIR; a measure of hydrocarbon chain branching</td>
<td>0.46 ± 0.01 (45)(^c)</td>
<td>0.43 ± 0.01</td>
</tr>
<tr>
<td>N</td>
<td>35</td>
<td>9</td>
</tr>
</tbody>
</table>

Meibum from donors with Sjögren’s syndrome (Mss) and dry eye, and meibum from donors without Sjögren’s syndrome or dry eye disease (Mn).

\(^a\) Phase transitional parameters from reference 117. Meibum from donors with Sjögren’s (Mss) and dry eye, and meibum from donors without Sjögren’s syndrome or dry eye disease (Mn).

There was no significant difference \((P > 0.05)\) between the values for Mn and Mss. \(^c\) From reference 93 where (45) is the number of samples.
The ocular surface temperature of people with SS was 0.71°C lower compared with people without SS or dry eye.\textsuperscript{132} The ocular surface temperature also varies with the ambient temperature.\textsuperscript{242} It is interesting that at cooler and ambient surface temperatures that are expected to increase tear film lipid order,\textsuperscript{117} tear breakup time decreases and tear film lipid layer thickness increases,\textsuperscript{241} strengthening the negative correlation between lipid order and tear breakup time.\textsuperscript{89}

MGD is prevalent in patients with SS\textsuperscript{243-248} as many patients with SS present with destroyed meibomian glands.\textsuperscript{246, 248} However, our data show that meibum lipid conformational order is not a biomarker for, or contributes to SS as it may be for age, MGD and dry eye due to hematopoietic stem cell transplantation\textsuperscript{128} or Parkinson’s disease.\textsuperscript{240} This result is not unusual as many biomarkers for patients with dry eye are different in patients with dry eye and SS. For instance, cytokine levels are higher in patients with dry eye and SS compared with patients with just dry eye.\textsuperscript{249} In addition, lymphocytes accumulate in the conjunctiva of SS patients that leads to inflammation.\textsuperscript{250-253} Changes in the ocular surface of patients with SS are often more severe compared to patients without SS but with dry eye.\textsuperscript{246, 248, 249} Reduced levels of goblet cell-specific mucin MUC5AC, which correlates with decreased levels of conjunctival MUC5AC mRNA are observed in patients with SS.\textsuperscript{254-256} A lack of correlation between meibum lipid phase transitional parameters and tear film stability with SS may also be due to the observation that reduced tear lacritin levels in SS patients are highly correlated with clinical signs of dry eye.\textsuperscript{257} Lacritin proteoforms prevent tear film collapse and maintain epithelial homeostasis.\textsuperscript{257} Thus, there are factors other than an increase in meibum lipid
conformation that are markers for, or that may contribute to dry eye in patients with SS which are not present in patients without SS and with dry eye.

4.5. Conclusions

In conclusion, the compositional differences between Mss compared to Mn did not result in differences in any of the nine meibum lipid phase transitional parameters measured. The compositional differences observed between Mss and Mn could be markers for or contribute to SS as the differences could lead to tear film lipid packing differences other than conformational differences.
CHAPTER V

RESEARCH SUMMARY AND FUTURE DIRECTIONS

5.1. SUMMARY

DED is a disease that currently has no cure. MGD accounts for about 86% of dry eye occurrences, while aqueous-deficiency accounts for ~ 14%. Although CE and WE make up about 80% of human meibum lipidome, their conformational relationships has not been thoroughly examined due to difficulty in separating CE from WE and their subsequent detection. Results from model studies using synthetic CE and WE are not completely applicable to human lipidome because human CE and WE for instance, contain variable amounts of hydrocarbon chain branching, saturation and chain lengths.

The projects outlined in this dissertation sought to bridge several gaps in knowledge about the correlation of CE/WE ratio with Sjögren syndrome. Interactions between HA and phospholipids, CE and WE, and their possible contributions to tear film characteristics were also ascertained. Adsorption column chromatography was used to completely separate CE from WE. $^1$H – NMR was used to monitor resonance changes in HA during lipid binding, and to confirm the presence of, and quantify molar ratios of CE/WE. FTIR was employed to measure the lipid phase transitions of CE and WE. The ensuing paragraphs informs the reader about the key conclusions attained in our research.
5.1.1. ANALYTICAL INSTRUMENTATION CONCLUSIONS

\(^1\)H–NMR and FTIR remains the de-facto instruments for our molar quantification analyses and phase transition measurements, respectively, due to their efficiencies as well as the capability of preserving sample integrity for multiple research investigations. Areas under the \(^1\)H-NMR spectral resonances sustains a direct correlation to lipid concentration. Adsorption column chromatography with magnesium oxide enabled us to completely separate cholesteryl esters from wax esters. Although both isocratic elution and gradient elution were successful, gradient elution was more seamless and enabled superior sample recovery. Subsequent attempts to speed up separation by reducing the column slurry height significantly by up to half, appeared to jeopardize separation efficiency. Recovery efficiency of the separation protocols is also worth investigating.

5.1.2. INFLUENCE OF HA-LIPID INTERACTIONS AND CE/WE INTERACTIONS ON CONFORMATION

Our spectroscopic studies yielded some major insights into the effect of biomolecular interactions in a sugar-lipid or lipid-lipid system: Accounting for normal physiological levels of HA and some of the phopholipids, our in-vitro studies using the binding profiles of lipids and HA indicated a binding of less than 5% of HA protons. Significant binding of a wax lipid, PP, to HA, suggested the application feasibility of this study to possible interaction of HA in eye drops with ocular tear film. Given that the concentration of HA in eye drops for dry eye can vary, the parallel of the results of this study with human tear film lipids as it applies to the effect on the conformation of TF lipids and rheology, remains to be investigated and established. For the lipid – lipid interactions, human WE added to CE decreased the phase transition temperature and hydrocarbon chain order. Our studies also showed that a more fluid CE in comparison to
WE, introduced to pure WE reduced the order, phase transition temperature and minimum frequency of pure WE. All these point to the decrease in cohesion properties of CE and WE which would theoretically cause an increase in fluidization in a mixture when compared to a stand-alone lipid type such as CE or WE. By extension, these proposed effects could be applied to tear film lipids. In the same vein, the influence of increasing amounts of CE on a mixture of CE and WE showed the contingency upon whether the CE was more or less ordered. For sjögren patients, meibum was found to contain higher cholesteryl ester/wax ester ratio and more straight chains compared with donors without SS or dry eye. However, this change was not reflected on any of the phase transitional parameters.

5.1.3. POSSIBLE BIOLOGICAL CONCLUSIONS

The feasibility of HA – lipid interactions in the ameliorating effects in DED eye drops is brought to attention as a result of our binding studies. Also, the possibility exists that decrease in the spreading of tear film lipid layer due to increase in meibum lipid order could occur. With this in mind, it is not farfetched based on our results, to conclude that WE is necessary to fluidize an otherwise ordered CE to enable more biologically favourable tear film mobility. Our data therefore support the idea that less CE in the meibum from donors with MGD would cause an increase in lipid order and decrease in tear film stability. Our data also fuels the idea that changes in hydrocarbon chain composition of WE and CE may play only a minor role in the observed changes in hydrocarbon chain order. Other factors besides CE/WE ratios could be influential in the incidence of these hydrocarbon chain order changes. Exploring this will be a worthwhile undertaking because although the four samples used in our pilot meibum separation study
are typical of native meibum lipids, the limited number of samples may not be representative of any classification of meibum donors.

5.2 FUTURE DIRECTION

HA-lipid binding studies provided insightful background to further investigate interactions between meibum lipids and active ingredients in eye drops. Currently, some of the additives in eye drops include glycerin, cellulose, HA, and androgens such as testosterone. Thus, it is not inconsequential to probe these biomolecules. Through ultrasoundication, miscibility between the lipids and HA compounds for our binding study was maximized sufficiently to give quantifiable NMR spectra. One can speculate that the constitution methodology of the samples involving other eye drop additive compounds in subsequent binding stud(ies) might be a little different, but equally feasible. This is because even with the higher molar mass of HA than all the other polysaccharides and testosterone, quality data was obtained. Also, testosterone is a steroid, more non polar compared to polysaccharides like HA, cellulose or glycerin. It is expected that lipid compounds (native or standard) will be more miscible with a steroid than sugar due to chemical similarity.

Studies to separate CE and WE from meibum are warranted to determine if/how meibum CE and WE conformation and composition change with age, gender, race and MGD and if the changes are related to tear film stability. It is beyond the scope of this study to explore compositional changes with just age and MGD. To measure the composition of meibum using $^1$H-NMR and the phase transition parameters of enriched CE, enriched WE and one mixture of the two, it would take 4,200 hours to study 40 samples of meibum from donors of different ages, and 20 samples of meibum from
donors with MGD. Recovery efficiency of our meibum separation protocol is also worth investigating. As can be expected, it is not farfetched to envision the loss of sample during fraction collection, and sample reconstitution for $^1$H–NMR analysis. As recovery was not specifically probed, future studies are warranted. Lastly, the FTIR studies employed in this dissertation required the mixing of different mole % of CE and WE together and their phase transitions investigated. Results supported by sufficient umbrella of statistical queries would suggest more well-rounded research and as such, would require duplicate or triplicate studies. This poses several difficulties:

i. This would take re-separation and re–amalgamation of both species of lipids for phase transition studies which will increase the chances of sample loss of an already – valuable and relatively small sample.

ii. In an unlikely case where same mixture samples are reused, it is important to remember that the ocular tear film physiology require the expulsion of tear lipids with tears after use as new melted meibum lipids emerge unto the tear film. As such, with each lipid expulsion and new lipid mixture reintroduced, a fresh mixture is introduced from the meibomian gland which is not mirrored in the repeated melting and cooling of same mixture samples from separated lipids.

iii. Each melting and cooling cycle could take away from the structural and/or conformational integrity of each stand-alone lipid, and could progressively introduce deviations in phase transition parameters, defeating the purpose of statistical finetuning.

Future studies are therefore warranted to answer this overarching query by acquiring many meibum samples for possible doublet and triplicate studies.
REFERENCES


43. Millar TJ, Schuett BS. The real reason for having a meibomian lipid layer covering the outer surface of the tear film – A review. *Experimental eye research* 2015;137:125-138.


77. Butovich IA, Millar TJ, Ham BM. Understanding and analyzing meibomian lipids--a review. *Current eye research* 2008;33:405-420.


143. Kóta Z, Debreczeny M, Szalontai B. Separable contributions of ordered and disordered lipid fatty acyl chain segments to ννCH2 bands in model and biological


191. You IC, Li Y, Jin R, Ahn M, Choi W, Yoon KC. Comparison of 0.1%, 0.18%, and 0.3% Hyaluronic Acid Eye Drops in the Treatment of Experimental Dry Eye. *J Ocul Pharmacol Ther* 2018;34:557-564.


211. Chen J, Nichols KK. Comprehensive shotgun lipidomics of human meibomian gland secretions using MS/MS(all) with successive switching between acquisition polarity modes. *J Lipid Res* 2018;59:2223-2236.


219. Nicolaides N. Magnesium oxide as an adsorbent for the chromatographic separation of molecules according to their degree of flatness, e.g. the separation of wax esters from sterol esters. *J Chromatogr Sci* 1970;8:717-720.


222. Urata K, Takaishi N. Cholesteryl ester compounds containing alkyl branched acyl groups — their preparations, properties and applications. *Lipid / Fett* 1997;99:327-332.


APPENDIX

LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>ABBREVIATION</th>
<th>DEFINITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADDE</td>
<td>Aqueous Deficient Dry Eye</td>
</tr>
<tr>
<td>CDCl$_3$</td>
<td>Deuterated Chloroform</td>
</tr>
<tr>
<td>CE</td>
<td>Cholesteryl Ester</td>
</tr>
<tr>
<td>CEo</td>
<td>Cholesteryl Ester from an older donor</td>
</tr>
<tr>
<td>CEy</td>
<td>Cholesteryl Ester from an older donor</td>
</tr>
<tr>
<td>Cer</td>
<td>Ceramides</td>
</tr>
<tr>
<td>Cho</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>DAG</td>
<td>Di-acylglycerol</td>
</tr>
<tr>
<td>DED</td>
<td>Dry Eye Disease</td>
</tr>
<tr>
<td>D$_2$O</td>
<td>Deuterated H$_2$O</td>
</tr>
<tr>
<td>DPPA</td>
<td>Dipalmitoyl Phosphatidic Acid</td>
</tr>
<tr>
<td>DPPC</td>
<td>Dipalmitoyl Phosphatidylcholine</td>
</tr>
<tr>
<td>ED</td>
<td>Evaporative Dry Eye</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty Acid</td>
</tr>
<tr>
<td>FFA</td>
<td>Free Fatty Acid</td>
</tr>
<tr>
<td>FID</td>
<td>Free Induction Decay</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared Spectroscopy</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic Acid</td>
</tr>
<tr>
<td>GlcA</td>
<td>Glucuronic Acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance Liquid Chromatography</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear Single Quantum Coherence</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
</tr>
<tr>
<td>LB</td>
<td>Langmuir - Blodgett</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid Chromatography</td>
</tr>
<tr>
<td>MGD</td>
<td>Meibomian Gland Dysfunction</td>
</tr>
<tr>
<td>MgO</td>
<td>Magnesium Oxide</td>
</tr>
<tr>
<td>N-GlcNA</td>
<td>Glucosamine</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NP</td>
<td>Normal - Phase</td>
</tr>
<tr>
<td>OAHFA</td>
<td>(O-acyl) – ω - Omega – Hydroxyl Fatty Acid</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic Acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PG</td>
<td>Palmitoyl glyceride</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PL</td>
<td>Phospholipids</td>
</tr>
<tr>
<td>PP</td>
<td>Palmitoyl Palmitate</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>pSM</td>
<td>Palmitoyl Sphingomyelin</td>
</tr>
<tr>
<td>RF</td>
<td>Radiofrequency</td>
</tr>
<tr>
<td>RP</td>
<td>Reverse - phase</td>
</tr>
<tr>
<td>SM</td>
<td>Sphingomyelin</td>
</tr>
<tr>
<td>SP</td>
<td>Stationary phase</td>
</tr>
<tr>
<td>SS</td>
<td>Sjögren Syndrome</td>
</tr>
<tr>
<td>TAG</td>
<td>Tri - acylglycerol</td>
</tr>
<tr>
<td>TF</td>
<td>Tear Film</td>
</tr>
<tr>
<td>TFLL</td>
<td>Tear Film Lipid Layer</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
</tr>
<tr>
<td>UV – VIS</td>
<td>Ultra violet – Visible</td>
</tr>
</tbody>
</table>
VH  Vitreous Humor

WE  Wax Ester

WEo  Older Donor Wax Ester from an older donor

WEy  Younger Donor Wax Ester from a younger donor
CURRICULUM VITA

NAME: Anthony Chigozie Ewurum

ADDRESS
Department of Chemistry
University of Louisville
2320 South Brook Street
Louisville, KY 40292

Department of Ophthalmology &
Visual Sciences
University of Louisville
301 E. Muhammed Ali Boulevard

DOB: Onitsha, Anambra, Nigeria - April 23, 1991

EDUCATION & TRAINING
B.S., Chemistry
Indiana University Southeast
2011-2016
Advisor: Dr. Victor Waingeh

Ph.D., Chemistry
University of Louisville, Kentucky
2016-2022
Advisor: Dr. Douglas Borchman

Graduate Teaching Assistant
Introduction to Chemical Analysis
III & IV
University of Louisville
2019-2021

AWARDS:
Dissertation Writing Awards
Spring 2022
Graduate Student Fellowship
PROFESSIONAL SOCIETIES

American Chemical Society  
2015-2016

Association for Research in Vision & Ophthalmology  
2020-2021

PUBLICATIONS:


NATIONAL MEETING PRESENTATIONS:


**Ewurum, A.; Borchman, D:** A Spectroscopic Study of the Composition and Conformation of Cholesteryl and Wax Esters Purified from Meibum. Presented at the ARVO annual Conference, USA, May 2–6, 2021.